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# The Evaluation of Response to Isolated Microspores Culture in Some Iranian Hexaploid Wheat (*Triticum aestivum* L.) Cultivars

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**Abstract**

The most important method of haploid plant production is isolated microspores culture. To be successful in this method, genetic potential of cultivars is very important. In this research, ten Iranian hexaploid wheat cultivars (exclusive of MV17) on the basis of their characters such as heat, drought and salinity resistant have been selected and their responses to isolated microspore culture were evaluated. Meanwhile the effects of two pretreatments (cold and heat + chemical) and embryo induction media (NPB99 and CHB-2) on androgenic traits including embryogenesis, total plant regeneration and green plant regeneration were studied in selected cultivars. There were significant interactions between cultivars and pretreatments and also between cultivars and media in most cases. The interaction between cultivars Chamran and Navid with 21 days cold pretreatment produced the highest number of embryos and the interaction between cultivar Hamoon and 21 days cold and heat + chemical pretreatments produced the most green plant regeneration percentage. Also, the results showed that the interaction between cultivar Chamran and CHB-2 medium produced the highest number of embryos while the interaction between cultivar Hamoon and NPB99 medium had the highest green plant regeneration.

**Keywords:** *Triticum aestivum* L, isolated microspores culture, embryo induction medium, plant regeneration, pretreatment, embryogenesis

**Abbreviations:** *HSP*: Heat shock protein, *2-HNA*: 2-Hydroxynicotinic acid, *OVCM*: Ovary-conditioned medium, *PAA*: Phenylacetic acid

## 1. Introduction

Upon stress, microspores can be switched from their normal gametophytic pathway to a sporophytic one, a process called androgenesis (Reynolds, 1997). The production of haploid and doubled haploid plants via androgenesis improves selection efficiency; facilitates plant genome mapping and transgenic studies (Kasha et al., 1990; Hu, 1997). The production of haploid plants exhibits a beneficial method for isolating recessive mutants. Recessive characters were expressed in haploid plants obtained from diploid species since there is only one set of chromosomes (Cistue & Kasha, 2005). Also the major advantage of doubled haploids in plant breeding is the achievement of complete homozygosity in a single generation. The breeders can omit the numerous cycles of inbreeding required by current methods and reduce population size required for selection.

The first success in the regeneration of wheat plants through anther culture was pertained early 1970s (Ouyang et al., 1973; Picard & De Buyser, 1973). Afterwards, production of haploid plants in wheat via microspores culture has advanced (Hu et al., 1995; Hu & Kasha, 1997; Liu et al., 2002). Isolated microspores culture has several excellences over other systems of production of haploid plants. In this system, microspores can be isolated in large quantities with a potentiality for embryogenesis (Touraev et al., 2001). Also microspore culture presents a method for the analysis of basic mechanisms of androgenesis at the molecular and biochemical levels (Reynolds, 1997) and the microspores are proper targets for genetic transformation, too (Zheng et al., 2001).

Numerous factors affect the success in microspore culture and may limit the efficiency and excellence of the system. The major factors which affect the microspore culture include genotype, donor plant physiology, microspore development stage, pretreatment, conditions for embryo induction and plant regeneration (Hu et al., 1995; Hu & Kasha, 1997). Response to androgenesis was strongly affected by genotype of the donor plants. Some genotypes react better than others in response to environmental changes (Liu et al., 2001). In anther

culture of hexaploid wheat, embryo induction, total plant regeneration and green plant regeneration inherited independently (Agache et al., 1988). This fitness provides the basis for introducing these traits into genotypes with low response.

Appropriate stress treatment to induce the embryogenesis in microspores is a prerequisite for success. To induce androgenesis, different pretreatments have been applied including physical, physiological and chemical pretreatments of excised spikes, anthers or microspores (Zoriniants et al., 2005). In general, it seems pretreatment acts by applying a stress that causes a shifting from gametophytic pathway to saprophytic pathway.

Production of albino plants that is peculiar to cereals reduces the efficiency of microspores culture. Therefore it provides a proper environment which is necessary to development of embryogenic microspores and production of embryo. Favorable factors during this phase may include both optimized physical conditions and proper elements in the media (Mejza et al., 1993; Gustafson et al., 1995).

In the present research, ten Iranian hexaploid wheat cultivars (exclusive of MV17) have been selected on the basis of their characters. In the first experiment, the effects of cultivar and two pretreatments including cold and heat + chemical were investigated. In the second experiment, the effects of cultivar and two embryo induction media (NPM99 and CHB-2) were studied by using cold pretreatment. These pretreatments and induction media have been chosen because they are efficiently used in androgenesis.

## 2. Materials and Methods

### *2.1 Growth of Donor Plants*

Cultivars used in this study including Azadi, Alvand, Pishtaz, Chamran, Golestan, Moghn2, Navid, Hamoon and MV17 were cultivated in an experimental field and also agronomic cares including irrigation, weeding and fertilization were carried out (Figure 1a).

### *2.2 Collecting Tillers*

The morphological features of tillers containing microspores at the mid to late-uninucleate were established for each cultivar via microscopic examination of microspores with acetocarmine stain (Figure 1b). Tillers containing microspores at proper stages were cut at the second node from the top of the tillers and were placed in a container containing distilled water.

### *2.3 Pretreatment of Spikes*

Tillers were pretreated according to the type of experiment. In this research, two pretreatments including cold and heat + chemical were investigated. In the cold pretreatment, four tillers were placed in a humid paper towel and were covered by aluminum foil for preserving its humidity (Hu & Kasha, 1999). The basal ends of tillers were placed in the beaker containing 200 ml of distilled water and incubated at 4°C for 21 days. In heat + chemical pretreatment, according to method of Liu et al. (2002), the basal of four tillers were placed in a small beaker containing 50 ml of sterile inducer formulation (100 mg $L^{-1}$ 2-HNA, $10^{-6}$ M 2,4-D and $10^{-6}$ M BAP). The plastic bag was placed over the spikes and sealed around the beaker with parafilm tape. The beaker was placed in an incubator at 33°C (ranging between 48-58 h depending on different cultivars).

