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Full Length Research Paper

Effects of embryo induction media and pretreatments in isolated microspore culture of hexaploid wheat (*Triticum aestivum* L. cv. Falat)

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Isolated microspores of many plants can be induced *in vitro* to switch their developmental process from the gametophytic to a sporophytic pathway under appropriate conditions and produce haploid plants. This research reports the effects of cold pretreatment with or without either mannitol or chemical + heat and also the effects of 5 embryo induction media (NPB-99, C17, W14, CHB-2 and P2) on embryo production, percentage of total and green plant regeneration in isolated microspore culture of an Iranian spring bread wheat, cultivar 'Falat'. The results showed that combination of 21 days cold (4°C) with mannitol (0.3 M) produced the highest number of embryos/spike while the combination of cold with chemical + heat produced the lowest number. In the case of total and green plant regeneration, "7 days cold + mannitol" was more superior than other pretreatments. NPB-99, W14 and C17 media produced the highest number of embryos/spike, while CHB-2 medium appeared to be a better medium for green plant regeneration.

Key words: Hexaploid wheat, haploid, isolated microspore culture, pretreatment, embryo induction medium, cultivar "Falat".

INTRODUCTION

Haploid plants posses the gametic number (n) of chromosomes and a doubled haploid is derived from a haploid by doubling its chromosome number (Kasha et al., 1995). Haploids/doubled haploids are important for production of genetically homozygous lines, genetic analysis, induction of mutation, genome mapping and gene transfer (Kasha et al., 1995; Zheng, 2003; Abdollahi et al., 2007). Nowadays, three plant biotechnology methods are used for producing of haploids in plants, androgenesis (anther and microspore culture), gynogenesis and chromosome elimination following wide hybridization. Microspore culture has advantages over other methods. Millions of potentially embryogenic single cells are provided by microspore culture (Touraev et al., 2001). This method offers an opportunity to investigate biochemical and molecular processes of microspore embryogenesis and also the developmental sequence from individual microspores to the whole plant can be followed directly (Reynolds, 1997; Indrianto et al., 2001). Embryos derived from microspore culture are almost haploid, while embryos might be obtained from the diploid somatic tissue in anther culture (Bonet and Olmedilla, 2000).

Since the first reports of isolated microspore culture in *Triticum aestivum* L. (Mejza et al., 1993; Tuvesson and Öhlund, 1993), there have been remarkable progresses in developing this system (Touraev et al., 1996; Hu and Kasha, 1997; Zheng et al., 2001; Liu et al., 2002). Some important factors influencing the efficiency of microspore culture include genotype, donor plant physiology, microspore developmental stage, stress pretreatments and medium composition (Hu and Kasha, 1997; Zheng et al.,

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Table 1. Studied stress pretreatments in isolated microspore culture of Falat spring wheat.

Pretreatments	Plant material	Abbreviation
Tillers, with the basal end in a beaker of water, were cold pretreated (4°C) for 3 weeks	Tillers	3 weeks cold
Spikes were immersed in 100×15 mm Petri dishes containing 40 ml of 0.4M mannitol and were cold pretreated (4°C) for 7 days	Spikes	7 days cold + mannitol
As in 7 days cold + mannitol, but spikes were cold pretreated for 3 weeks	Spikes	3 weeks cold + mannitol
As in 3 weeks cold but tillers were cold pretreated for 7 days and then were heat pretreated (33°C) for 48 h, with their basal end in an autoclaved sterile flask, containing 50 ml sterile inducer formulation (100 mg l^{-1} 2-HNA, 10^{-6} mol l^{-1} 2,4-D and 10^{-6} mol l^{-1} BAP)	Tillers	Cold + (chemical + heat)

2,4-D: 2,4-dichlorophenoxyacetic acid; 2-HNA: 2-hydroxynicotinic acid; BAP: 6-benzylaminopurine

2001). Different stress pretreatments including cold shock (Gustafson et al., 1995; Hu and Kasha, 1999), sugar starvation alone and in combination with cold shock or heat shock (Mejza et al., 1993; Touraev et al., 1996; Hu and Kasha, 1997, 1999) and inducer chemicals alone or in combination with heat shock (Zheng et al., 2001; Liu et al., 2002), and various embryo induction media including CHB-2, FMN6 (Mejza et al., 1993), A2 (Touraev et al., 1996), MMS3 (Hu and Kasha, 1997), NPB-99 (Liu et al., 2002), etc. have been used in wheat isolated microspore culture. However, the number of published reports on the comparison of different stress pretreatments and media in isolated microspore culture of plants, especially in wheat, is very low. On the other hand, the number of publications in the case of Iranian wheat cultivars is too low. Therefore, in the present research, we examined the effects of some stress pretreatments [3 weeks cold, 7 days cold + mannitol, 3 weeks cold + mannitol and cold + (chemical + heat)] and embryo induction media (NPB-99, C17, W14, CHB-2 and P2), on efficiency of microspore embryogenesis, total and green plant regeneration in *Triticum aestivum* cv. Falat. These stress pretreatments and media compositions have been chosen because they are widely used in androgenesis and also they are easy to handle.

