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## **Response of Some Egyptian and Introduced Wheat Hybrids to Androgenic Process**

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### **ABSTRACT**

Wheat anther culture produces homozygous doubled haploid plants, in a very short time span as comparing with traditional breeding methods. It is affected with several different factors, such as suitable spike stage as a donor for anthers, anthers cold pretreatment duration, rate of embryogenic callus formation and the time of anthers transferring from callus induction to regeneration medium. The effects of these factors on androgenic process were studied in this work the response of five F<sub>1</sub> wheat hybrids, of ten varieties, differing in their salt tolerance, was investigated in anther culture system for haploid plantlets regeneration. Anthers were plated on P4S medium for callus induction, anthers were taken from different spikes ages (as in a distance between the top of the spike and flag leaf), different duration of cold pretreatment and different time of transferring anthers from callus induction to regeneration medium (190-2) were studied. Significant differences between genotypes on androgenic process were detected. Genotype influence was significant on the suitable spike length which has high percentage of proper stage of mid-uninucleate stage, consequently on both of callus induction and plant regeneration (green, albino and total plantlets). On the other hand, there was no significant influence in the embryogenic callus formation per 100 anthers between the hybrids under same duration (14, 28 and 56 days). The suitable duration for staying on induction callus medium was 28 days as the green regeneration plants increased. This is considered as accurate parameter for evaluation efficiency of androgenesis for anther culture.

**Key words:** Wheat, F<sub>1</sub> hybrids, anther culture, plant regeneration, spike length, callus induction, haploid production

### **INTRODUCTION**

Anther culture is a competitive technique for traditional methods that plant breeders use. This technique can produce haploids to improve breeding in both mono and dicotyledonous plants to get new lines characterizing with both high yield and disease resistance in a short time (Ramakrishnan *et al.*, 2005). Production of haploid plants with androgenesis in cereals has been applied such as: wheat (Simmonds *et al.*, 1993), Barely (Luckett and Smithard, 1992), Triticale (Charment and Bernard, 1984), Rice ( Miah *et al.*, 1985), Maize (Petolino and Thompson, 1987) etc. Doubled haploid production technology, inducing homozygosity, shortens breeding

program and permitting variation between genotype within any generation which attracts breeders attention (Maria *et al.*, 2006), reducing cost of cultivar development (Hu and Yang, 1986; Hu, 1997; Liu *et al.*, 2002). In wheat breeding programs obtaining haploid plants resultant from anthers of F<sub>1</sub> hybrids through anther culture that accelerates new cultivars development when reduces many years and permitting easier and more efficient selection process (Camargo *et al.*, 1999; Ramos *et al.*, 2000).

Before eighteenth century, developing crops by doubled haploid plants was limited. Androgenesis process can be explained as formation of pseudo embryos (embryoids) which can germinate into plants giving genetically true doubled haploid (DH) plants. This is an important and attractive goal for plant breeders, geneticists and biotechnology researches. DH progeny enables getting inherited traits of different gene combinations (Konzak *et al.*, 1987). Anther culture technique gives a very important advantage of obtaining more than thousand haploid plants/anther culture (Devaux, 1988). In comparing to other methods, all of it are restricted to only one plant/floret and consequently to a low number of responsive genotypes while others remain without changing. Therefore, the urgent need for methods help to increase androgenesis process of microspores in a great number of genotypes (Hu *et al.*, 1995).

In wheat, may be through crosses androgenic response can be passed to progeny in great numbers as it is inherited (Henry and Buyser, 1981; Lazar *et al.*, 1984; Moieni and Sarrafi, 1995). Thus, formation of haploids and their regeneration is affected by three related traits; embryo formation rate, their regeneration capability and ratio of green to albino plants (Henry and Buyser, 1985; Ghaemi *et al.*, 1995; Moieni and Sarrafi, 1995). Regeneration frequency also is affected by the time of transferring anthers from induction medium to regeneration conditions (Zheng and Konzak, 1999; Redha *et al.*, 2000).

Developing of microspore-anther culture methods was getting when stress treatment was applied basing on the transferring from preprogrammed gametophytic to sporophytic pathway for getting androgenesis (Touraev *et al.*, 1996; Touraev *et al.*, 1997; Hu and Kasha, 1999; Zhou and Konzak, 1997; Simonson *et al.*, 1997; Reynolds, 1997; Zheng and Konzak, 1999; Ohnoutkoua *et al.*, 2000).