### *2.4 Microspore Isolation*

After the spikes were pretreated, they were disinfected through immersion in 1.5% (w/v) sodium hypochlorite for 15 min. Thereafter, three rinses were performed with sterile distilled water for 3 min. Microspores were isolated according to the method of Liu et al. (2002) with some modifications. The spikes were aseptically moved in the laminar air flow. Awns (if present) were removed. Spiklets were isolated from every four spikes and allowed to drop into the sterilized blender cup (2-speed Warring, Chiristion). Forty milliliters of 0.3 M autoclaved mannitol solution was added to the blender cup. The spikes were blended at slow speed for 15 second and blended slurry was filtered through a 100 μm sterilized stainless steel mesh and blender cup was rinsed with 10 ml of a 0.3 M autoclaved mannitol solution and this solution was filtered, too. The filtrate was collected with 5 ml sampler in two 50 ml sterilized tubes and centrifuged at 100×g at 4°C for 3 min. Supernatant was discarded and pellets were resuspended in 2 ml of 0.3 M autoclaved mannitol solution. The suspension pellets were layered over 5 ml of a 0.58 M sterilized maltose solution and centrifuged as above. Three milliliters of upper band was collected and dropped into 10 ml of a 0.3 M autoclaved mannitol solution in a 15 ml tube and centrifuged as above. The supernatant was discarded and the previous step was repeated with 10 ml of culture medium (according to experiments, CHB-2 or NPB99). The supernatant was discarded and pellets were resuspended in 2 ml of medium and microspores density was determined by a hemocytometer. Medium was added in microspores suspension to obtain a proper density (1×104 microspores per ml).

*2.5 Culture of Isolated Microspores*

Isolated microspores were cultured in the liquid NPB99 (Liu et al., 2002) and CHB-2 (Chu et al., 1990) media. Thereafter, 5 ml of these media were aliquoted per 55 by 10 mm plastic Petri dish. Immature ovaries were added to each Petri dish at the density of two per milliliter. The immature ovaries were cut from disinfected spikes of the same cultivar. Petri dishes were sealed with parafilm and incubated in the dark at 27°C for 30-40 days. Afterwards, the number of embryos per spike was counted (Figure 1c) and embryos (2 mm in diameter or larger) were transferred to the solid 190-2 medium (Zhuang & Xu, 1983) (50 embryos in each microspores culture) and incubated under a 16/8-h (day/night) photoperiod at 25°C and 300-400 Lux light intensity. After approximately 2 weeks, the numbers of total and green regenerated plants were determined (Figure 1d).

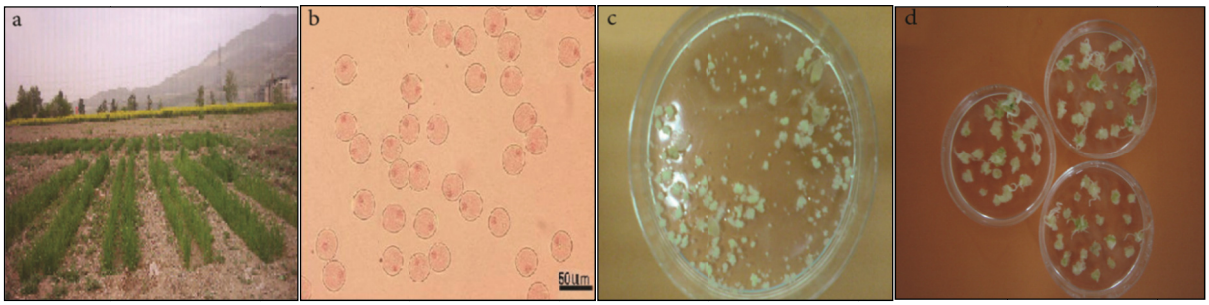


Figure 1. The process microspore culture. (a) Donor plants in an experimental field (b) Microspores in mid to late-uninucleate stages. (c) Obtained embryos after 28 days (d) Green and albino regenerated plants

*2.6 Data Analysis*

Experiments were analyzed as a factorial experiment based on completely randomized design (CRD). The first experiment was analyzed with two factors (cultivar and pretreatment) and three replications. The second experiment was also analyzed with two factors (cultivar and embryo induction medium) and three replications. In the both experiments, each microspores culture was considered as one replication. In each experiment, percentage of total and green plants regeneration were determined and analyzed with three replications (each replication was consisted of 50 embryos). Plant regeneration was not analyzed in the cultivars with low embryogenesis (<150 embryos in all three replications).

**3. Results**

*3.1 Experiment 1*

Analysis of variance showed a significant difference between cultivars and interaction between cultivars and pretreatments for embryo yield per spike. Embryo yield differed according to cultivars and pretreatments (Figure 2). A large number of embryos per spike were obtained from cultivars Chamran and Navid using 21 days cold pretreatment (197.5 and 192.16 embryos per spike, respectively) and the least embryogenesis was obtained from cultivar Golestan using the same pretreatment (2.08 embryos per spike). The embryo yields of cultivars Chamran, Navid, Pishtaz and Shole using the cold pretreatment were more than the heat + chemical pretreatment. In contrast, embryo yields of cultivars Moghan2, Alvand, MV17 and Golestan using the heat + chemical pretreatment were more than cold pretreatment. Effects of two above mentioned pretreatments in two cultivars Hamoon and Azadi were not significant.

