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Development of Homozygous Lines by *In Vivo* Doubled Haploid Technique in Hybrid Maize Breeding

İbrahim Cerit[1,a,*], Gönül Cömertpay[1,b], Bülent Çakir[1,c], Rüştü Hatipoğlu[2,d], Hakan Özkan[2,e]

[1]*Eastern Mediterranean Agricultural Research Institute, Adana, Türkiye*
[2]*Çukurova University, Faculty of Agriculture, Department of Field Crops, Adana, Türkiye*
[*]*Corresponding author*

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ABSTRACT

The maize breeding programs focuses on the development of homozygous parental lines for hybrid breeding to obtain heterosis. The Improvement of homozygous lines in a effective time is crucial for hybrid maize breeding. Objectives of this research were to obtain homozygous lines of maize in a short time by using of *in-vivo* maternal haploid (DH) technique and characterizing them morphologically. The experiments were carried out at the Eastern Mediterranean Agricultural Research Institute(EMARI). Inducers, RWS, RWK-76 and their hybrid RWS X RWK-76 were used as male parent. As female parent, 56 F_2 segregated material obtained by selfing of 66 F_1 hybrids developed in maize breeding program of Eastern Mediterranean Agricultural Research Institute (EMARI) and 2 F_2 segregated material selected from the open pollinated plants of 9 commercial maize variety. As a result of the experiment, 29 doubled haploid lines (DH) were developed. In the developed DH lines, days to tasseling, plant height, height of first ear , ear length, ear diameter, number of kernel per ear and thousand kernel weight varied as 57-78 days, 151-248 cm, 43-112 cm, 11.20-24.50 cm, 24-45 mm, 224-537 kernels/ear, and 180-320 g, respectively. In conclusion, in-vivo maternal DH technique is a highly effective method for obtaining homozygous lines.

[a] *ibrahim.cerit@tarimorman.gov.tr* https://orcid.org/0000-0001-6646-6653
[b] *gonul.comertpay@tarimorman.gov.tr* https://orcid.org/0000-0002-6522-4596
[c] *bulent.cakir@tarimorman.gov.tr* https://orcid.org/0000-0003-4672-7582
[d] *rhatip@cu.edu.tr* https://orcid.org/0000-0002-7977-0782
[e] *hozhan@cu.edu.tr* https://orcid.org/0000-0003-3530-2626

Introduction

Maize is an important cereal crop used for human and animal nutrition as well as a raw material in industry. The cultivation area of maize in Turkey is 911.885 ha, the production is 8.500.000 tons and the yield is 9.321 tons/ha (TUIK 2022). In Turkey, foreign hybrid maize varieties are widely cultivated. Therefore, a significant royalty fee is paid for foreign varieties every year. The current global yield level of maize has been achieved by the development of high-yielding hybrid maize varieties and the use of modern agricultural techniques.

Since classical plant breeding is highly dependent on environmental conditions, breeding studies are difficult and it takes a long time to reach the result. Although it varies according to the plant species, it takes about 10 to 14 years to breed a crop variety. Continuous development of new homozygous lines is required to develop hybrid varieties that yield high yield and quality crop in maize. Inbred line development is the main subject of hybrid maize breeding programs. At least 6-7 years are needed to obtain these homozygous lines with traditional methods, and at the end of this period, it is still not possible to reach 100% homozygosity. Therefore, plant breeders use the new technologies to shorten this process. The haploid plant production techniques provide significant advantages in shortening this period. The potential of doubled haploid lines obtained by the haploid plant production techniques in maize breeding has long been demonstrated (Chase, 1969). By using doubled haploid lines in maize breeding studies, 100% homozygous lines can be obtained in as little as two years, thus the breeding process in breeding studies can be shortened and the efficiency of breeding studies can be increased. Moreover, significant advantages can be provided in terms of cost reduction in breeding studies. Two techniques, *in-vitro* and *in-vivo*, are used to obtain haploid plants. It has been reported that most of the commercially developed doubled haploid lines have been obtained with the *in-vivo* haploid technique, while other techniques are less effective in developing doubled haploid lines (Geiger and Gordillo, 2009). In the *in-vivo* haploid plant production technique, lines developed in recent years