In view of the above, present work was planned to study the androgenetic process of anther cultures for F<sub>1</sub> hybrids of several wheat parents differing in salt tolerance level under different parameters i.e., (1) Proper stage of donor spikes development (in centimeters) as a morphological character (2) Duration of anthers cold pretreatment ( days) (3) Induction of embryogenic callus and embryos formation and (4) The time of transferring anthers from callus induction medium to regeneration medium.

## **MATERIALS AND METHODS**

**Plant materials and salt screening:** Salt screening and field crossing were done in 2009 and 2010, laboratory work was extending till Dec. 2011.

Ten varieties of bread wheat (*Triticum aestivum* L.), six were local (Sids 4, Sakha 93, Gemmeiza 9, Sids 1, Sakha 8 and Giza 162) and four introduced (Golan 2, Cham 6, Bow and Mexipak 69), were chosen to test the transactions affected by levels of salinity. The experiment was conducted in Petri dishes containing quarter strength of Hoagland solution (Hoagland and Arnon, 1950) under five salinity levels: 0, 8,000, 10,000, 12,000 and 14,000 ppm of 2:1 (NaCl:KCl) as a source of salinity stress. Each treatment was performed by 5 petri dishes (15 cm in diameter) in 3 replicates, each one contained 100 seeds. The germination percentage was calculated after 2 weeks. Seeds were scored as germinated when the primary roots were greater than 3 mm and the shoots were longer than the seed itself. Seeds contaminated by fungi during the test were ignored

Table 1: Crosses between selected parents for obtaining F<sub>1</sub> hybrids as donor plants for anthers

Cross number	Cross name	Parents
1	A	Gemmeiza 9 * Sakha 8
2	B	Gemmeiza 9* Cham 6
3	C	Sakha 8 * Cham 6
4	H1	Giza 162 * Bow
5	H5	Bow * Mexipak 69

(Ye *et al.*, 1987). On this base, six parents had been chosen, five of them were most resistant to salinity, while the sixth variety (Gemmeiza 9 is sensitive to salinity but high yielder) to set some crosses as shown in Table 1. The materials of this study were kindly provided by Dr. Samy. A.A. Attia who performed the crosses.

Laboratory work of anther culture, haploid production and regeneration was performed in Genetics and Cytology Department, Genetic Engineering and Biotechnology Division, National Research Center, Egypt.

**Determining suitable spike age for anther culture:** Anthers of F<sub>1</sub> hybrid plants from primary and three secondary tillers were collected when microspores from the anthers in the central region of the spikes were at the mid to late uninucleate stage before the first pollen mitosis (Barnabas *et al.*, 1991; Navarro-Alvarez *et al.*, 1994; Redha *et al.*, 1998). The developmental stage of microspores was checked by acetocarmine staining of anthers from the central region of the spike and subsequent light microscope observation. The sampled spikes at different ages as a distance between the top of the spike and flag leaf were 0 = 7.5, 10, 12.5 or 15 cm.

**Cold pre treatment:** Suitable spikes were placed in flasks containing tap water and stored in dark at 4°C for 2-7 or 14 days as an anther stress treatment.

**Induction of embryogenic callus and embryos formation:** Spikes were surface sterilized in 80% ethanol for 2 min intervals and the enclosed anthers were excised with fine forceps (Zheng and Konzak, 1999). Anthers were then placed aseptically in 50 mL glass jars containing 15 mL of P4S medium (Ouang *et al.*, 1983) to induce embryogenic callus formation at 26±2°C in dark. The largest embryos (>2 mm) produced were then counted and transferred into regeneration medium 190.2 (Zhuang and Jia, 1983), green and albino plantlets were recorded.

**The time of transferring anthers from callus induction medium to regeneration medium:** The following parameters were recorded to show the effects of the time of transferring anthers from callus induction medium to regeneration medium: embryogenic callus formation plant/100 anthers, regenerated green plants/100 anthers, regenerated albino plants/100 anthers and total regenerated plants/100 anthers after 14, 28 or 56 days on induction medium (P4S) to regeneration medium (190-2). Cytological examination for haploids was done.

## RESULTS AND DISCUSSION

**Salt screening:** As shown in Fig. 1, the ten different wheat varieties had emerged the effect of different levels of salinity on their germination rate were among the terms of higher and lower tolerance salinity. It is noticed that Mexipak 69, Bow Giza 162, Sakha 8 and Cham 6 gave the highest percentage of germination under all salinity levels.