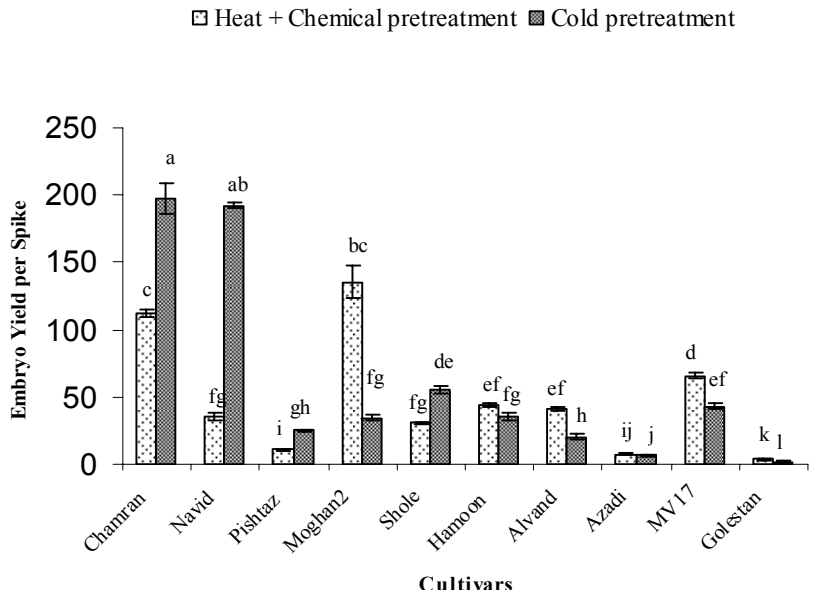


Figure 2. Comparison of means for interactions between cultivars and pretreatments for embryo yield per spike (the same letters are not significant different on the basis of 5% LSD analysis)

Analysis of variance for plant regeneration trait showed significant differences between cultivars and also between pretreatments. Percentage of total plant regeneration differed according to cultivars (Figure 3). Cultivars Hamoon, Chamran and MV17 produced the highest total plant regeneration, respectively while cultivars Moghan2 and Alvand had the least total plant regeneration. Plant regeneration in heat + chemical pretreatment was more than that of cold pretreatment (Figure 4).

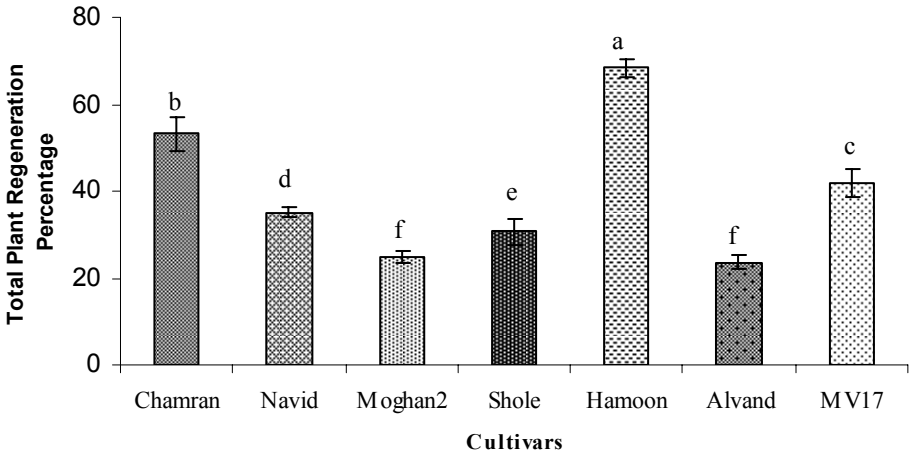


Figure 3. Comparison of means of cultivars for total plant regeneration percentage (the same letters are not significant different on the basis of 5% LSD analysis)

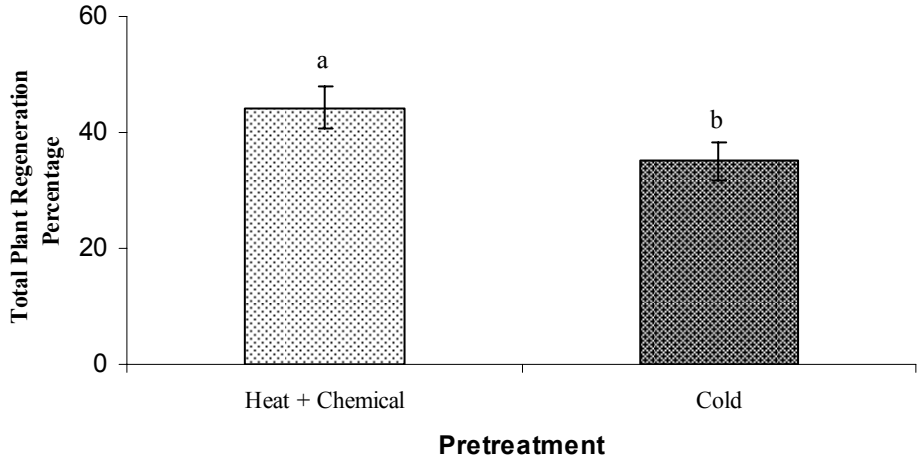


Figure 4. Effect of pretreatments on total plant regeneration percentage

Based on the analysis of variance for green plant regeneration, the simple effects of cultivar, pretreatment and also their interaction were significant. The interaction between cultivar Hamoon and heat + chemical pretreatment produced the highest percentage of green plant regeneration while interactions between cultivars Shole and MV17 and cold pretreatment together with interactions between cultivars Moghan2 and Alvand with the both pretreatments showed a low green plant regeneration (Figure 5). The green plant regeneration in Hamoon, Chamran, MV17, Navid and Shole cultivars in the heat + chemical pretreatment was more than that of cold pretreatment. Overall, the number of green plants produced by cultivar Hamoon using the two studied pretreatments was more than other cultivars (Figure 5).

