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Effect of Parental Genotypes and Their Reciprocal Crosses on Haploid Plant Production by Anther Culture and Confirmation of Double Haploids by Flow Cytometry in Bread Wheat

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ABSTRACT

Double haploids (DHs) production and utilization is an important aspect of wheat breeding programs in worldwide, because, it provides many advantages over the conventional breeding program. Therefore, in vitro and in vivo responses of anther derived two bread wheat cultivars and their F1 crosses were investigated. Results showed significant genetic variation among the tested bread wheat genotypes. The pre-cold treated anther derived embryonic calli and shoot induction (%) were obtained from all genotypes that ranged 44 - 76% and 32 - 58% respectively. The highest shoot induction from embryonic calli was achieved from F1 cross of Zubkov x Atay-85, whereas, lowest induction was obtained from Atay-85 cultivar. The F1 cross (Zubkov x Atay-85) showed a better response in anther culture in term of shoots per plant and root induction than their respective parental genotypes.

The present results indicated positive and significant heterotic effects of F1 crosses for calli and shoot induction that also showed less albino plant regeneration after colchicine treatments. Total number of 114 anthers driven DHs lines from two genotypes and their F1 crosses were regenerated, confirmed by flow-cytometry and evaluated under field condition. Some DHs lines were found to be significantly superior for agronomical traits including seed yield than the parental genotypes as well as local bread wheat (control) cultivars.

Keywords: Anther culture, Bread wheat, Colchicine, Double haploid, *Triticum aestivum* L.

1. Introduction

Bread wheat (*Triticum aestivum* L.) is one of the most cultivated cereals crop in worldwide including Turkey. The global wheat production is 761.5 million tonnes during 2019 (FAO 2020). Hexaploid (2n=6x=42) bread wheat is an important source of protein, vitamin, and mineral, as well as carbohydrate content for human consumption (Sun et al. 2014). Bread wheat genetic structure is highly affected under adverse agro-ecological conditions in the term of its yield potential. Therefore, there is always a need for wheat varieties that adapt well to regional ecologies, resistant to diseases and pests, tolerant to stress factors such as drought, cold and salinity (Mahmood & Baenziger 2008; Chauhan & Khurana 2011; Başer et al. 2020; Gebremariam et al. 2020; Liu et al. 2020). In conventional wheat breeding programs, many factors affect the development of homozygous lines (variety) after hybridization i.e. improper selection of parents for breeding purposes, genetic dependence of available genetic resources, and success of interspecific and intraspecific crosses for desire agronomical traits. Therefore, there is need to develop efficient double haploid (DH) technology that accelerates the wheat breeding for desirable agronomic traits including yield (Lantos et al. 2013; Asif et al. 2014).

In this aspect, development of haploid plants from hybrids and followed by chromosome doubling by colchicine, paved the way for accelerating the process of pure wheat lines development (Hansen & Andersen 1998; Jarzina et al. 2017). These DHs lines can be utilized as a recombinant wheat genotype with desired gene combinations. Moreover, the double haploid technique can be supplemented with modern technology i.e. gene transformation (Chauhan & Khurana 2011), development of a functional marker CAPS (Yue et al. 2015), identify the molecular markers (Abd El-Fatah et al. 2017) and applied CRISPR system for induction of improve starch quality (Liu et al. 2020) for further improvement and releasing of new bread wheat varieties.

It is well-established fact that anther culture is useful for DHs production in many cultivated crops including wheat. The microspore and anther culture have been widely used to produce haploid followed by DHs plants in wheat program (Gurel et al. 1993; Jauhar et al. 2009; Lantos et al. 2013; Asif et al. 2014; Jarzina et al. 2017). Moreover, chromosome elimination through wide-crosses between genetic incompatible species (wheat and maize or barley) can produced wheat haploids, therefore, well-trained person with controlled conditions required for embryo rescue techniques for fertile and haploid plant regeneration (Jarzina et al. 2017). Doubled haploids developed through colchicine-induced chromosomal doubling leads to the completely homozygous lines production in a single generation and saves at least 5-6 growing season of self-pollinated crop and labour cost for developing completely homogygous lines (Hassawi et al. 2005). On the other hand, anther culture has many difficulties such as, high genotype dependency as well as low frequency of haploid plant regeneration due to complicated in vitro manipulation steps including anther culture condition, growth regulators and media combinations (Gurel et al. 1993; Hansen & Andersen 1998; Redha et al. 2000; Patel et al. 2004; Jauhar et al. 2009; Lantos et al. 2013; Asif et al. 2014; Jarzina et al. 2017).