and called as inducers are used. Inducer lines are used as pollinators and, they allow the formation of haploid seeds as well as diploid seeds on the cobs of the plant pollinated by them. These haploid seeds are selected and germinated, chromosome doubling occurs with the application of colchicine to the germinated seeds, and as a result, 100% homozygous fertile doubled haploid lines can be obtained (Geiger and Gordillo, 2009). Two methods, "maternal" and "paternal haploid", are used to obtain haploid plants with the *in-vivo* haploid technique. The method of using the inducer line as a pollinator, that is, the father, is referred to as maternal haploid (Coe Jr, 1959; Kermicle, 1969). The haploid induction rate obtained by the maternal haploid method is higher than that by the paternal haploid method (Lashermes and Beckert, 1988). With the modern inducer lines developed as today's inducer, the haploid induction rate has increased to 6-14% (Geiger, 2013). As a matter of fact, the inducer RWS line developed at Hohenheim University is one of the most effective inducer lines developed in recent years, and it is a line that is well adapted to temperate climates but can also adapt to tropical climates. The haploid induction rate from the hybrid of the RWS inducer line is about 8% (Röber et al., 2005). Apart from the RWS inducer line, the RWK-76 line and its hybrid RWS X RWK-76 hybrid with the RWS line were also developed at the Plant Breeding Institute of Hohenheim University and are used to obtain haploid plants (Röber et al., 2005). Haploid induction rate from the RWK-76 line and the RWS X RWK-76 hybrid is approximately 9 -10% (Geiger and Gordillo, 2009).

In addition to the method of determining the ploidy level of the plant with the flow cytometer device, haploid seeds can be identified much more quickly, simply and cheaply with the help of the R1-nj color marker. In the selection of haploid seeds after pollination with inducer lines, haploid seeds can be easily distinguished with the help of dominant anthocyanin genes, which are defined as "red crown" or "navajo", which give red color in the embryo and endosperm of the seed (Röber et al., 2005). According to the color marker, seeds with anthocyanin color in their endosperm but colorless embryos are haploid. Seeds selected as haploid have n=10 chromosomes but plants from them are not fertile. In order to obtain the fertile plants from haploid seeds, chromosome doubling is done by applying colchicin to the seedlings obtained by germination of haploid seeds

The aims of this study were to obtain homozygous lines of maize in a short time by using of in-vivo maternal haploid (DH) technique and characterizing them morphologically to use them in the maize breeding program of the Eastern Mediterranean Agricultural Research Institute as parent.

Materials and Methods

Materials

In the study, RWS, RWK-76 lines and their hybrid RWS X RWK-76 hybrid were used as the inducer line (pollen donor) to obtain doubled haploid lines. As the female parent, a total of 58 F_2 plant materials, including 56 F_2 segregating materials obtained by selfing of 56 F1 hybrids obtained within the scope of Eastern Mediterranean Agricultural Research Institute maize breeding programs, and 2 genotype selected from F_2 progeny obtained by open pollination of commercial cultivars (pollen collector) was used.

Method

The study of the development of doubled haploid lines was carried out using the in-vivo maternal haploid technique, according to the method applied by Geiger and Gordillo (2009).

Obtaining haploid seeds by crossing inducer lines with starting material

The field experiments were carried out in the research area of the Eastern Mediterranean Agricultural Research Institute during the 2014 maize growing season. Inducer lines and female plants were grown in the plots including two rows with 70 cm row spacing and 5 m length. Seeds of the female plants were sown with 25 cm intra-row spacing. To synchronize the pollen shed of male plants with the silk emergence of the female plants, the female plants were sown at two different date such as 23 March 2014 and 2 April 2014, and the male plants were sown at three different date such as 11 April, 15 April and 24 April 2014. The plants were grown under the conventional maize growing technique in the region. The hybridization process of the inducer lines and the starting material F_2 was carried out according to the method applied by Russell and Eberhart (1975). In the hybridization process, the cobs of the F_2 plants selected as female parent were covered with kraft paper bags before silk emergence to prevent uncontrolled pollination of them and hybridization was done on at least 5 cobs for each combination. The tassels of inducer lines were insulated with a kraft paper bag just before pollen shed. Pollination was carried out by giving pollen of the isolated paternal lines to the silks of the female plants when the silks began to emergence. The hybridized cobs were kept in a kraft paper bag insulation until harvest.

Selection of haploid seeds and germination of seeds

After the hybridization of the starting materials with inducer lines, the cobs on which the grains reached harvest maturity were harvested by hand and haploid seeds were selected with the help of color marker defined by Röber et al. (2005). Three different categories of seeds were formed in the cobs obtained after hybridization of the starting materials with inducer lines. In the first category, seeds with colorless embryo and colorless endosperm were included. These are diploid seeds that have received foreign pollen due to contamination or are formed by the self-pollination. Seeds in this category are generally very rare. Seeds with purple embryo and purple endosperm were included in the second category. These are the seeds formed through the fertilization of the mother plant with pollens of the inducer line, and they are diploid. Seeds in this category have the highest total percentage of seeds. In the third category, seeds with colorless embryo and purple colored endosperm were included. These are seeds containing haploid embryos formed pathogenetically in the female flower of the mother plant as a result of abnormal fertilization with pollen of the inducer line. Seeds in these categories are shown in Figure 1a, 1b and 1c. Seeds germinated to be treated with colchicine for chromosome folding are considered haploid in the third category (Figure 1c).

a **b** **c**

Figure 1. a) Colorless embryo colorless endosperm (diploid), b) Colored embryo colored endosperm (diploid), c) Colorless embryo colored endosperm (haploid).