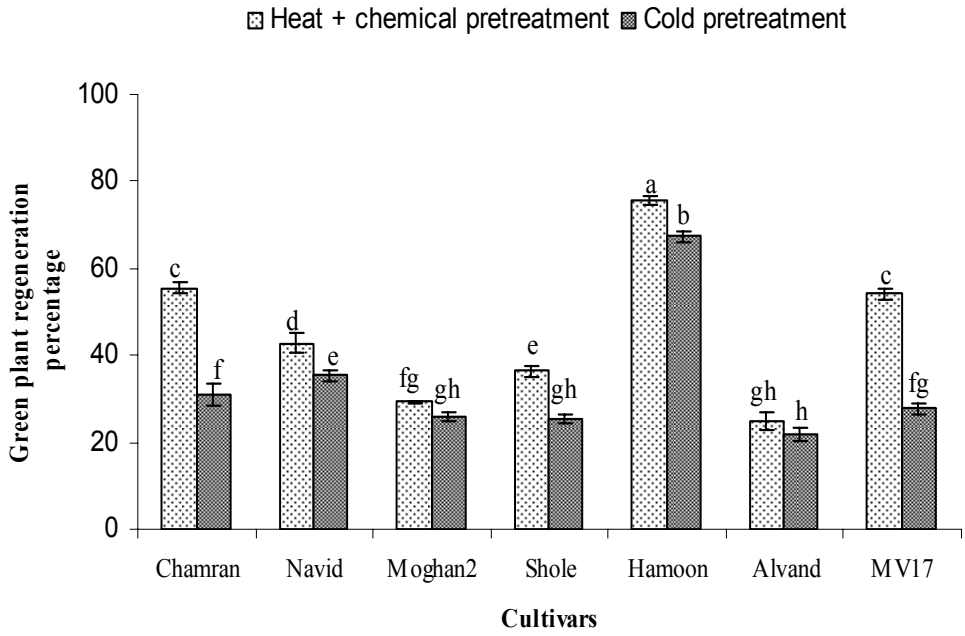


Figure 5. Comparison of means for interactions between cultivars and pretreatments for green plant regeneration percentage (the same letters are not significant different on the basis of 5% LSD analysis)

*3.2 Experiment 2*

Analysis of variance showed significant differences between cultivars, embryo induction media and their interactions for embryo yield per spike. Interaction between cultivar Chamran and CHB-2 medium showed the highest embryo yield per spike (320.42 embryos per spike). Embryo yield in the cultivars Chamran and Navid was more than other cultivars in the NPB99 medium. Among cultivars, cultivar Golestan did not produce any

embryo in the CHB-2 medium (Figure 6). Cultivars Chamran and Shole produced more embryos using CHB-2 medium while in cultivars Navid and MV17, NPB99 medium produced higher embryos than CHB-2 medium.

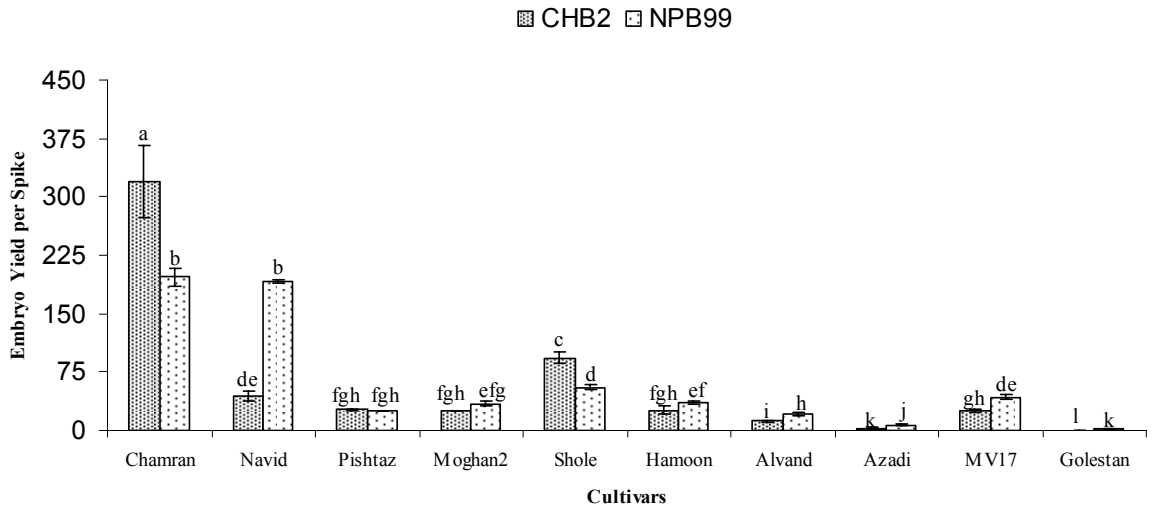


Figure 6. Comparison of means for interactions between cultivars and embryo induction media for embryo yield per spike (the same letters are not significant different on the basis of 5% LSD analysis)

Analysis of variance showed significant differences between cultivars and interactions between cultivars and media for percentage of total plant regeneration. The embryos obtained from interaction Hamoon cultivar and NPB99 medium produced the highest percentage of plant regeneration (64.5%) (Figure 7). Overall, the embryos obtained from interaction between cultivars Hamoon, Chamran and MV17 and NPB99 medium produced higher percentage of plant regeneration than CHB-2 medium. In contrast, in cultivar Navid, CHB-2 medium increased the plant regeneration (Figure 7).

Analysis of variance for percentage of green plant regeneration showed significant differences between cultivars, media and their interactions. Interactions between cultivar Hamoon and NPB99 and CHB-2 media produced the highest percentage of green plant regeneration (67.41% and 59.38%, respectively) (Figure 8). In cultivars Chamran, Navid, Shole and MV17, CHB-2 medium increased the percentage of green plant regeneration while the NPB99 medium decreased it. In two cultivars Pishtaz and Moghan2, there was not a significant difference between both used embryo induction media.