Almost all the researchers working on the development of haploid plants emphasised that anther culture is a tedious work due to genotype effect, which is the main limiting factor of in vitro androgenesis and makes this technique too expensive for routine purposes (Yermishina et al. 2004). Therefore, it is suggested that tissue culture responsive genotypes should be utilized for anther culture (Andersen et al. 1988). Although, responsiveness of parental genotype to anther culture also affects the response of hybrid combinations, there are evidence that F1 hybrids have a higher androgenetic capacity than the parental genotypes (Zamani et al. 2003). Therefore, this study aimed to screen in vitro anther culture responses of Atay-85 and Zubkov cultivars and their reciprocal crosses.

2. Material and Methods

2.1. Plant materials

In the present study, four hexaploid bread winter wheat genotypes namely Zubkov and Atay-85 cultivars, F1 hybrids of Zubkov × Atay-85 and Atay-85 × Zubkov were used as plant materials. Twenty-five seeds of each genotype were sown in pots filled with sterilized soil under glass-house condition at Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey. The bread winter wheat genotypes were obtained from Central Research Institute of Field Crops, Ministry of Food, Agriculture and Livestock, Ankara, Turkey. All the necessary care (irrigation, fertilizer) were taken for the healthy growth of the donor wheat plants. The plants were foliar sprayed with commercial foliar fertilizers NPK (20:20:20) solution.

2.2. Harvesting of spikes

One of the most important factors affecting the rate of double haploid production through anther culture is the microspore development and selection phase at the time when the donor plant spikes were harvested. For cytological examination, a drop of 1.0% acetocarmin was placed on the anthers taken from flowers and viability were examined under a light microscope. On the basis of morphological observation, the spikes were harvested when the distance between the flag leaf and below leaf around 3-5 cm on the donor plants. The spikes were collected at the uninucleate microspores stage (after tetrad stage) and placed in a plastic beaker filled with sterilized double distilled water and subjected to cold treatment (4 °C) for 4 days.

2.3. Anther isolation

After the cold treatment, the spikes were surface-sterilized with 2% commercial bleach (sodium hypochlorite) in a laminar air flow for 20 minutes and rinsed 3×5 minutes with sterilized double distilled water. More than 300 sterilized anthers of each genotypes were aseptically isolated and transferred to disposable petri dish (60 mm x 15 mm) containing 5 mL of CHB3 liquid medium supplemented with 2 mg/L 2,4-D, 2 mg/L kinetin and 90 g/L maltose (Table 1). The cultures were incubated at 25±1 °C under dark condition for 4-5 weeks.

Table 1- Composition of the embryogenic callus induction medium (CHB3), Shoot regeneration medium (SRM4) and Rooting medium (RM2)

*Chemical Compounds**	*Embryogenic callus-induction media (CHB3)* (mg/L)	*Regeneration media (SRM4)* (mg/L)	*Shoot elongation and rooting media (RM2)* (mg/L)
Vitamins			
Myo-inositol	300	100	100
Pyridoxine HCl	0.5	5	0.4
Nicotinic acid	0.5	5	0.4
Thiamine HCl	2.5	1	0.4
Pantothenic acid	0.25	-	-
Ascorbic acid	0.25	-	0.4
Biotine	0.25	-	0.4
Macro-elements			
KNO_3	1415	1000	1900
$(NH_4)SO_4$	232	-	-
KH_2PO_4	200	300	170
$MgSO_4.7H_2O$	93	71	370
$CaCl_2.2H_2O$	83	-	440
NH_4NO_3	-	1000	164
$Ca(NO_3)4H_2O$	-	500	-
KCl	-	65	-
Micro-elements			
KI	0.4	0.75	0.83
$MnSO_4.4H_2O$	5	4.9	22.3
$Na_2MoO_4.2H_2O$	0.012	0.2	0.25
$ZnSO_4.7H_2O$	5	2.7	8.6
$CuSO_4.5H_2O$	0.025	0.076	0.025
$CoCl_2.6H_2O$	0.025	0.05	-
H_3BO_3	5	1.6	6.2
Iron source			
$FeSO_4.7H_2O$	20	20	20
Na_2EDTA	20	20	20
Hormones			
2,4-Dichlorophenoxyacitic acid	2	-	-
Kinetin	2	-	-
IAA	-	1	1
Amino acid			
Glycine	1	2	-
Glutamine	993.5	-	750
Carbon source			
Maltose	90000	-	-
Sucrose	-	20000	20000
Agarose	-	6500	-
Other Material			
Agar	-	-	6000
pH	5.4	5.9	5.8