Seeds in these three categories were classified separately, and the rate of obtaining haploid seeds in the third category was calculated. The rate of obtaining haploid seeds varies according to environmental factors, inducer lines, genotypes used as female and pollination time.

The haploid induction rate (HIR) was calculated according to the formula below.

HIR (%) = Haploid seeds/Total seeds harvested×100

Filter paper was used for the germination process and putative haploid seeds were placed on wet filter papers at equal intervals and rolled into rolls, and they were germinated in the dark climate chamber for 2-3 days at 26 °C. They were kept until the coleoptile length was 2 cm. Haploid seeds have n=10 chromosomes, and plants from them will be sterile. In order to make the plants from the haploid seeds 2n=20 chromosomes, that is, to make fertile, they must be subjected to chromosome doubling by applying colchicine. For this, colchicine was applied to the plantlets from the putative haploid seeds. The ideal coleoptile length for colchicine application is 2 cm, and the longer coleoptiles are shortened. Chromosome doubling of haploid seedlings was performed according to the method of Deimling et al. (1997) and Gayen et al. (1994). When the coleoptile length of the seedlings, which were obtained by germination of haploid seeds in petri dishes at 26 ºC for 2-3 days under greenhouse conditions, reached 20-30 mm, they were kept in a solution containing 0.06% colchicine and 0.5% DSMO (dimethylsolfoxid) at 18 ºC for 12 hours. Then, they were washed with top water for 20 minutes and planted in viols, the viols were kept constantly moist in order to prevent excessive water loss until new roots formed, and the humidity of the plant growth cabinet was fixed at 75%. The plantlets were kept in the plant growth cabinet until they had 3-4 leaves, and then they were transferred to the field. The chromosome doubled D0 plants were planted in the rows with a row spacing of 70 cm and an intra-row spacing of 25 cm. The plants were grown under conventional growing management practices.

Self-fertilization in doubled haploid plants

The selfing process in plants identified and labeled as doubled haploid was carried out using the technique applied by Russell and Eberhart (1975). In the selfing process, the cobs of the selected lines were covered with pelur paper bags to prevent pollination. The tassels of the same plants were isolated with a kraft paper bag just before pollen shed. Selfing was carried out by giving the pollen from the isolated tassel to the silks of the previously isolated cob of the same plant when the silks emerged. Inbred cobs were kept in a kraft paper bag in isolation until harvest. During selfing, after selfing each plant, hands were washed with alcohol to prevent the pollen from the previous line from passing to the next line. Cultural practices for the selfed plants, were as given below in the subtitel 2.2.4. When D1 grains of the selfed D0 plants maturated, grains were harvested from the cobs of each selfed D0 plants.

Determination of morphological and phenological characteristics of doubled haploid plants

The doubled haploid D1 grains obtained from each selfed D0 plants were sown in 4, 3, 2, and 1 row depending on the amount of the grains obtained from each D0 plant with 5 m row length, 70 cm row spacing, and 20 cm intra-row spacing. In the D1 lines some agro-morphological characteristics such as days to tasseling (days), plant height (cm), first ear height (cm), ear length (cm), ear thickness (mm), number of grains per ear (grains/cob), thousand-grain weight were studied by the methods given by Moll et al. (1982).

Results and Discussion

Results on Obtaining Haploid Seeds

In the study, it was determined that 914 of 20038 seeds obtained by crossing 58 F_2 female individual used as female parent with 3 inducer genotypes were putative haploid according to the color marker. In addition, chromosome counts in some of the seedlings obtained from putative haploid seeds selected according to the color marker were made according to the Feulgen method described by Hatipoğlu (1991), and the accuracy was confirmed (Figures 2a,b). An average haploid induction rate of 4.56% was obtained in the study. Geıger (2013) reported that the mean haploid induction rate ranged from 6-14% in studies with the inductor genotypes RWS, RWK-76, and RWS x RWS-76. The rate of 4.56% found in this study is below the haploid induction rate range reported by Geiger (2013). Cengiz and Zararsız et al. (2019) used RWS, RWK-76, RWSxRWK-76 hybrid, and WS14 lines as inducer lines in their research to obtain doubled haploid plants, the highest haploid induction ratio (HIR) was

20,42% with RWK-76. In this study, the haploid induction ratio (HIR) of 7.79% was obtained from the RWK-76 line. However, Röber et al. (2005) explained that the haploid induction rate in haploid induction with inducer lines differs significantly depending on the genotypes used as female parent and environmental conditions. The genotypes used as female parent in the study and the ecological conditions in which the research was conducted can be seen as the reason for the low haploid induction rate detected in this study.

The number of haploid seeds obtained varied according to the female genotype and the inducer paternal line. The number of hybrid combinations to obtain haploid plants, the number of haploid seeds obtained and haploid induction ratios are given in Table 1.