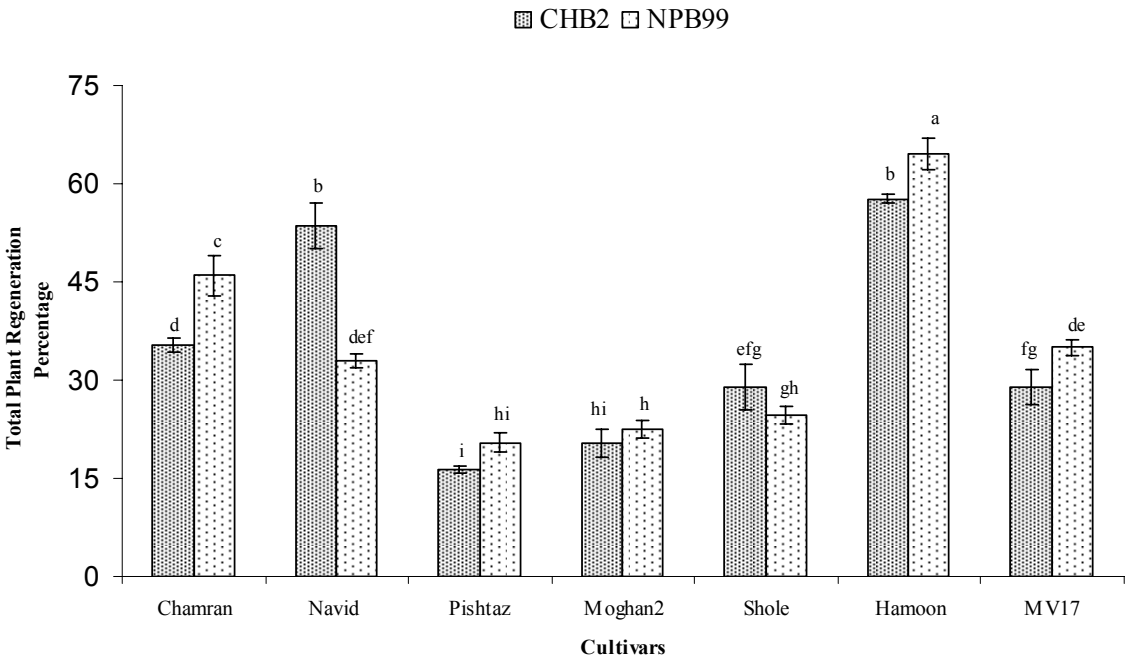


Figure 7. Comparison of means for interactions between cultivars and embryo induction media for total plant regeneration percentage (the same letters are not significant different on the basis of 5% LSD analysis)

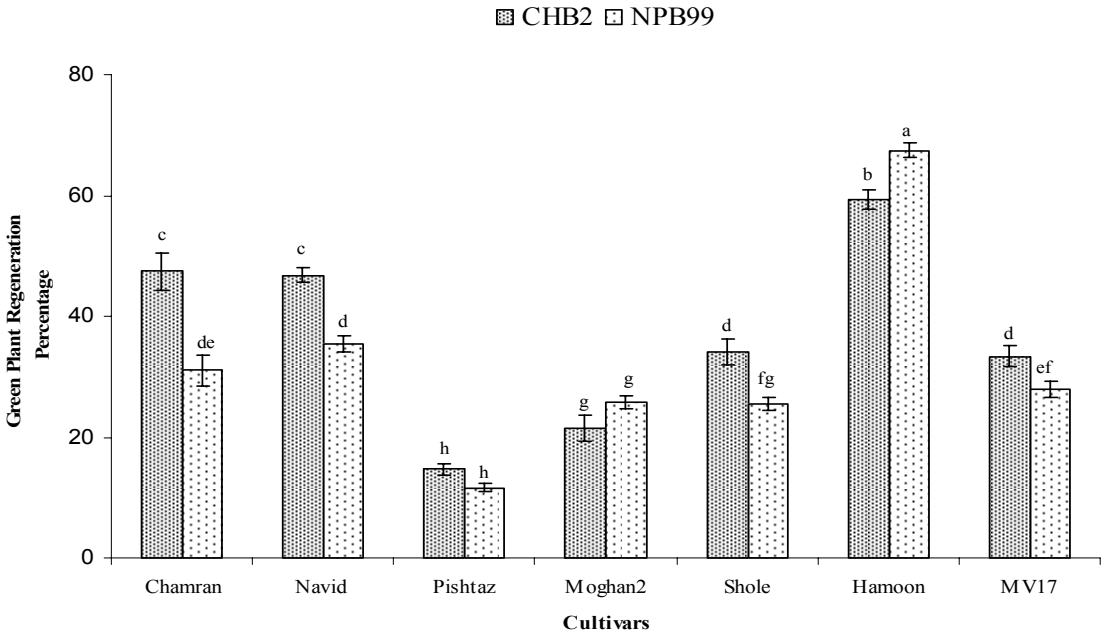


Figure 8. Comparison of means for interactions between cultivars and embryo induction media for green plant regeneration percentage (the same letters are not significant different on the basis of 5% LSD analysis)

## 4. Discussion

### *4.1 The Effect of Cultivar*

It has been recognized that both environmental and genetic factors interfere to the microspore embryogenesis response (Kasha et al., 1990). In the present research, the effect of cultivar was significant. Also the previous studies showed that response to androgenesis is strongly affected by the genotype (Tuvesson et al., 1989; Masojc et al., 1993). In this study, significant interactions between cultivars and pretreatments and also between cultivars and embryo induction media for response to androgenesis are observed. These findings were consistent with

other researches including Mentenwab and Sarrafi (1997) and Ghaemi et al. (1994), respectively. Some of the cultivars showed a better response than others to environmental changes on microspores. Zhang and Li (1984) showed that the chromosomes 2A and 2D consist of major genes for androgenesis and chromosomes 2B, 4A, 5A and 5B consist of minor genes acting to inhibit embryo production. Several authors have believed that the presence of the D genome in wheat (*Triticum aestivum* L.) is important for responding to androgenesis. Important genes for controlling embryo induction, total plant regeneration and green plant regeneration were located on chromosomes of D genome (De Buyser et al., 1992; Ghaemi et al., 1995). Information of effective genes and their interactions for response to androgenesis may help to overcome problems caused by the genotypic dependence.