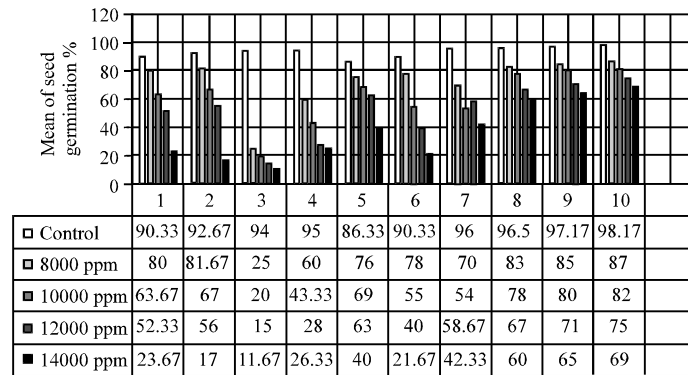


Fig. 1: Seed germination percentages of the ten studied cultivars under five salinity levels.  
 1: Sids 4, 2: Sakha 93, 3: Gemmeiza 9, 4: Sids 1, 5: Sakha 8, 6: Golan 2, 7: Cham 6,  
 8: Giza 162, 9: Bow, 10: Mexipak 69

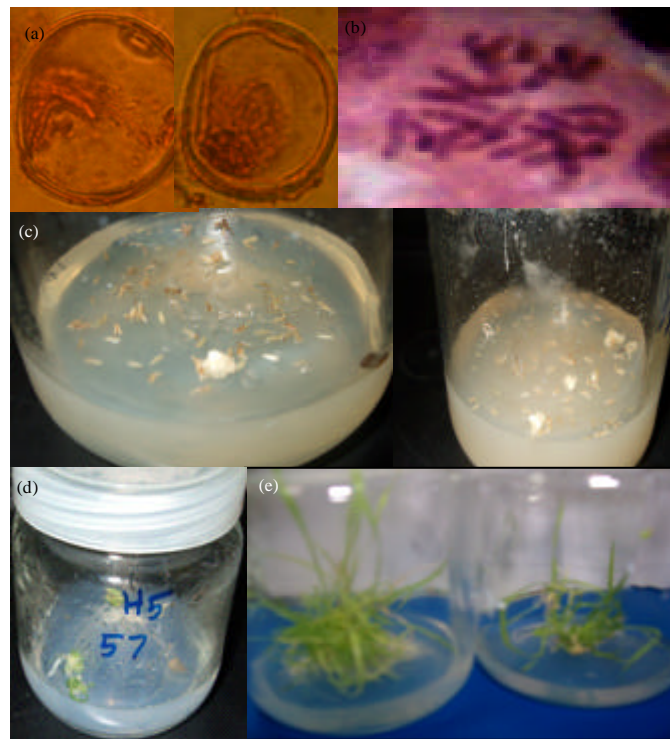


Fig. 2(a-e): Developmental stages of anther culture, (a) Suitable stage of anther culture (mid-uninucleate stage), (b) Chromosome number of regenerated haploid plantlets  $1n = 3x = 21$ , (c) Callus formation from anther culture, (d) Initiation of shoot tip, (e) Regenerated plantlets from embryogenic callus

**Suitable spike age for anther culture:** According to Ding-Gang and Jun-Wen (1984) and Lantos *et al.* (2006) the most critical steps of wheat microspore anther cultures is the choice of ideal microspore development stage (Fig. 2a). It is the first important step of androgenesis induction.

Table 2: Percentage of microspores/spike at mid-uninucleate stage induced at different spike stages of the five wheat hybrids (crosses)

Genotype	Spike length (cm)					Mean
	0	7.5	10	12.5	15	
A	10.8	70.0	63.0	50.0	37.0	46.16
B	9.7	77.0	59.0	45.6	33.9	45.08
C	8.0	39.0	41.0	48.1	29.0	33.02
H1	4.0	21.8	45.2	32.6	25.7	25.86
H5	11.4	39.8	46.2	62.0	30.2	37.88
Mean	8.78	51.02	52.88	50.18	31.16	

Table 3: Percentage of wheat embryogenic callus/100 anthers of the five crosses in response to cold pretreatment durations