As seen in Table 1, a total of 20,038 seeds were obtained from crosses of 58 genotypes with 3 different inducer lines. According to the color marker, 914 haploid seeds were obtained from the crosses. The number of diploid seeds was 16,029 and the contamination was 3,492. The highest haploid seed induction rate of 7.79% was obtained from crosses with the RWK-76 line. The lowest haploid seed induction rate of 3.05% was obtained from crosses with the RWSXRWK-76 hybrid inducer. The lowest haploid induction rate of the RWSXRWK-76 line may be due to the desynchronization of flowering times of some materials used as the female parent in the study.

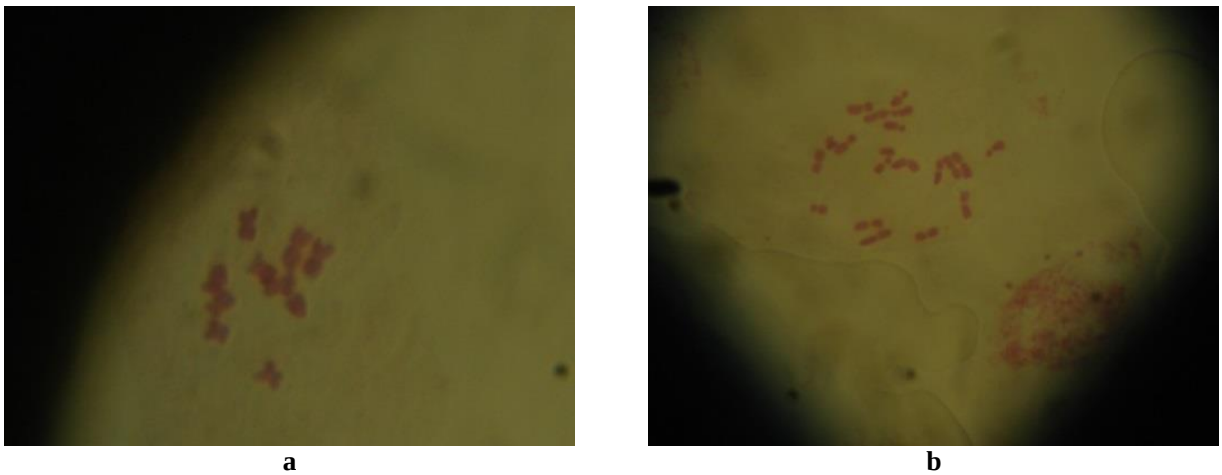
Results on Obtaining Doubled Haploid Plants

From the 914 putative haploid seeds obtained from 58 F2 female genotypes by crossing with three inducer line, 906 putative seeds from 42 female genotypes were germinated. Thus, germination rate of the putative haploid seeds was 99.1 %. Zararsız et al. (2019) reported that averaged germination rate of putative haploid seeds from 7 female genotypes was 81.8 %. Prasanna et al. (2012) gave a germination ratio of 85-90 % for putative haploid seeds in maize. This germination rates are lower than that in our research. After colchicine treatment, 432 of the 906 colchicine treated plantlets died due to toxic effect of the colchicine on the plantlets. That is, the surviving ratio of plantlets treated with colchicine was 52.3 %. Zararsız et al. (2019) was determined this ratio as 66.3% for the putative haploid seeds obtained from 7 female genotype. Geıger (2013) reported that only 70-80% of the seedlings survive after colchicine application, and 20-30% die due to the toxic effect of colchicine. In our study, the mortality rate of seedlings due to colchicine application was 47.7%. This rate is higher than the rate reported by Geıger (2013). This may be related to the genotype of the seedlings treated with colchicine and the colchicine application conditions.

A total of 474 plantlets survived after colchicine treatment belonging to the 38 different F2 female parent were transplanted into the field on 11.Mayıs 2015. Since each of the 474 plants that were transplanted into the field were of different genetic structures, they were labeled with a separate code.

Inbreeding in doubled haploid plants (D_0)

Some of the 474 doubled haploid plants (100 of them) belonging to 38 F_2 female parent that were transplanted into the field were died 7-10 days after they were transplanted, although all necessary maintenance procedures were carried out in the field. Thus, 78.9 of the transplanted putative doubled haploid plants survived under field conditions. This rate is lower than that (86.8 %) reported by Zararsız et al. (2019).



a **b**

Figure-2. a) Number of chromosomes in Haploid plants obtained (n= 10), b) Chromosome number of doubled haploid plants (2n=20)

Table 1. Number of hybrid combinations to obtain haploid plants and obtained haploid seed, haploid induction ratios (HIR), and non-haploid seed numbers

Inducer	Number of females genotypes crossed	Number of haploid seeds	Number of diploid seeds	Number of seeds from contamination	HIR (%)	Total
RWS	17	271	2.886	716	6,99	3.873
RWK-76	13	246	2.841	69	7,79	3.156
RWS X RWK-76	28	397	10.302	2.707	3,05	13.009
Total	58	914	16.029	3.492	4,56	20.038