*: Modified chemical composition of the medium (Picard & De Buyser 1973)

2.4. Embryogenic callus induction and plant regeneration

The anthers were swollen and whitened after one week of culture inoculation. The appearance of embryogenic callus on the anther surface was observed after 4-5 weeks of culture. The embryonic calli were then transferred to agarose solidified regeneration medium (SRM4 medium; Picard & De Buyser 1973) supplemented with 20 g/L sucrose and 1 mg/L IAA in petri dishes and incubated at 25±1 °C with 16/8 h light/dark cycle for 7-8 weeks. The green and healthy plantlets were transferred for shoot elongation and rooting medium (RM2) for 3-4 weeks. The regenerated green plantlets with poor root systems were subcultured on fresh RM2 medium.

2.5. Acclimatization of in vitro plantlets

The plantlets with well established root-shoot system and reach a height of 10-12 cm in the culture vessels were transferred to pots containing peat-moss and perlite mixture (2:1), under 16/12 °C day/night temperature with 80% relative humidity for 2 weeks. The relative humidity was periodically decreased and successfully acclimatized plants were transferred to larger pots containing sterilized field soil.

2.6. Colchicine treatment and production of doubled haploid

The healthy haploid plants were taken from the pots (soil grown) and thoroughly washed with tap water, especially root parts. The root and shoots were trimmed and then immersed in 0.2% colchicine solution for 4 h at room temperature (Pauk et al. 2003; Tadesse et al. 2013). After colchicine treatment, the plants were rinsed with running tap water for 3-4 hours. Thereafter, the plants were transplanted in pots containing peat-moss and perlite mixture (2:1) and covered with transparent polythene bags to create relative humidity. All survived plants were grown until they reach physiological maturity. DH plants were confirmed by Flow Cytometry. The percentage of DHs were calculated by number of regenerated shoots per explant and haploid plant development. The seeds were harvested from mature plants and kept separately.

2.7. Determination of DHs plant through flow cytometry

Determination of ploidy levels in DHs plants were confirmed by flow cytometry (Sysmex UF-100, TOA Medical Electronics/Europe GmbH, Hamburg, Germany) device (Battistelli et al. 2013). The DNA were extracted from the young leaf of the DHs plant and haploid (control) plant. For DNA extraction, leaves were crushed in 0.4 mL of nuclei extraction buffer and incubated for 1 minute at room temperature. The solution was filtered through cellTrics (Sysmex Partec GMBh, Germany) and added 1.6 mL staining buffer (DAPI), and then incubated for 1 min in dark condition. The prepared samples were loaded on the cytometry device and data were recorded.

2.8. Statistical analysis

Each treatment had 15 replicates with 20-30 anthers. Data given in percentage were normalized through arcsine ($\sqrt{X}$) transformation (Snedecor & Cochran 1967) before statistical analysis, and all data were analyzed with ANOVA and compared via Duncan's multiple range test using SPSS statistical (IBM® SPSS® statistics 24.0 for Windows) analysis at the 5% level of significance.

3. Results and Discussion

The biotechnological techniques especially tissue culture have excellent potential to improve the bread wheat quality through genetic transformation, somaclonal production and DHs production (Tadesse et al. 2013; Jarzina et al. 2017). However, the dedifferentiation (callus induction) and redifferentiation (plant regeneration) is influenced by many factors including extrinsic supply of macro and micronutrients, and growth regulators in culture medium (Barpete et al. 2020). Table-1 depicts that CHB3 medium (Picard & De Buyser 1973) for embryogenic callus induction from sterilized anthers, nutrient media for plant regeneration (SRM4) and shoot elongation and rooting media (RM2). Analysis of variance results showed a significant difference among genotypes ($P \leq 0.05$) on embryonic callus formation (%), shoot induction (%), number of regenerated shoots and roots induction (%) and production of double haploid plants (Table 2).