Genotype cross	Durations of cold treatment ( days)							Mean
	2	3	4	5	6	7	14	
A	15.11	18.3	21.13	29.60	38.0	47.0	49.7	31.26
B	11.6	15.0	19.30	26.12	29.9	36.4	40.2	25.502
C	10.0	17.0	20.10	23.20	25.0	30.9	28.4	22.08
H1	12.3	12.7	13.50	14.60	19.7	32.8	33.3	19.89
H5	25.6	28.6	31.10	36.30	43.2	58.4	56.6	39.97
Mean	14.92	18.32	21.05	25.96	31.16	41.0	41.64	

Data in Table 2 showed the percentage of microspores/spike at mid-uninucleate stage in the central part of spikes of the 5 used hybrids expressed in centimeter as distance between the top of the spike and the flag leaf auricles. Table 2 revealed that spikes at <10 cm stage for the five used F<sub>1</sub> hybrids possessed the highest mean frequency of microspore cells in the mid-uninucleate stage (52.88) as compared to the other four spike ages, i.e., 0, 7.5, 12.5 or 15 cm where, their mean values reached 8.78, 51.02, 50.18 and 31.16, respectively. It seems that genotype effect has significant influence on the differences among the mean percentage of microspores/spike in the proper stage of the 5 genotypes. These result is in agreement with Ding-Gang and Jun-Wen (1984), Maluszynski *et al.* (2001) and Lantos *et al.* (2006), who stated that percentage of microspores in mid-uninucleate stage in a certain age of spike morphology depend on the genotype. So, correlation between developmental stage of microspores and morphological characters of spike such as distance between the top of the spike (last floret) and flag leaf auricles is important to define the suitable spike age for anther culture. These results also are supported by findings of Mehmet *et al.* (2008), who concluded that the spike length is significantly correlated with callus number and green plantlet.

**Effect of cold pretreatment of spikes on callus induction:** Low temperature ~4°C for several days, prior to normal culture procedure, was used under speculation that it might has a dual function: interrupting normal gametophytic development and the nursing effects of anther tissue on microspores (Fazar *et al.*, 1985; Armstrong *et al.*, 1987; Henry and Buysler, 1981). Table 3 showed the response of the 5 used crosses to cold treatment durations as percentage of callus produced from 100 anthers.

The results in Table 3 indicated that cold treatment of spikes for duration shorter than 7 days could not have enough impact on starving microspores to switch their normal pathway to form embryos. On the contrary, starving microspores for 7 or 14 days was highly recommended for the used crosses. Table 3 presented that the five crosses significantly varied as the percentage of cross

Table 4: Number and percentage of callus and embryoids from anthers of the five crosses on inductions (P4S) medium

Crosses	Parameters			
	No. of cultured anthers	No. of callus	Callus (%)	Embryoids
A	3000	1065	55.5	2130
B	3000	924	30.8	2005
C	3000	84	2.8	865
H1	3000	219	7.3	2343
H5	3000	1098	36.6	2382

H5 reached 58.4 and 56.6% under cold duration of 7 and 14 days, respectively. It was followed by cross A which reached 47 and 49.7%, respectively. While, cross C showed the lowest response, its percentages were 30.9 and 28.4, respectively, also the two crosses B and H<sub>1</sub> produced intermediate levels of anther response. The diverse of the five genotypes responses for cold pretreatment were attributable to varying genotypes, pretreatment durations and the genotype x pretreatment durations. Present results are in agreement with the findings of Lazar *et al.* (1984), Armstrong *et al.* (1987) and Henry and Buyser (1981). In contrary, Khiabani *et al.* (2008) included that cold pretreatment was found ineffective on androgenic ability of genotypes where they showed low response to cold treatments.

**Induction of embryogenic callus and embryos formation:** Embryogenic callus formation (Fig. 2c) of the five crosses on induction medium (P4S), their percentages and No. of embryos are in Table 4 which showed that the percentages ranged from, the lowest percentage (2.8) for cross C and the highest (55.5) for the cross A. While crosses H<sub>5</sub>, B and H<sub>1</sub> ranged in between the previous crosses where, their callus formation percentages were 36.6, 30.8 and 7.3%, respectively. These differences among crosses could be attributed to the effects of genetic make-up and or the culture composition, where genotype affected anther culture response significantly (Lu *et al.*, 1991; Konieczny *et al.*, 2003; Bagheri and Jelodar, 2008; Zheng *et al.*, 2001).

Others suggest that these differences in androgenic response could be referred to the presence of several genes located on the different wheat genome (Szakacs *et al.*, 1988; Sibikeeva and Sibikeev, 1996). Others referred the difference in androgenic responses, of used genotypes, to their differences in endogenous hormones levels, which in turn in different requirements for the culture medium.