Moieni and Sarrafi (1995) showed a significant genetic variability and relatively high heritability for androgenic traits (embryo yield, total plant regeneration and green plant regeneration) in hexaploid wheat. Among androgenic traits, albino plant regeneration has showed the lowest heritability value (Zheng, 2003). This case may cause to reduce albino by the control of culture conditions. Agache et al. (1988) showed that all three androgenic traits inherited dependently. Dominant and additive genes that affect on these traits may cause to convert important cultivars with low response into cultivars with high response to androgenesis.

### *4.2 The Effect of Pretreatment*

The type of stress used (applied as a pretreatment) plays an important role not only in embryogenesis induction but also in the production of green plants (Touraev et al., 1996). In the present research, two pretreatments (cold and heat + chemical) were used. These pretreatments differed in intensity and nature. These characteristics are important in blocking normal pathway of microspores and induction embryogenesis. Some of cultivars in cold pretreatment and some others in heat +chemical pretreatment had better response. This finding indicates the presence of interaction between cultivars and pretreatments. These results were consistent with findings of Mentenwab and Sarrafi (1997).

Both insufficient and over-extended pretreatments are destructive for the production of green plants. The aim of pretreatment is not only to maximize the number of embryogenic microspores, but also to produce proper embryos that can regenerate green plants (Zheng, 2003).

Cold pretreatment is a common system to induce embryogenesis. In the present study, some of cultivars after pretreating their tillers at 4°C for 21 days, showed a proper response to microspore culture that was consistent with findings of Armstrong et al. (1987) and Tuvesson et al. (2000). Cold pretreatment can increase the total content of free amino acids which cause to adaptation of microspores to the metabolic changes and induction of embryogenesis (Claparols et al., 1993; Xie et al., 1997). Also during the cold pretreatment, two HSP genes (tom 66 and tom 111) are expressed that may protect microspores against cold damages (Sabehat et al., 1998). In general, cold pretreatment cannot actually be considered a stress. It is seemed cold pretreatment generally plays as role protective while the induction of embryogenesis is started by other stresses. In the current research, the cultivars that showed a low response to cold pretreatment may be required to cold pretreatment with a high intensity or other pretreatments.

Chemical inducers consist of 2-HNA helps to trigger embryogenesis and to maintain microspores viability. These characters are important for an efficient androgenesis (Zheng et al., 2001). In the present study, cultivars that showed a low response to heat + chemical pretreatment required pretreatment with low intensity. In these cultivars, frequency of dead microspores after applying heat + chemical pretreatment was high. A fine pretreatment must decrease the frequency of dead microspores because the dead microspores can stop the production of embryos by releasing phenolics and changing medium composition (Liu et al., 2002). Less intensity of this pretreatment may be achieved by decreasing concentration of 2-HNA, temperature and period of pretreatment.

Stress conditions may help to the induction of embryogenesis but these stresses may also be caused to alterations leading to low plant regeneration and albino plants production. This subject can be related to the differences observed in total plant and green plant regenerations in the present study. Previous studies have shown that stability of plastid ribosomes was highly temperature-dependent. Seedlings of rye, barley and oat grown at temperature of 32-34°C were deficient to from 70s ribosomes (Hess et al., 1992) and the expression of plastid genes were chiefly depressed at low temperature (Yoshida et al., 1996). These effects may differ according to genotype. In general, the choice of stress related to its efficiency and practicality in androgenesis.

### *4.3 The Effect of Embryo Induction Medium*

After embryogenesis induction, the optimal environment require for embryogenic microspores to grow into embryos and subsequently regenerate to green plants. In isolated microspore culture, after the induction of

embryogenesis, an ongoing series cell divisions are necessary for success. These cell divisions were established in response to the use of different media, hormone combinations, conditioning factors, amino acid and other additives in the medium (Jähne & Lörz, 1995).

The present results showed the significant interactions between cultivars and embryo induction media for embryo yield and green plant regeneration. These results are consistent with finding of Moieni and Sarrafi (1996) and Lantos et al. (2005). The composition of medium is critical for embryogenesis induction and green plant regeneration. The principal differences in two used media in the present research included myo-inositol, amino acids (especially glutamine), PAA and hormone combinations.

The concentration, combination and exogenous hormones balance in the medium are critical. Growth condition of donor plants and stresses to them may greatly affect the endogenous hormone levels and subsequently the hormone levels required in embryo induction medium (Cistue & Kasha, 2005). Profitable effects of PAA have been reported on barley and wheat androgenesis. Ziauddin et al. (1992) reported that medium containing PAA has produced a slightly higher embryo than medium containing 2,4-D. They also reported a significant increase in green plant regeneration for wheat anther culture and barely microspores culture by medium containing PAA. In contrast, our data did not show a dramatic effect of presence of PAA in NPB99 medium for green plant regeneration. It seems the effect of optimal hormonal combination on embryo production was affected by cultivar, growth condition of donor plants and pretreatment.

The presence of myo-inositol in NPB99 medium may be useful because inositol plays a regulative role for $Ca^{2+}$ in the internal cell which is important in cell cycle (Hetherington & Brownlee, 2004). The higher plant regeneration obtained by CHB-2 medium may be due to concentration of the organic nitrogen especially glutamine used in this medium (Mordhorst & lörz, 1993). It has been showed that certain amino acids can be beneficial for embryo maturation and plant regeneration (Trottier et al., 1993).

In media without the presence of immature ovaries, cell division was blocked in the developing pathway microspores for embryo formation. The presence of immature ovaries provides nurse factors for androgenesis. We suggest using a combination of OVCM and ovary co-culture for low-responsive cultivars according to the idea of Zheng et al. (2001). It seems OVCM provides nurse factor earlier than immature ovaries in beginning microspores culture. It is important particularly for cultivars with low response.

In this research, ten Iranian hexaploid wheat cultivars (exclusive of MV17) were evaluated. These cultivars were used as the parents in the breeding programs due to some suitable characters such as heat, drought, salinity and disease resistance. The results showed that in the most of the cultivars, the interaction effects were significant and a considerable number of the embryos or the green plant regeneration could be produced after determining a suitable interaction effect.