Table 2- The effect of genotype on callus induction, plant regeneration and doubled haploid plant production

Genotypes and crosses	*Embryogenic callus- induction (%)*	*Shoot-induction (%)*	*Number of regenerated shoots per embryogenic calli (%)*	*Root induction (%)*	*Double Haploid plants production (%)*
	*CHB3 media**	*SRM4 media**		*RM2**	
Zubkov	74a	54ab	5.7a	10.6a	4.6
Atay-85	44b	32c	2.4b	5.4b	1.6
F1 (Zubkov x Atay-85)	76a	58a	6.3a	12.4a	5.3
F1 (Atay-85 x Zubkov)	66a	44bc	3.0b	2.8b	1.0

*: Values shown in a columns followed by different letters are statistically different using Duncan's multiple range test at 0.05 level of significance

3.1. Embryogenic callus induction

Anthers of four bread winter wheat genotypes were cultured on modified CHB3 medium supplemented with 2 mg/L 2,4-D and 2 mg/L kinetin for embryogenic callus induction. The results showed a significant difference among cultivars and their F1 hybrid ($P \leq 0.05$) on embryogenic callus induction (Table 2). The embryogenic callus induction was initiated after 3-4 weeks of culture inoculation in CHB3 medium, thereafter, it was observed that the callus size increased significantly. The calli size in the term of anther's genotypes were not significantly differ among wheat genotypes (Figure 1d, e and f). The embryogenic callus induction ranged from 44 to 76% depending on genotypes. The highest callus induction was noted on F1 hybrid of Zubkov x Atay-85, whereas, lowest induction was recorded on Atay-85 cultivar. Moreover, comparing Zubkov and Atay-85 cultivars, Zubkov and their F1 hybrid were superior for embryonic callus development and further culture growth. The present results are in line with the previous studies by Ahmet & Adak (2007), El-Hennawy et al. (2011), Xynias et al. (2014) and Yorgancılar et al. (2016). They also noticed that wheat F1 hybrids were superior to its respective parents.

