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Impacts of F_2 derived winter bread wheat progenies on callus production and regeneration frequencies through anther culture

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Anther culture is one of the most feasible techniques to obtain doubled haploid (DH) plants. In this study, anther culture responses of F_2 derived bread wheat hybrids of Yektay406 × Altay 2000, Demir2000 × Soyer02, Atlı2002 × Müfitbey, Müfitbey × Atlı2002, Müfitbey × Eser, Müfitbey × Bezostaya were assessed to clarify the genotype-based reactions under tissue culture conditions. Firstly, twenty five spikes which were grown in dry land, collected from F_2 hybrid plants. A total of 10.963 inoculated F_2 anthers formed to 188 callus in the primary hormone-free MN6 media. Some of the calli produced hybrid groups did not generate any haploid plantlet in regeneration media. Regeneration frequencies of Demir2000 × Soyer02 and Müfitbey × Atlı2002 hybrids were 30.76% and 63.63% respectively. F_2 anthers of Müfitbey × Atlı2002 produced 121 callus, 10 albino and 77 haploid plantlets. In contrary, Atlı2002 × Müfitbey produced only 26 callus, 3 albino and zero haploid plantlets. Other corresponding hybrids of Müfitbey × Eser and Müfitbey × Bezostaya generated 9 and 13 callus, while the least number of callus was detected in Yektay406 × Altay 2000. A correlation between callus number and haploid plantlet number per 100 anther revealed a positive relationship for each F_2 progeny ($r = 0.988$, $P < 0.01$). Our results significantly indicated that Müfitbey cultivar might be used an effective anther source for DH wheat production.

Key words: anther culture, bread wheat, F_2 plants, haploidy, regeneration

Rapid narrowing in the pool of agricultural areas is one of the results of environmental effects such as salinity, desertification, drought and hard epidemic diseases. Also, soil productivity is reducing day by day in the last a few decades. To overcome these problems, development of stress resistant genotypes with the high grain quality has become an essential point for sustainable agriculture (Glantz et al., 2009). In addition to the conventional plant breeding, tissue culture and other biotechnology approaches have the potential to serve feasible solutions for improving crops. Beyond the positive impacts on generating homozygous lines, DH production helps to save time and give exact traits of interest only within the two generations. Especially, haploidy production via anther culture technique has been demonstrated in many of the species, such as barley (Savaskan et al., 1999;

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Lazaridou et al., 2005; Belinskaya EV 2010), wheat (Craig 1974; Schaeffer et al., 1979; Kim and Baenziger 2005), rice (Niizeki and Oono 1968; Enriquez et al., 2000), maize (Tsay et al., 1986; Pescitelli et al., 1990; Zhongchen et al., 2000) and cotton (Ozyigit et al., 2007) for generating haploid plants.

To produce homozygous plants, all of the developed tissue culture methods can be applied to the whole plant, cells, tissues and various organs for obtaining exact results in a short time (Ahmet and Adak 2007). Also, it is approved that callus induction and plant regeneration during wheat tissue culture is depended on the source of explant, type of nutrient medium and genotype (Ozgen et al., 1998). Moreover, the response of anthers to the culture media is sometimes genotype-specific (Sears and Deckard 1982; Kondiã and Šesek 1999; De Buyser et al., 1992; Murigneux et al., 1994). In the last decade, some groups of wheat parental lines have been investigated to reveal the anther culture responses and plant regeneration (Balla et al., 2012; Dogramaci et al., 2001; Duran and Savaskan 2011; Hassawi 2004; Kım and Baenzıger PS 2005; Lantos et al., 2006; Zamani et al., 2003). Additionally, numerous endogenous and exogenous factors have been defined for the response of anthers. In detail, genotype, physiological state and donor plant growth condition, stage of pollen development, pre-treatment of flower buds or anthers, culture medium and conditions, together with their interactions, are accepted as all factors that greatly affect the response of anthers to *in vitro* culture (Wang et al., 2000, Germana 2011). Also, cytoplasmic and nuclear genes modified by the environment are reported as important factors during anther culture (Heberle-Bors 1985; Ekiz and Konzak 1991). Previously, Orlov et al., (1993) demonstrated the variation in performance of genotypes regarding to their ability to produce regenerated callus and/or embryos and plantlet.

In the current study, different F_2 hybrids originated from Turkish winter bread wheat cultivars were tested for their applicability to anther culture and their responses during callus production and haploid plant regeneration were also investigated.