HIR: haploid induction ratio

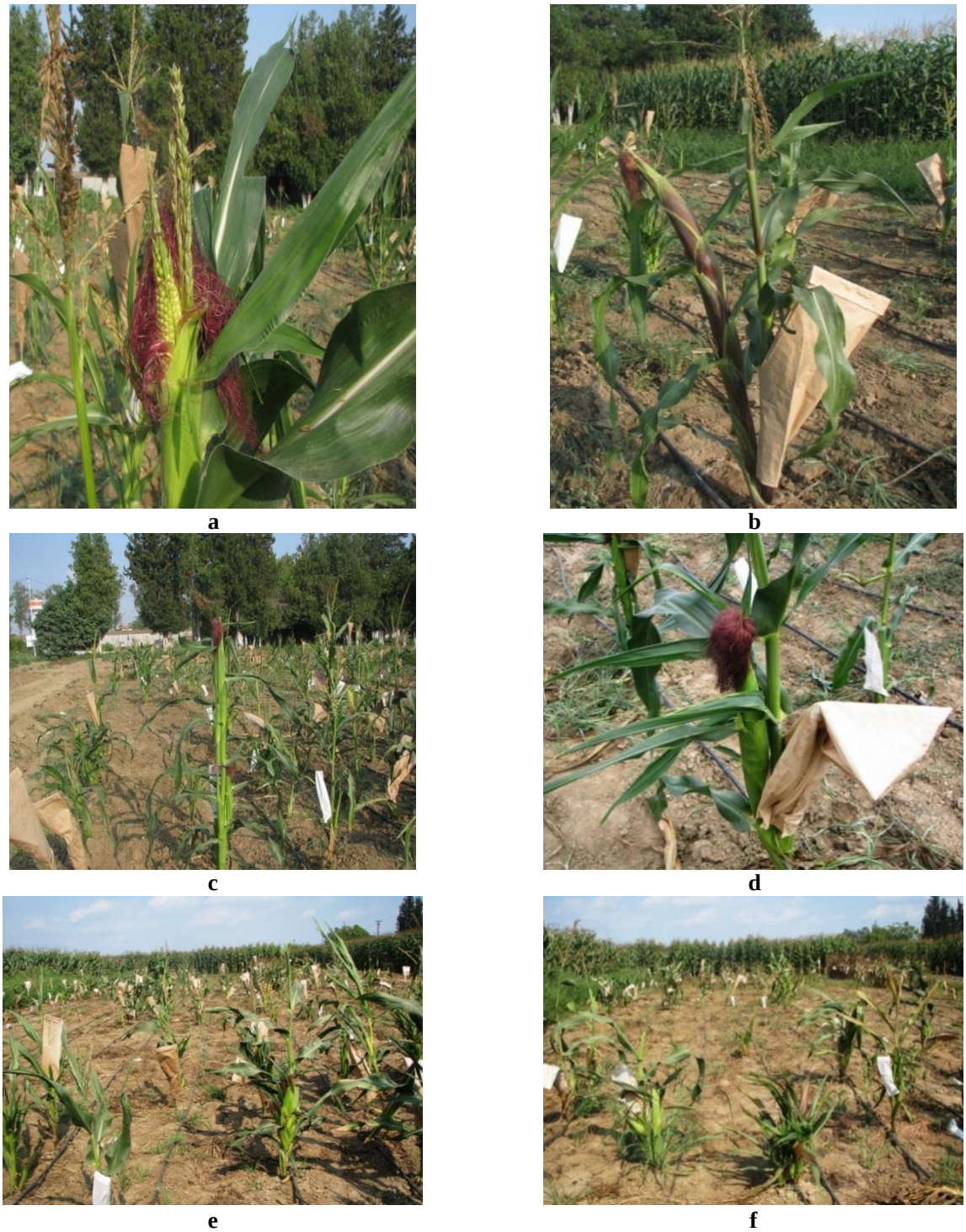


Figure 3. Abnormal plant appearances due to the toxic effect of colchicine application

a) abnormal plant appearance with ear and tassel coming out of the same sheath, b) abnormal plant appearance ear emerging from the 1st node of the plant, c) abnormal plant appearance with 3-4 ears formed on a single plant but no tassels, d) abnormal plant appearance the ear of the plant formed in the first node, e) numerous spike-shaped ears and infertile tassels protruding from the 1st node of the plant, f) abnormal plant appearance that has many tillering from a single seed but not formed ears and tassels.