**References**

Agache, S., Buyser, J., Henry, Y., & Snape, J. W. (1988). Studies of the genetic relationship between anther culture response and somatic tissue culture abilities in wheat. *Plant Breed, 100*, 26-33. http://dx.doi.org/10.1111/j.1439-0523.1988.tb00213.x

Armstrong, T. A., Metz, S. G., & Mascia, P. N. (1987). Two regeneration systems for the production of haploid plants from wheat anther culture. *Plant Sci., 51*, 231-237. http://dx.doi.org/10.1016/0168-9452(87)90197-X

Chu, C., Hill, R., & Brule-babel, A. (1990). High frequency of pollen embryoid formation and plant regeneration in *Triticum aestivum* L. *Plant Science, 66*, 255-262. http://dx.doi.org/10.1016/0168-9452(90)90211-6

Cistue, L., & Kasha, K. J. (2005). Gametic embryogenesis in *Triticum*: a study of some critical factors in haploid (microspore) embryogenesis. In A. Mujib, & J. Samaj (Eds.), *Somatic Embryogenesis* (pp. 321-342). Berlin Heidelberg.

Claparols, I., Santos, M. A., & Torne, J. M. (1993). Influence of some exogenous amino acids on the production of maize embryogenic callus and on endogenous amino acid content. *Plant Cell Tiss Org Cult., 34*, 1-11. http://dx.doi.org/10.1007/BF00048457

De Buyser, J., Hachemi-Rachedi, S., Lemee, M. L., Sejourne, S., Marcotte, J. L., & Henry, J. (1992). Aneuploid analysis of anther response in wheat. *Plant Breed, 109*, 339-342. http://dx.doi.org/10.1111/j.1439-0523.1992.tb00194.x

Ghaemi, M., Sarrafi, A., & Alibert, G. (1994). The effects of silver nitrate, colchicine, cupric sulfate and genotype on the production of embryoids from anthers of tetraploid wheat (*Triticum turgidum*). *Plant Cell Tiss Org Cult., 36*, 355-359. http://dx.doi.org/10.1007/BF00046093

Ghaemi, M., Sarrafi, A., & Morris, R. (1995). Reciprocal substitution analysis of embryo induction and plant regeneration from anther culture in wheat (*Triticum aestivum* L.). *Genome, 38*, 158-165. http://dx.doi.org/10.1139/g95-020

Gustafson, V., Baenziger, P. S., Wright, M. S., Stroup, W. W., &Yen, Y. (1995). Isolated wheat microspore culture. *Plant Cell Tiss Org Cult., 42*, 207-213. http://dx.doi.org/10.1007/BF00034239

Hess, W. R., Blank-Huber, M., Fieder, B., Börner, T., & Rüdiger, W. (1992). Chlorophyl synthethase and chloroplast tRNAglu are present in heat-bleached, ribosome-deficient plastids. *J. Plant Physiol, 139*, 427-430. http://dx.doi.org/10.1016/S0176-1617(11)80489-X

Hetherington, A. M., & Brownlee, C. (2004). The generation of $Ca^{2+}$ signals in plants. *Plant boil., 55*, 401-427.

Hu, H. (1997). *In vitro* induced haploids in wheat. In S. M. Jain, S. K. Sopory, & R. E. Veilleux (Eds.), *In vitro Haploid Production in Higher Plants* (pp. 73-97). Kluwer Academic Publishers, Dordrecht, Netherlands. 4.

Hu, T. C., & Kasha, K. J. (1997). Improvement of isolated microspore culture of wheat (*Triticum aestivum* L.) through ovary co-culture. *Plant Cell Rep., 16*, 520-525. http://dx.doi.org/10.1007/s002990050271

Hu, T. C., Ziauddin, A., Simion, E., & Kasha, K. J. (1995). Isolated microspore culture of wheat (*Triticum aestivum* L.) in a defined media. *In Vitro Cell Dev Biol., 31*, 79-83.

Hu, T., & Kasha, K. J. (1999). A cytological study of pretreatments used to improve isolated microspore culture of wheat (*Triticum aestivum* L.) cv. Chris. *Genome, 42*, 432-441.

Jähne, A., & Lorz, H. (1995). Cereal microspore culture. *Plant Science, 109*, 1-12. http://dx.doi.org/10.1016/0168-9452(95)04149-O

Kasha, K. J., Ziauddin, A., & Cho, U. H. (1990). Haploids in cereal improvement: anther and microspore culture. In: J. P. Gustafson (Ed.), *Gene Manipulation in Plant Improvement II* (pp. 213-235). Plenum, New York. http://dx.doi.org/10.1007/978-1-4684-7047-5_11

Lantos, C., Jancso, M., & Pauk, J. (2005). Microspore culture of small grain cereals. *Acta Physiologia Plantarum, 27*, 631-639. http://dx.doi.org/10.1007/s11738-005-0067-6

Liu, W., Zheng, M. Y., & Konzak, C. F. (2001). Improving green plant production via isolated microspore culture in bread wheat. *Plant Cell Rep., 20*, 821-824.

Liu, W., Zheng, M. Y., Polle, E., & Konzak, C. F. (2002). Highly efficient doubled-haploid production in wheat (*Triticum aestivum* L.) via induced microspore embryogenesis. *Crop Sci., 42*, 686-692. http://dx.doi.org/10.2135/cropsci2002.0686

Masojc, P., Lukow, O. M., Mc Kenzie, R. I. H., & Howes, K. (1993). Responsiveness to anther culture in cultivars and F1 crosses of spring wheat. *Can. J. Plant Sci., 73*, 777-783. http://dx.doi.org/10.4141/cjps93-100

Mejza, S. J., Morgant, V., Dibona, D. E., & Wong, J. R. (1993). Plant regeneration from isolated microspores of *Triticum aestivum*. *Plant Cell Rep., 12*, 149-153. http://dx.doi.org/10.1007/BF00239096

Mentenwab, A., & Sarrafi, A. (1997). Influence of genotype and cold pretreatment on the production of embryoids and their regeneration in tetraploid and hexaploid wheats. *J. Genet Breed., 51*, 59-62.