Material and Methods

Plant material

As parent plant material, six registered Turkish winter bread wheat cultivars (Table 1) were used and they were crossed to obtain F_1 plant population in the field conditions. Subsequently, F_1 seeds of Yektay406 × Altay 2000, Demir2000 × Soyer02, Atlı2002 × Müfitbey, Müfitbey × Atlı2002, Müfitbey × Eser, Müfitbey × Bezostaya crosses were planted to develop F_2 plants between the seasons of 2008-2009 years in the field. All plant material including parents and their hybrids were originally grown in the field trials of Transitional Zone Agricultural Research Institute, Eskisehir, Turkey. Twenty five spikes were randomly collected prior to anthesis stage from F_2 derived plants which were cultivated in dry land conditions and they were taken into to anther culture immediately.

Anther culture

Before picking up anthers, each spike was gently cut its edge of spikelets (Figure 1a) and they were surface sterilized in 20% sodium hypochloride solution for 10 minutes by continuous shaking to remove to surface contaminants (fungi, bacteria) and then all spikelets were rinsed in sterilized distilled water for five times. All experiments were performed in aseptical conditions. To prevent anther injury, each spikelet cut one third rate (Figure 1b), after then, anthers were transferred to the modified hormone free MN6 nutrient media (Figure 1c) according to the Ouyang (1987). There was also two modified forms of 190-2 media used for rooting and plant regeneration (Zlniang and Jia 1983). For cold pretreatment, anthers in the MN6 media were incubated at +4^{o}C overnight and the latter day, for induction of mitotic division of microspores in anthers, plates were incubated for 30-40 days at +30^{o}C in

dark (Figure 1d-f). A total of 10.963 anthers cultured from 6 different F_2 hybrids. For obtaining the vegetative plant parts, newly formed structures (callus, embryoids) on anthers (Figure 1g, h) were incubated at +25°C, 16h day/8h dark period in plant growth chambers under the light intensity of 50 µmol s^{-1} m^{-1} in the 190-2 modified regeneration media.

A total number of 703 haploid emryoids placed in 403 petri dishes were screened according to their growth potentials. Observations were made according to both callus induction and haploid plantlet production (Figure 1i) after 30 days. Regeneration frequencies were calculated by proportion of the number of regenerated callus to the number of callus forming embryoid according to the Ahmet and Adak (2007). Standart excell program were used for correlation analysis between callus and anther numbers.