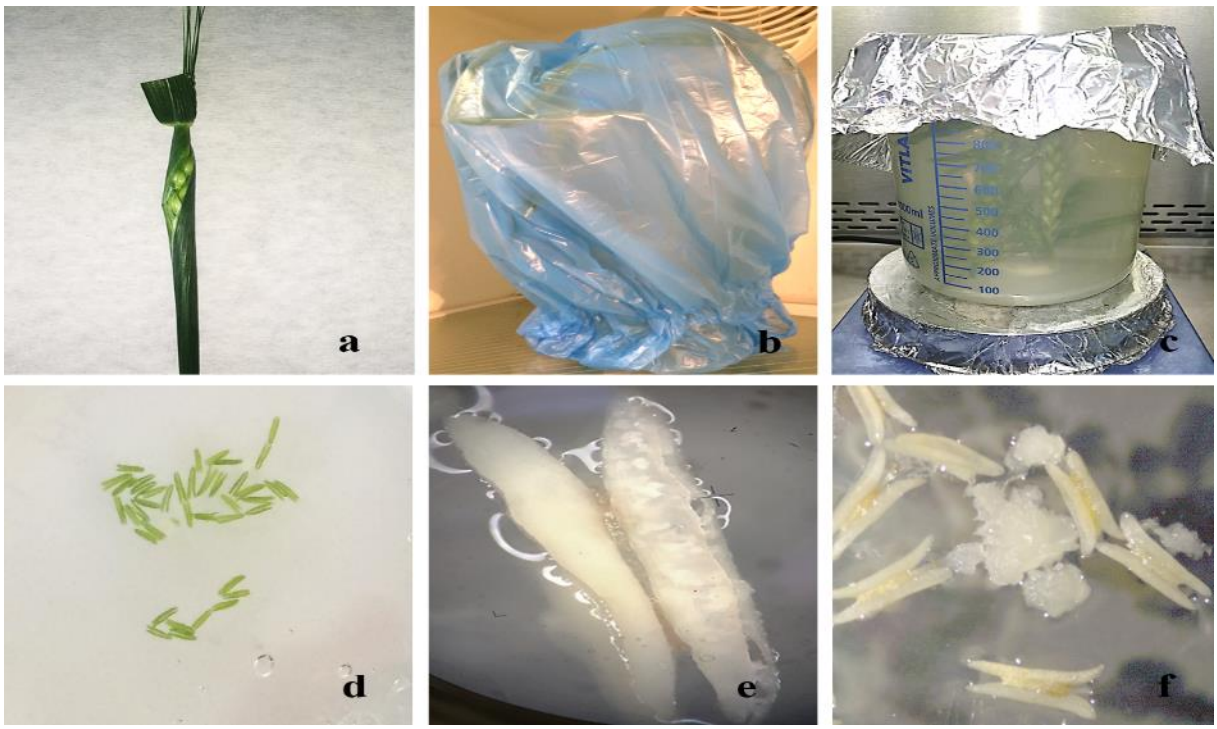


Figure 1- Anther culture technique: a) harvesting of spikes from donor plants, b) cold treatment of spikes, c) surface sterilization of spikes, d) isolation of anthers, e & f) in vitro culture and initiation of embryonic callus

3.2. Shoot induction from anther derived calli

The CHB3 medium was also very responsive for shoot initiation on embryogenic callus and small shoots were observed approximately 5-6 weeks of culture inoculation (Figure 2a, b and c). The callus derived small shoots were further transferred to shoot regeneration (SRM4) media. The analysis of results showed a significant difference among cultivars and F1 hybrids ($P \leq 0.05$) on shoot induction (%) and number of shoots per explant that were ranged from 32–58% and 2.4–6.3, respectively (Figure 2d, e and f). It was well established that generally F1 hybrids always exhibited positive heterosis over their parent when they intraspecific crossed (Ozbay & Özgen 2010). Therefore, F1 hybrid of Zubkov x Atay-85 bread winter wheat showed superior for embryonic callus developments. The highest shoot induction and number of shoots per explant were recorded on F1 hybrid of Zubkov x Atay-85 cultivars. Whereas, the lowest shoot regeneration and number was recorded on Atay-85 cultivar, but, there were no inhibitory effect on growth of regenerated shoots. The all regenerated shoots were healthy and green are in agreement with Ahmet & Adak (2007) and Jarzina et al. (2017). The researchers also recovered green and healthy shoots from anther derived calli of Polish winter and spring wheat varieties and their F1 hybrid. In the present study, rooting was significantly promoted in F1 hybrid (Zubkov x Atay-85) when subcultured on RM2 medium containing 1 mg/L IAA followed by Zubkov cultivar. Root induction were ranged from 2.8 to 12.4% and minimum root induction was noted on F1 hybrid of Atay-85 x Zubkov. Similar results were obtained by Konieczny et al. (2003), Barakat et al. (2012), and Al-Shaker (2013) on bread wheat varieties. They also reported that F1 hybrid always superior for rooting then their respective parental genotypes.