Moieni, A., & Sarrafi, A. (1995). Genetic analysis for haploid-regeneration responses of hexaploid-wheat anther cultures. *Plant Breed, 114*, 247-249. http://dx.doi.org/10.1111/j.1439-0523.1995.tb00803.x

Moieni, A., & Sarrafi, A. (1996). The effects of gibberlic acid, phenylethylamine, 2,4-D and genotype on androgenesis in hexaploid wheat (*Triticum aestivum* L.). *Cereal Research Communications, 24*, 139-145.

Mordhorst, A., & Lörz, H. (1993). Embryogenesis and development of barley microspores are influenced by the amount and composition of nitrogen sources in culture media. *Plant Physiol, 142*, 485-492. http://dx.doi.org/10.1016/S0176-1617(11)81256-3

Ouyang, J. W., Hu, H., Chuang, C. C., & Tseng, C. C. (1973). Induction of pollen plants from anthers of *Triticum aestivum* L. cultured *in vitro*. *Sci Sinica, 16*, 79-95.

Picard, E., & De Buyser, J. (1973). Obtention de plantules haploids de *Triticum aestivum* L. a partir de cultures d'anthers *in vitro*. *C R Acad. Sci., 277*, 1463-1466.

Reynolds, T. L. (1997). Pollen embryogenesis. *Plant Molecular Biology, 33*, 1-10. http://dx.doi.org/10.1023/A:1005748614261

Sabehat, A., Lurie, S., & Weiss, D. (1998). Expression of small heat-shock proteins at low temperatures: a possible role in protecting against chilling injuries. *Plant Physiol., 117*, 651-658. http://dx.doi.org/10.1104/pp.117.2.651

Touraev, A., Indrianto, A., Wratschko, I., Vicente, O., & Heberle-Bors, E. (1996). Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperatures. *Sex Plant Reprod., 9*, 209-215. http://dx.doi.org/10.1007/BF02173100

Touraev, A., Pfosser, M., & Heberle-Bors, E. (2001). The microspore: a haploid multipurpose cell. *Advances in Botanical Research, 35*, 53-109. http://dx.doi.org/10.1016/S0065-2296(01)35004-8

Trottier, M. C., Collin, J., & Comeau, A. (1993). Comparison of media for their aptitude in wheat anther culture. *Plant Cel Tissue and Organ Culture, 35*, 59-67. http://dx.doi.org/10.1007/BF00043940

Tuvesson, I. K. D., Perdersen, S., & Andersen, S. B. (1989). Nuclear genes affecting albinism in wheat (*Triticum aestivum* L.). *Theor App Genet., 78*, 879-883. http://dx.doi.org/10.1007/BF00266675

Tuvesson, S., Ljungberg, A., Johansson, N., Karlsson, K. E., Suijs, L. W., & Josset, J. P. (2000). Large-scale production of wheat and triticale double haploids through the use of a single-anther culture method. *Plant Breed, 119*, 455-459. http://dx.doi.org/10.1046/j.1439-0523.2000.00536.x

Xie, J. H., Gao, M. W., Liang, Z. Q., Shu, Q. Y., Cheng, X. Y., & Xue, Q. Z. (1997). The effect of cool-pretreatment on the isolated microspore culture and the free amino acid change of anthers in Japonica rice (*Oryza sativa* L.). *Journal Plant Physiol, 151*, 79-82. http://dx.doi.org/10.1016/S0176-1617(97)80040-5

Yoshida, R., Kanno, A., Sato, T., & Kameya, T. (1996). Cool temperatureinduced chlorosis in rice plants: I. Relationship between the induction and a disturbance of etioplast development. *Plant Physiol, 110*, 997-1005. http://dx.doi.org/10.1104/pp.110.3.997

Zhang, Y. L., & Li, D. S. (1984). Anther culture of monosomics in Triticum aestivum. *Hereditas, 6*, 7-10.

Zheng, M. Y. (2003). Microspore culture in wheat (*Triticum aestivum*) - doubled haploid production via induced embryogenesis. *Plant Cell Tiss Org Cult., 73*, 213-230. http://dx.doi.org/10.1023/A:1023076213639

Zheng, M. Y., Liu, W., Weng, Y., Polle, E., & Konzak, C. F. (2001). Culture of freshly isolated wheat (*Triticum aestivum* L.) microspores treated with inducer chemicals. *Plant Cell Rep., 20*, 685-690. http://dx.doi.org/10.1007/s00299-001-0393-0

Zheng, M. Y., Weng , Y., Liu, W., & Konzak, C. F. (2001). The effect of ovary-conditioned medium on microspore embryogenesis in common wheat (*Triticum aestivum* L.). *Plant Cell Rep., 20*, 802-807.

Zhuang, J. J., & Xu, J. (1983). Increasing differentiation frequencies in wheat pollen callus. In H. Hu, & M. R. Vega (Eds.), *Cell and Tissue Culture Techniques for Cereal Crop Improvement*. Beijing, China: Science Press.

Ziauddin, A., Marsolais, A., Simion, E., & Kasha, K. J. (1992). Improved plant regeneration from wheat anther and barley microspore culture using phenylacetic acid (PAA). *Plant Cell Rep., 11*, 489-498. http://dx.doi.org/10.1007/BF00236263

Zoriniants, S., Tashpulatov, A. S., Heberle-Bors, E., & Touraev, A. (2005). The role of stress in the induction of haploid embryogenesis. In C. E. Palmer, W. A. Keller, & K. J. Kasha (Eds.), *Haploids in crop improvement II* (pp. 35-52). Berlin Heidelberg: Springer. http://dx.doi.org/10.1007/3-540-26889-8_3