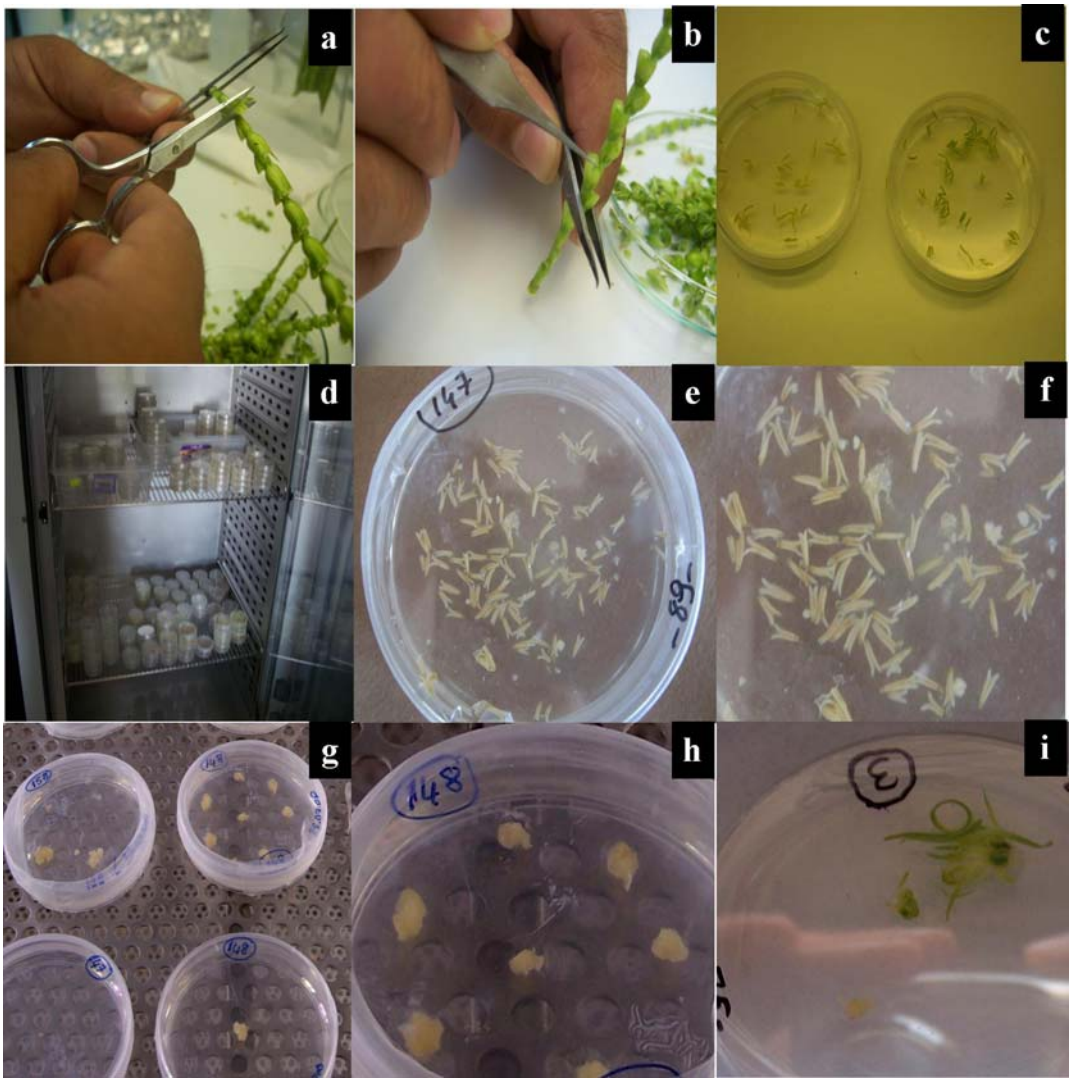

Figure 1. Flow-chart of wheat anther culture, a- spikes are gently cut their edges, b- dissection of anthers from each spikelet, c- transferring anthers two modified nutrient media (MN6 and 190-2), d- Incubation phase in the growth chamber, e, f- anthers ıncubated in the petridishes, g, h- newly formed callus tissue, i- regenerated green plantlet.

Results and Discussion

To study the responses of anthers, six F_2 derived bread wheat hybrids were analyzed during anther culture and comparisons were done according to the results of callus production rates, haploidy number and regenerated plantlets. In the second round of recombination, genetic segregation in F_2 progenies are more evident than that of F_1 population. Hence, genetic variation reduction during doubled haploid plant production might be partially eliminated. So, we have tested the regeneration ability of Turkish bread wheat hybrids by using their F_2 progenies via anther culture. However, *Datura stramonium* was reported as first naturally observed haploid plant (Blakeslee et al., 1922), one of the first studies for wheat anther culture was performed in the early 1970s (Quyeng 1973). Studies mostly continued with bread wheat, but durum wheat is also used to produce doubled haploid plants (Cistue et al., 2006 and 2009). Until now, haploid plant regeneration was studied in an extended range of plant species from Solanaceae to Gramineae (Forster and Thomas, 2005).

Our findings showed that anthers of different F_2 spikes originated from different hybrids (Table 1) generated different regeneration frequencies and callus production (Table 3). Changing environmental conditions are presented to limit callus induction and plant regeneration. There are different factors reported as affecting plant regeneration in tissue culture. Pelletier and Ilami (1972) has been reported that diffusion of nutrients through the anther walls often considered to be one of the reasons affecting microspore embryogenesis. Also, the use of one parent as male or female could lead to change the production of green plants from the F_1 hybrids (Yıldırım et al., 2008). In the current work, corresponding F_2 hybrids of Müfitbey × Atlı2002 produced 77 haploid plantlets from callus, while there was no regenarated haploid plantlets observed for the Atlı2002 ×Müfitbey. According to Zhou and Konzak (1991), cytoplasmic effects have been caused significant response on embryo/callus and plantlet production, although nuclear genes were found to be predominant in these studies. Obviously, our data might be proved with the maternal effects which were occurred after application of a reciprocal crosses between Müfitbey and Atlı2002. Moreover, choice of parental line as female or male donor is an important step during anther culture studies. Previously, F_2 and backcrosses found that mainly additive effects have been influenced callus induction (Deaton et al., 1987).

In the present study, development of green plantlets was observed after 30 days of anther culture. While regeneration frequencies of haploid plantlets were 30.76% for Demir2000 × Soyer02, this proportion was found as 63.63% for Müfitbey × Atlı2002 progeny (Table 3), other hybrids did not produced any haploid plantlet from callus. Due to this reason, regenaration frequencies could not been calculated for the remaining genotypes. Callus was obtained from anthers of all crosses at different amounts. According to the counts of callus number, Müfitbey × Atlı2002 crosses produced the vast majority of callus (121), while Yektay406 × Altay2000 crosses had the least callus number (6). In addition, the average response per spike was 1.25 callus (Table 3). According to calculations per 100 anther, the highest number of callus and haploid plantlet were observed in Müfitbey × Atlı2002, 6.87 and 4.37 respectively. Tuvesson et al., (2000) observed 3.3 haploid plantlets for every 100 anther while there was no green haploid plantlet found after anther culture of ten durum wheat cultivars (Dogramaci-Altuntepe et al., 2001). Callus regeneration rates are found significantly diffent between bread and durum wheat genotypes due to the genome differences and also reclacitrant behaviour of durum wheat (Kasha et al., 1990; Cistue et al., 2009). In the recent work, a correlation between callus number and haploid plantlet number per 100 anther revealed a positive relationship for each F_2