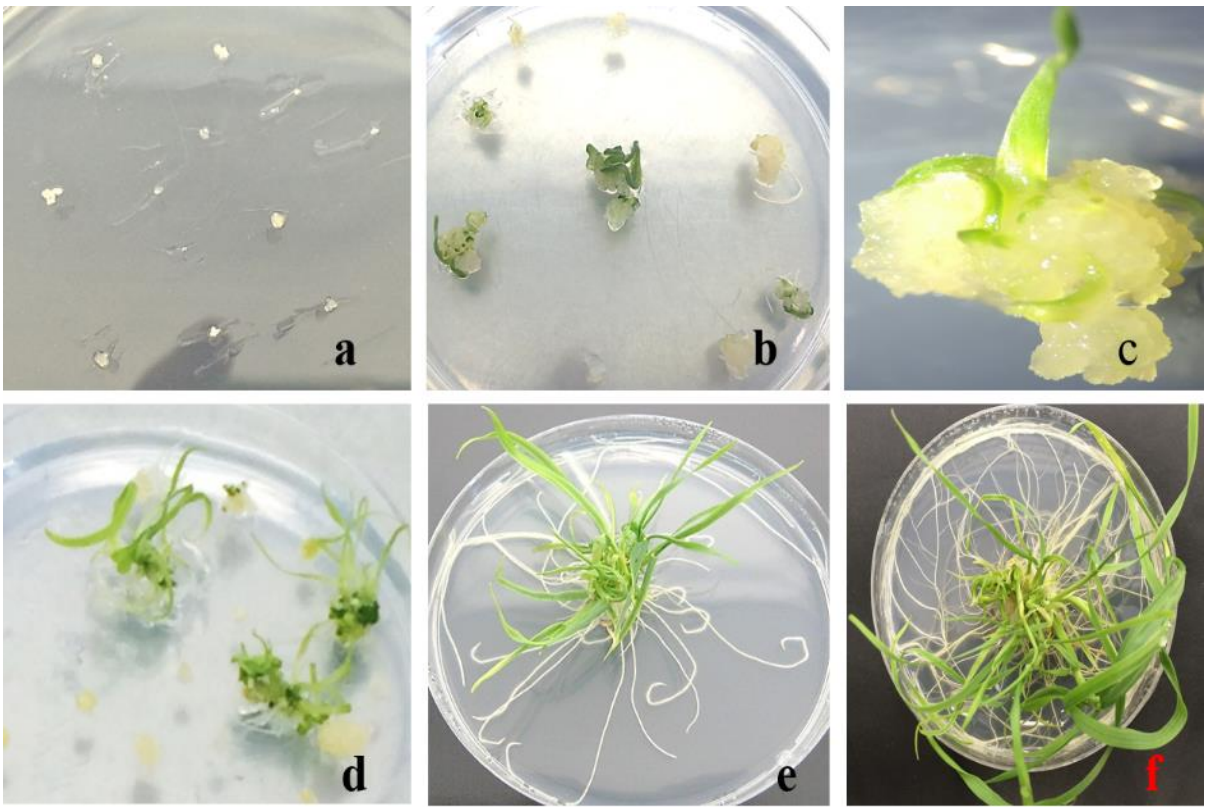


Figure 2- In vitro anther culture of different wheat genotypes and shoot regeneration; a) embryonic callus induction, b, c and d) regeneration of shoots on embryonic callus, e and f) plantlets growing and rotting on SRM4 and RM2 media

3.3. Hardening, colchicine treatment and doubled haploid plant production

The healthy and well rooted in vitro plantlets were transferred to pots for hardening in greenhouse under ambient daylight condition with 80-90% relative humidity (Figure 3a and b). The healthy acclimatized plants with 5-6 leaves were screened and taken from the pots and thoroughly rinsed with running tap water. After washing, root and shoot were trimmed and immersed in 0.2% colchicine solution (Tadesse et al. 2013) and kept at room temperature for 4 hours (Figure 3c). After colchicine treatment, the plants were washed with running tap water for 3-4 hours and then transferred into large pots containing sterilized field soil (Figure 3d, e and f) in a greenhouse and covered with transparent plastic bags for 48 hours. To avoid outcrossing, fertile heads are covered with brown bags before anthesis. Proper care taken for fertilization and irrigation until the plant physiological maturity.

The plant regeneration rate varied significantly depending on genotypes. The genotype Zubkov and F1 of Zubkov x Atay-85 showed higher plant regeneration rates than Atay-85 and F1 of Atay-85 x Zubkov. The rate of regeneration of green plants varied greatly depending on the genotypes. The Zubkov and F1 hybrid of Zubkov x Atay-85 showed higher green plant regeneration rates compared to the genotype Atay-85 and F1of Atay-85 x Zubkov. Moreover, the rate of albino plant also varied after colchicine treatment. The F1 hybrid of Atay-85 x Zubkov showed higher rate of albino plant than the Zubkov winter wheat cultivar.