**Effect of transferring wheat anthers (from induction to regeneration medium) on the androgenic response, of the five studied crosses:** Data in Table 5, showed the percentages of embryogenic callus, green regenerated plants (Fig. 2d-e), albino regenerated plants and total number of regenerated plants/100 anther after 14, 28 or 56 days on induction medium (P4S) to regeneration medium (190-2). From Table 5, we notice that, there were no significant differences in the embryogenic callus formation per 100 anthers between the five studied crosses under the same duration (14, 28 or 56 days). On the other hand, we find that 28 days duration is the proper time of transferring for obtaining higher percentages of green regenerated plants as well as for albino and total regenerated plants (haploid 1n = 3x = 21, Fig. 2b). Data showed great increment percentage of green regenerated plants at 28 days than 14 or 56 days, where for cross (a) the

Table 5: Percentages of embryogenic callus, green, albino and total regenerated plantlets per 100 anthers

Time of transfer (day)	A				B				C				H <sub>1</sub>				H <sub>5</sub>			
	Em./ An.	GRP/ An.	ARP/ An.	TRP/ An.	Em./ An.	GRP/ An.	ARP/ An.	TRP/ An.	Em./ An.	GRP/ An.	ARP/ An.	TRP/ An.	Em./ An.	GRP/ An.	ARP/ An.	TRP/ An.	Em./ An.	GRP/ An.	ARP/ An.	TRP/ An.
14	10.63	5.19	4.34	9.53	9.73	4.29	3.44	7.73	6.43	5.99	0.14	6.13	10.03	4.58	3.63	8.21	10.62	5.17	4.45	9.62
28	43.61	14.51	10.54	25.05	42.71	11.61	9.64	21.25	39.40	10.30	6.33	16.63	41.90	11.91	9.62	21.53	43.70	14.62	9.97	24.59
56	51.90	4.97	10.17	15.14	51.00	4.07	9.27	13.34	41.70	4.76	5.96	10.72	51.30	4.37	9.53	13.9	51.70	4.76	9.16	13.92

Em.: Embryos, An: Anthers, GRP: Green regenerated plants, ARP: Albino regenerated plants, TRP: Total regenerated plants

increment reached 2.8 and 2.9 fold, respectively; while for cross (b) it reached 2.7 and 2.85 fold; for cross (c) it was 1.71 and 2.16 fold, respectively; for cross (H<sub>1</sub>) it increased 2.6 and 2.72 fold; while for cross (H<sub>5</sub>) it was the highest increasing 2.82 and 3.07 fold.

In terms of regeneration, 28 days duration was the best as the percentage of green regenerated plants is considered as accurate parameter for efficiency evaluation of anther culture.

On the other hand, in comparing with 14 days, the percentages of the albino regenerated plants increased greater with 28 days, while the greatest increment was with 56 days for all the studied crosses which reflects as a problem for the application of anther culture technique in wheat breeding programs as Saidi *et al.* (1997) stated that, production of albino plants was affected by genotype and medium composition. They added that albinism might occur by mutation in chloroplast DNA. While Caredda *et al.* (1999) referred albinism to the probability of the abnormalities of plastids features during embryogenesis process.

The early transfer of anthers (that formed callus) from the induction medium to regeneration medium caused a strong reduction in the production of embryogenic callus, while the overall regeneration of plants/100 anthers showed an improvement for delaying. These results are in agreement with previous findings on wheat (Henery and Buyser, 1985; Redha *et al.*, 2000). The reduction of embryogenic callus production with the early transfer, suggesting that, the earlier transfer interferes with the development of the embryos. However, it had a positive effect on the quality of the embryos and, as a result, the regeneration of the plants improved considerably. When the embryos are left for longer time (56 days) on induction medium, the quality of the embryos and their totipotency to regenerate may be lower (Redha *et al.*, 2000).

## CONCLUSION

Anthers of five F<sub>1</sub> wheat hybrids, their parents differ in salt tolerance showed a variation in the productive response of callus formation and regeneration capacity (green, albino and total plant numbers) under different studied factors. It was concluded that androgenic response is affected mainly by genotype which has significant influence on the differences among the mean percentage of microspores/spike in the anther proper stage of the 5 used genotypes. While, embryogenic callus formation was better in response to 7 days of anthers cold pretreatment durations. Also the proper time of transferring calluses from callus induction medium to regeneration medium affected androgenic process.

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