progeny ($r = 0.988$, $P < 0.01$). From a total of 188 callus, only 13 albino plants generated in the groups of two F_2 bread wheat progenies (Table 3). In durum wheat, Aiti et al., (1999) reported only three albino plants from a total of 87 callus and embryoids.

Table 1. Quality traits of donor bread wheat genotypes

Genotypes	Quality traits
Demir2000	High quality and resistant to yellow rust
Altay2000	Resistant to yellow rust, root node and virus
Soyer02	Modaretly resistant to yellow and stem rust
Bezostaya	High quality
Yektay406	Sensitive to different rust disease
Müfitbey	High quality and resistant to yellow and brown rust

Table 2. List of macro and micro constituents for MN6 and 190-2 culture media

Macroelement stock solution (MN6) 1000 ml	Amount	Macroelement stock solution (190-2) 1000 ml	Amount
KNO_3	14.15 gr	KNO_3	10 gr
$CaCl_2$ x2H_2O	0.83 gr	$Ca(NO_3)$ x 4H_2O	1 gr
$MgSO_4$ x 7 H_2O	0.93 gr	$MgSO_4$ x 7H_2O	2 gr
KH_2PO_4	2.00 gr	KH_2PO_4	3 gr
(NH_4) 2SO_4	2.32 gr	$(NH_4)_2SO_4$	2 gr
		KCI	0.4 gr
Microelement stock solution (MN6) 1000 ml	**Amount**	**Microelement stock solution (190-2) 1000 ml**	**Amount**
$ZnSO_4$ X 7 H_2O	0.05 gr	$ZnSO_4$ x 7H_2O	0.06 gr
$MnSO_4$ X 4H_2O	0.05 gr	$MnSO_4$ x 4H_2O	0.16 gr
H_3BO_3	0.05 gr	H_3BO_3	0.06 gr
KI	0.004 gr	KI	0.01 gr
Vitamin stock solution (MN6) 100 ml	**Amount**	**Vitamin stock solution (190-2) 100 ml**	**Amount**
Glycine	0.01 gr	Glycine	0.02 gr
Thymine HCI	0.025 gr	Thyamine HCI	0.01 gr
Pyridoxin HCI	0.005 gr	Pyridoxin HCI	0.005 gr
Nicotinic acid	0.005 gr	Nicotinic acid	0.005 gr
H- Biotin	0.0025 gr	Inositol	1 gr
Ascorbic acid	0.005 gr		
Ca-pantotenate	0025 gr		
Inositol	3 gr		

Table 3. Regeneration of green plantlets and callus production rates for F_2 derived bread wheat plants

F_2 progeny	Number of collected spikes	Number of inoculated anthers	Number of produced callus	Number of albino plants	Number of haploid plantlets	Regeneration Frequency of callus (%)
Yektay406 x Altay 2000	25	1755	6	-	-	-
Demir2000 x Soyer02	25	1707	13	-	4	30.76
Atlı2002 x Müfitbey	25	1888	26	3	-	-
Müfitbey x Atlı2002	25	1760	121	10	77	63.63
Müfitbey x Eser	25	1997	9	-	-	-
Müfitbey x Bezostaya	25	1856	13	-	-	-

Conclusions

Anther response, callus production, plantlet regeneration and haploid plantlet production are very important steps to be thought before doubled haploid (DH) wheat development. Studies which were performed to identify the most appropriate DH plant, have numerous benefits for sustainable agriculture. However there is no general protocol or well established culture conditions for routine useage in wheat, it is possible to combine some promising parameters to select the best practical way during anther culture. Evaluation of different wheat genotypes according to their responses during haploid plant production might be accelarete the selection process. So, there is an urgent need to identify the wheat lines, cultivars and landraces according to their responses to anther culture. By the frame of this work, F_2 progenies of six registered Turkish bread wheat cultivars were categorized according to their callus production, plant regeneration and haploid plantlet production rates. In the present work, Müfitbey defined as a suitable female donor for anther culture studies and $F_{1:2}$ derived F_2 spikes of it might be used as an applicable anther source for haploid wheat production during wheat improvement programs.

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