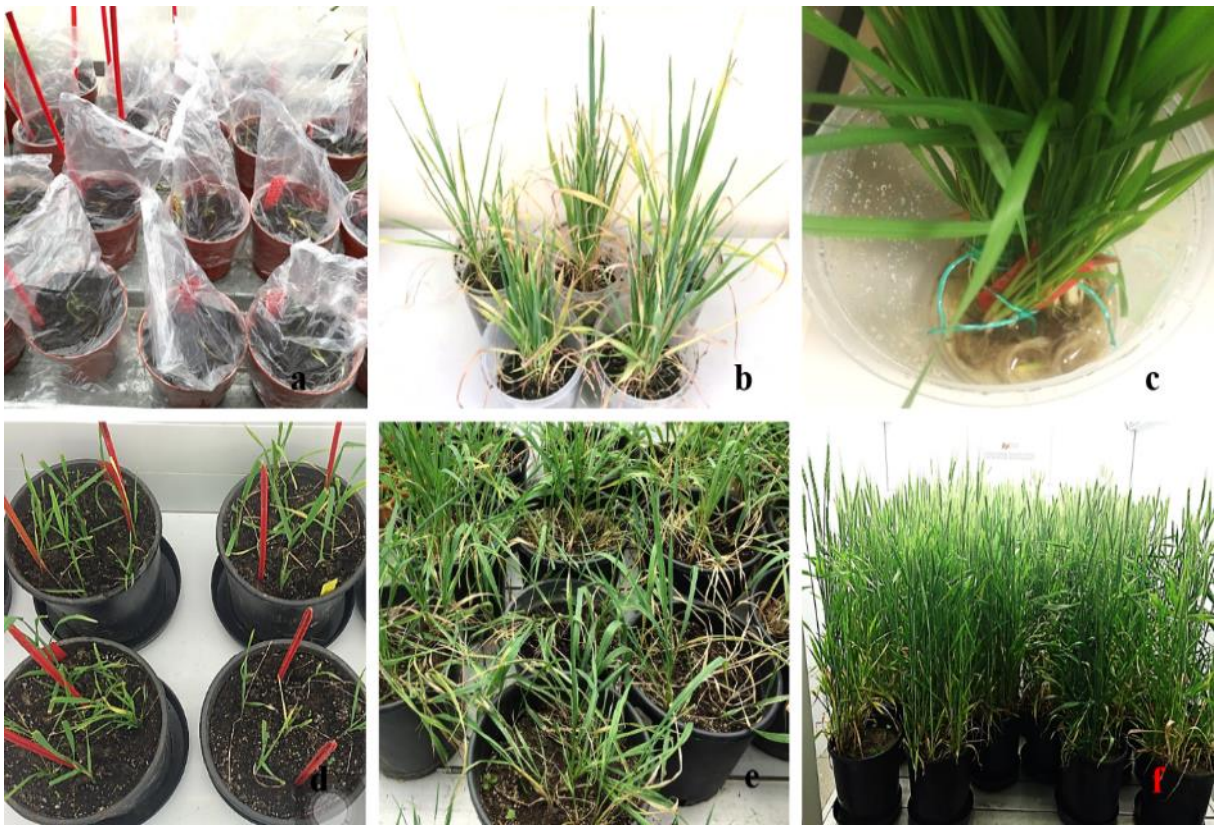
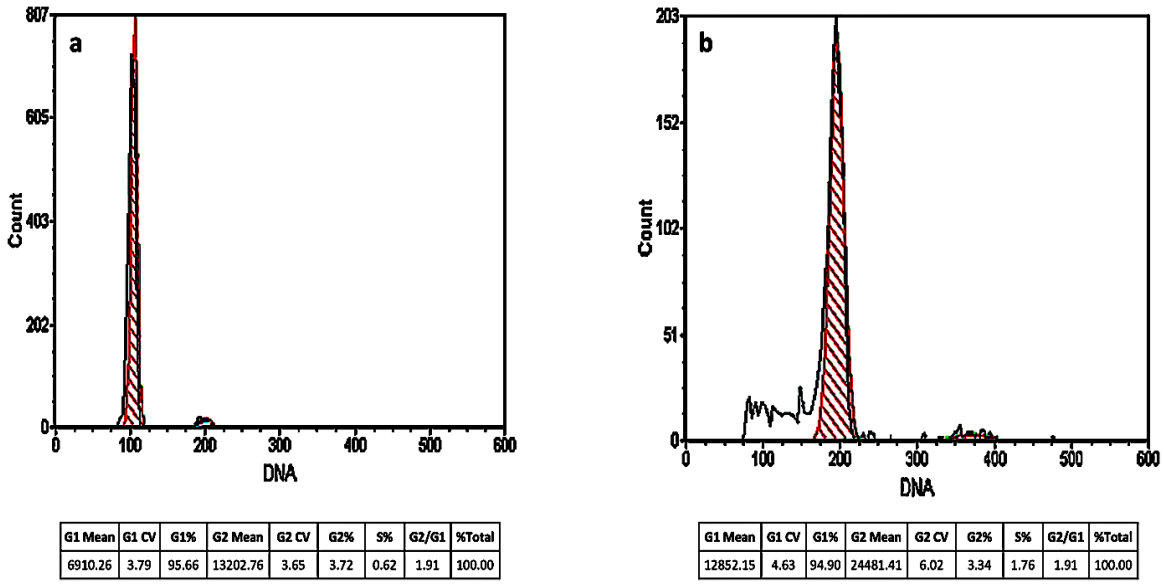