Fertile pollen was detected in only 111 of the plants that reached the tasseling period, and inbreeding was done in these plants. Thus, 29.7 % of the survived plants under field conditions was fertile. Cengiz and Korkut (2020) reported that fertile plants made up 57% of live plants. Zararsız et al. (2019) reported this ratio as 34.3 %. These rates are higher than those of the present study. Infertility of the survived putative doubled plants may be due to some chromosomal abnormalities. Indeed, Zhang et al. (2008) determined that 55.4% of the haploid seedlings obtained by crossing 4 corn lines with the HZII inducer line showed the chromosome numbers varied between 9-21, and 45.5% of the seedlings were normal haploid plants with n=10 chromosomes. As a result of chromosome doubling being made in seedlings showing chromosomal abnormality in this way, normal corn plants with 2n=20 chromosomes cannot be obtained, so these plants are not likely to produce fertile pollen. Out of 111 inbred plants, 89 formed ears with grains, and 22 formed ears without grain. In this case, inbred grain was obtained in 23.8% of 374 putative

doubled haploid plants that were transplanted into the field after chromosome doubling and continued to survive. Geiger (2013) reported that 20-30% of plants that continue to survive after colchicine application and selfed can give the seed. The rate of the selfed plants with fertile ear determined in the study is within the limits reported by Geiger (2013). The fact that 22 of 111 inbred plants did not form seeds can be explained by the fact that pollen used in selfing may not be fully functional due to possible cytological instability. Since colchicine is a highly toxic substance, different abnormalities may occur due to many different reasons in the plant. For this reason, the usability of some substances that can be an alternative to colchicine is among the research topics (Häntzschel and Weber 2010). Again, on this issue, Prasanna et al. (2012) reported that germination percentage of haploid seeds before any application was between 85% and 90%, almost half (40-80%) was lost due to the toxic effect of colchicine applied to germinated seeds. They stated that depending on the genotype 0% to 40% of the colchicine treated plants can give both pollen and cob silks, and the rest show abnormalities, but only 30-50% of those can produce ears with grain. In this study, the problems arising due to the toxic effect of colchicine (such as plant death, morphological anomalies, and tassel anomalies) were similar. Similar to our study, Prigge and Melchinger (2012) stated that in their optimization studies of in vivo doubled haploid technology in corn, tassel abnormalities were frequently seen in plants where chromosome doubling could not be completed due to the toxic effect of colchicine. In addition, (Prigge and Melchinger 2012) also mentioned anomalies that may occur as a result of chromosome doubling in parallel with our study and reported that partially fertile (anther and pollen formation in only a small part of the crown tassel) and completely sterile (anther and pollen-free tassel) tassels can occur. Morphological anomalies that may occur as a result of colchicine application have also emerged in other plant species. An example of this is the study conducted by Tiwari and Mishra (2012) to determine the effect of colchicine application on the morphological characteristics of the plant in annual phlox (*Phlox drummondii)*. In the aforementioned study, they reported that colchicine application affected the survival rate of the plant negatively, the plants were shortened in height, and the number of leaves in the plant decreased while the number of tillers increased. In our study, morphologically similar abnormalities were observed in plants (Figure-3). For this reason, the inbreeding process could not be done in these plants with abnormal structures and therefore no seeds could be obtained. On the other hand, it was determined that grains of 55 out of 89 doubled haploid plants with fertile ears have color markers in both their endosperm and embryos, and that 55 plants were false positive because grains of them were selected as haploid during haploid seed selection according to the color marker, but the color marker was not very clear. For this reason, 55 of 89 inbred plants with grains on their cobs were excluded from the trial and the experiment was continued with 34 doubled haploid plants.

Depending on the genotype, the number of grains from the 34 doubled haploid lines changed between 3 and 324.

The pedigrees of the lines (D_0) developed as doubled haploids and the number of doubled haploid seeds obtained by the selfing of them are given in Table 2. The doubled haploid lines (D_1) and pedigrees obtained within the scope of the project are given in Table 3.

Morphological and phenological characteristics of doubled haploid plants

To study the morphological and phenological characteristics of D1 doubled haploid lines, D1 grains from each of 34 D0 doubled haploid lines were sown in the rows with 70 cm row spacing and 25 cm intra-row spacing on May 2016 under field conditions. Grains of 5 doubled haploid line did not germinated. Therefore, all measurement were done on the plants of 29 doubled haploid lines. The plants were grown under conventional growing management methods in the region. The mean values determined for the characteristics studied are given in Table 4.

Table 2. Pedigrees of the lines (D_0) developed as doubled haploids and the number of doubled haploid seeds obtained