Figure 3- Double haploid plant production from bread wheat anther culture; a and b) haploid plants at acclimatization stage in a greenhouse, c) colchicine treatment of haploid wheat plants, d, e) acclimatization of DHs wheat plants, f) Growing DH wheat lines after confirmation by flow cytometry in controlled condition

A total number of 114 DH lines were obtained as a result of chromosome doubling from Atay-85 and Zubkov cultivars and their reciprocal crosses. The anther derived each DHs lines had 22-87 seeds/plant after physiological maturity. Seeds from individual DHs lines were separately harvested for genetic purity and stability in anther derived DHs lines are in contradictory with Kisana et al. (1993). They found anther-derived DHs wheat lines had cytologically unstable. Whereas, no significant differences found in anther derived and intergeneric crosses made for DHs wheat lines by Bjornstad et al. (1993). On the other hand, El-Hennawy et al. (2011) evaluated and identified highly stable and superior genotypes from anther derived DHs wheat lines. The present study also showed that anther-derived DHs lines are superior over the national control cultivar in agreement with Konieczny et al. (2003), Barakat et al. (2012), and Al-Shaker (2013).

3.4. Confirmation of DH plants by flow cytometry

The winter wheat plants with different ploidy levels including haploids and DHs were identified using flow cytometry analysis. The Figure 4a depicts that the G1 and G2 peak of haploid plant having a DNA concentration of 3.79 and 3.65 pg/2C respectively. Whereas, double haploids plant showed a DNA concentration of 4.63 and 6.02 pg/2C in G1 and G2 peaks respectively. The Figure 4a and 4b histogram displays single high peaks considered the haploid and double haploid plants. Based on the amount of DNA, the duplication (double haploid) was confirmed in regenerated plants. The rate of duplication of F1 hybrids of Zubkov x Atay-85 was also higher compared to the donor genotypes. Whereas, F1 hybrid of Atay-85 x Zubkov shown contradictory results that indicating double haploids production varies between genotypes and its combination. However, several plants (during haploid to DH stage) did not survive till the sample collection as well as physiological maturity. It may be due to toxic effect of colchicine for chromosomal doubling in agreement with Battistelli et al. (2013). A total number of 352 plants were treated with colchicine and 114 plants were survived that taken for flow cytometry analysis. The overall success rate of DH (chromosomal duplication) plant production were ranged from 1.0 to 5.33%. However, the chromosomal duplication phase was most difficult to obtain DHs plant due to higher mortality rates through anther culture in agreement with Grauda et al. (2010), Tadesse et al. (2013) and Jarzina et al. (2017).



G1 Mean	G1 CV	G1%	G2 Mean	G2 CV	G2%	S%	G2/G1	%Total
6910.26	3.79	95.66	13202.76	3.65	3.72	0.62	1.91	100.00

G1 Mean	G1 CV	G1%	G2 Mean	G2 CV	G2%	S%	G2/G1	%Total
12852.15	4.63	94.90	24481.41	6.02	3.34	1.76	1.91	100.00

Figure 4- Flow cytometry histograms for quantification of DNA content from bread wheat leaves. a) haploid, and b) doubled haploid plants

4. Conclusions

In conclusion, we found that the donor plant genotype is one of the most important factors affecting the success rate of DHs lines production during in vitro culture and haploid plant development in bread winter wheat. The in vitro culture condition and stage of anther were more suitable for development of full fertile DHs lines. However, F1 hybrids exhibited positive heterosis over their parent when they reciprocal crossed. The outcomes of the study produced a significant number of fertile DHs wheat lines that can be used in bread wheat breeding programmes. Additionally, improved protocol and operational simplicity for production of DHs lines reduced labor requirements and cost effective.

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