No	FP	MP	HSD	No	FP	MP	HSD
1	SA2001/1X2004/3223A/8	RWS	3	18	96/13X00/315/A/3	RWS	141
2	SA2001/1X2004/3223A/10	RWS	175	19	SA2001/56X00/313B1/5	RWK-76	56
3	96/6-KX2004/3223A/4	RWS	30	20	96/6X00/313B1/1	RWS	259
4	96/6-KX2004/3223A/12	RWS	29	21	01/POP/12A2X00/313B1/	RWS	242
5	97/8BX2004/3223A/1	RWK-76	20	22	SA2001/19X00/315B1/1	RWK-76	52
6	97/13X2004/31N27/2	RWSXRWK-76	166	23	SA2001/19X00/315B1/2	RWK-76	13
7	97/13X2004/31N27/3	RWSXRWK-76	77	24	96/5-KX96/22A/1	RWSXRWK-76	159
8	97/13X2004/31N27/4	RWSXRWK-76	147	25	96/25X01/POP/1/1	RWK-76	3
9	01/POP/01X2004/31N27/1	RWSXRWK-76	36	26	96/25X01/POP/1/2	RWK-76	6
10	2001/1X2004/31N27/2	RWSXRWK-76	3	27	96/25X01/POP/1/3	RWK-76	5
11	2001/1X2004/31N27/6	RWSXRWK-76	20	28	SA2001/56X POP/14B/1	RWSXRWK-76	18
12	2001/1X2004/31N27/8	RWSXRWK-76	37	29	SA2001/56X POP/14B/1	RWSXRWK-76	66
13	SA2001/56X2004/32D99A/2	RWSXRWK-76	290	30	M1X13/1	RWSXRWK-76	224
14	SA2001/56X2004/32D99A/9	RWSXRWK-76	17	31	M1X13/2	RWSXRWK-76	288
15	SA2001/56X2004/32D99A/13	RWSXRWK-76	11	32	M1X13/4	RWSXRWK-76	322
16	SA2001/56X2004/32D99A/17	RWSXRWK-76	124	33	31G98(F2)/2	RWSXRWK-76	324
17	96/13X00/315/A/2	RWS	24	34	ES-CALİENTE(F2)/3	RWSXRWK-76	93

FM: Female Parent, MP: Male Parent, HSD: Haploid Seeds Doubled

Table 3. Doubled haploid lines (D_1) and pedigrees obtained within the scope of the project

No	DHLC	FPP	MPP	No	DHLC	FPP	MPP
1	DH-1/5-2	SA2001/1X2004/3223A/10	RWS	16	DH-16/24-18	96/13X00/315/A/3	RWS
2	DH-2/6-3	96/6-KX2004/3223A/4	RWS	17	DH-17/31-19	SA2001/56X00/313B1/5	RWK-76
3	DH-3/6-4	96/6-KX2004/3223A/12	RWS	18	DH-18/38-20	96/6X00/313B1/1	RWS
4	DH-4/7-5	97/8BX2004/3223A/1	RWK-76	19	DH-19/41-21	01/POP/12A2X00/313B1/1	RWS
5	DH-5/9-6	97/13X2004/31N27/2	RWSXRWK-76	20	DH-20/44-22	SA2001/19X00/315B1/1	RWK-76
6	DH-6/9-7	97/13X2004/31N27/3	RWSXRWK-76	21	DH-21/55-24	96/5-KX96/22A/1	RWSXRWK-76
7	DH-7/9-8	97/13X2004/31N27/4	RWSXRWK-76	22	DH-22/60-26	96/25X01/POP/1/2	RWK-76
8	DH-8/11-9	01/POP/01X2004/31N27/1	RWSXRWK-76	23	DH-23/64-28	SA2001/56X POP/14B/1	RWSXRWK-76
9	DH-9/13-10	2001/1X2004/31N27/2	RWSXRWK-76	24	DH-24/64-29	SA2001/56X POP/14B/1	RWSXRWK-76
10	DH-10/13-12	2001/1X2004/31N27/8	RWSXRWK-76	25	DH-25/65-30	M1X13/1	RWSXRWK-76
11	DH-11/17-13	SA2001/56X2004/32D99A/2	RWSXRWK-76	26	DH-26/65-31	M1X13/2	RWSXRWK-76
12	DH-12/17-14	SA2001/56X2004/32D99A/9	RWSXRWK-76	27	DH-27/65-32	M1X13/4	RWSXRWK-76
13	DH-13/17-15	SA2001/56X2004/32D99A/13	RWSXRWK-76	28	DH-28/68-33	31G98(F2)/2	RWSXRWK-76
14	DH-14/17-16	SA2001/56X2004/32D99A/17	RWSXRWK-76	29	DH-29/71-34	ES-CALİENTE(F2)/3	RWSXRWK-76
15	DH-15/24-17	96/13X00/315/A/2	RWS				

DHLC: Double Haploid Line Code, FPP: Female Parent Pedigree, MPP: Male Parent Pedigree,

Table 4. Some morphological and phenological features of doubled haploid lines (D_1) that were obtained within the scope of the project

No	DHLC	TFT (day)	PH (cm)	HFE (cm)	EL (cm)	ED (mm)	NKPE (number/ear)	TKW (g)
1	DH-1/5-2	72	151	63	12.8	39	303	220
2	DH-2/6-3	74	172	74	14.3	37	259	220
3	DH-3/6-4	75	208	83	13.7	39	367	200
4	DH-4/7-5	78	159	62	15.5	36	414	180
5	DH-5/9-6	64	209	78	17.7	38	396	220
6	DH-6/9-7	64	179	71	14.2	36	347	220
7	DH-7/9-8	64	180	57	15.0	36	240	260
8	DH-8/11-9	68	141	43	16.2	43	280	320
9*	DH-9/13-10*	75	170	55	12.5	24	*	*
10	DH-10/13-12	76	216	76	15.7	41	262	220
11	DH-11/17-13	63	201	68	17.2	41	383	280
12	DH-12/17-14	57	174	48	17.3	40	227	260
13	DH-13/17-15	68	211	86	24.5	43	312	300
14	DH-14/17-16	61	183	48	17.7	45	475	260
15	DH-15/24-17	76	170	70	12.8	32	237	220
16	DH-16/24-18	71	172	79	15.7	33	380	180
17	DH-17/31-19	71	221	96	11.2	38	267	200
18	DH-18/38-20	68	248	112	18.5	43	537	220
19	DH-19/41-21	66	245	110	17.7	42	362	280
20	DH-20/44-22	72	167	52	17.5	39	384	200
21	DH-21/55-24	71	206	77	16.0	45	421	260
22	DH-22/60-26	72	245	90	12.5	37	364	220
23	DH-23/64-28	59	169	54	18.2	42	224	300
24	DH-24/64-29	65	212	62	16.0	43	264	260
25	DH-25/65-30	64	202	60	16.5	37	317	280
26	DH-26/65-31	62	215	70	16.5	47	443	300
27	DH-27/65-32	62	204	70	16.5	40	400	260
28	DH-28/68-33	59	232	76	17.0	41	376	280
29	DH-29/71-34	65	226	68	16.5	41	258	300

DHLC: Double Haploid Line Code, TFT: Tassel Flowering Time, PH: Plant Height, HFE: Height of First Ear, EL: Ear Length, ED: Ear Diameter, NKPE: Number of Kernels Per Ear, TKW: Thousand Kernel Weight; *: Since one seed was taken on the line with code number DH-9/13-10, the number of kernels per ear and thousand kernel weight values could not be measured.

When Table 4 is examined, it is seen that days to tasseling of the doubled haploid lines varied between 57-78 (days), the plant height between 151-248 (cm), the height of the first ear between 43-112 (cm), the length of the ear 11.2-24.5 (cm), the ear diameter between 24-45 (mm), the number of kernels per ear between 224-537 and the weight of thousand kernels between 180-320 (g). It has been determined that the doubled haploid lines have a wide variation in terms of the investigated properties.

Conclusion

This research was carried out in the research area of the Eastern Mediterranean Agricultural Research Institute between 2014 and 2017 to obtain homozygous lines by in vivo haploid technique in a short time and to determine the morphological characteristics of these lines in corn. A total of 20,038 seeds were obtained from the crossing of 58 F_2 plants as female parent with 3 different inducer lines. According to the color marker, 914 haploid seeds were obtained from the crosses. The highest haploid seed induction rate was obtained from the RWK-76 line with 7.79%, and the lowest haploid seed induction rate was obtained from the RWSxRWK-76 hybrid inducer line with 3.05%. The lowest haploid seed induction rate of the RWSxRWK-76 inducer line was due to the problem of flowering synchronization of some materials used as the female parent in the study. The average haploid induction rate within the scope of the study was 4,56 %. The genetic structure of the inducer genotypes, the genetic structure of the female genotypes, environmental factors and the time of pollination also affect the haploid seed induction rate of the inducer lines. As a result of the study, 29 doubled haploid lines were developed. The days to tasseling of doubled haploid lines varied between 57-78 (days), plant height between 151-248 (cm), height of first ear between 43-112 (cm), the ear length between 11.2-24.5 (cm), ear diameter between 24-45 (mm), number of kernels per ear between 224-537 (pieces/cob) and thousand kernels weight between 180-320 (g). It has been determined that the doubled haploid lines have a wide variation in terms of the investigated properties. While line development studies can be carried out in at least 7 years in classical corn breeding studies, 100% homozygous lines have been developed in a short period of 2 years with in vivo doubled haploid plant production technique. As a result, it has been demonstrated that the in vivo maternal haploid plant production technique can be successfully applied in corn breeding studies.

Author Contributions

İ.C, G.C, and R.H, concept; İ.C, G.C, and R.H, design;., İ.C, G.C, and R.H resources; İ.C, G.C, and R.H; materials; İ.C, G.C, R.H, H.Ö, and B.Ç, data collection or processing; İ.C, G.C, R.H, and H.Ö data validation; İ.C, G.C, R.H, H.Ö, analysis or interpretation; İ.C, G.C, and R.H literature search; İ.C and R.H, writing; İ.C, G.C, and R.H critical reviews; İ.C, project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Declarations

Conflict of interest: Authors declare no conflict of interest.

Ethical Approval: This manuscript does not contain any studies with human participants or animals performed by any of the authors.

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