MATERIALS AND METHODS

Plant material

The spring bread wheat, *Triticum aestivum* L. cv. Falat, was used as donor plant for isolated microspores culture. Seeds were kindly provided by seed and plant improvement institute, Karaj, Iran. Donor plants were grown in the field conditions.

Stress pretreatments

Tillers containing the microspores at the mid to late-uninucleate stages were cut at the second node from the top of the tiller, then all leaves were cut except the flag leaf and the base of tillers were immersed in 400 ml beaker containing 200 ml fresh tap water (Liu et al., 2002). In this study, two experiments were done, separately. In the first experiment, the effects of some stress pretreatments on the spikes or tillers (Table 1) were investigated in comparison with the control in which microspore were cultured immediately after spikes collection, without any pretreatment. In the second experiment, the collected tillers were cold pre-treated (4°C) for 3 weeks and then the effects of 5 embryo induction media were studied.

Microspore isolation

Microspore isolation was conducted according to the protocol described by Liu et al. (2002), with some modifications. Following pretreatments, pretreated spikes and the fresh spikes in the case of control, were disinfected through immersion in 1.5 (w/v) sodium hypochlorite solution for 15 min, followed by twice rinse with sterile distilled water over 3 min. Then, florets were separated and blended (2 speed warring, Christison) with low speed for 20 s. Florets obtained from 4 spikes were used for each run of blending in 50 ml of autoclaved 0.3 M mannitol solution. To eliminate the large debris, resulting slurry was passed through a 100 µm stainless steel mesh filter. The blender cup was rinsed twice using 5 ml of 0.3 M mannitol each time and this solution was also poured into the filter. Then the filtrate was pipetted into two 50 ml sterile centrifuge tubes and centrifuged at 100 × g for 3 min. After discarding the supernatant, the 2 pellets were combined and resuspended in 2 ml of 0.3 M mannitol solution. The resuspended pellets were layered over 5 ml of a 0.58 M sterile maltose solution and centrifuged at 100 × g for 3 min. Upper band was collected and resuspended in 10 ml of 0.3 M mannitol solution in a 15 ml centrifuge tube and was centrifuged at 100 × g for 3 min. The supernatant was discarded and the pellet was resuspended in 10 ml of embryo induction medium and then centrifuged at 100 × g for 3 min. The supernatant was discarded again and the pellet resuspended in 2 ml of embryo induction medium. Microspore culture density was estimated using hemocytometer, and adjusted to 1×10^4 microspores per ml.

Microspore culture and plant regeneration

In the first experiment, NPB-99 medium (Liu et al., 2002) was used as embryo induction medium and in the second experiment, different induction media including NPB-99 (Liu et al., 2002), C17 (Wang and Chen, 1986), W14 (Ouyang et al., 1988), CHB-2 (Chu et al., 1990) and P2 (Chuang et al., 1978) supplemented with 90 gl^{-1}

Table 2. Studied plant growth regulators and adjusted pH of embryo induction media in isolated microspore culture of Falat spring wheat.

Media	Plant growth regulators	pH
NPB-99	0.2 mg l^{-1} 2,4-D + 0.2 mg l^{-1} Kinetin + 1 mg l^{-1} PAA	7.0
C17	0.2 mg l^{-1} 2,4-D + 0.2 mg l^{-1} Kinetin + 1 mg l^{-1} PAA	5.8
W14	2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kinetin	5.8
CHB-2	0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kinetin	5.4
P2	1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kinetin	5.8

PAA: phenylacetic acid

Table 3. Effects of different pretreatments on mean performance of studied androgenic traits in isolated microspore culture of the cultivar Falat.

Pretreatment	Means		
	EM	TR	GR
control	83.00c	29.00b	21.26c
3 weeks cold	190.00b	60.66a	23.40c
7 days cold + mannitol	216.08b	55.33a	40.33a
3 weeks cold + mannitol	772.25a	63.66a	27.83b
Cold + (chemical + heat)	6.91d	-	-

EM: Produced embryos per spike; TR: Total plant regeneration per 100 cultured embryos; GR: Green plant regeneration percentage.
Means within the same column having a different letter are significantly different at P=0.05 (LSD test).

maltose were used (for plant growth regulators and pH see Table 2. An aliquot of 5 ml medium per 55 × 15 mm plastic Petri dish was used. Also, 10 ovaries (from the same wheat cultivar) were added into each Petri dish. The Petri dishes were sealed with Parafilm and incubated in the dark at 28°C. After 30 to 40 days from micro-spore culture, 100 large embryos (≥ 2 mm) were transferred into 190 - 2 solid plant regeneration medium (Zhuang and Xu, 1983) containing 3 g l^{-1} phytagel and 30 g l^{-1} sucrose at a density of 10 to 15 embryos per plastic Petri dish (55 × 15 mm). The transferred embryos were kept at 25°C with a 16 h photoperiod (4000 lux).

Data analysis

Experiments were analyzed as a completely randomized design with 3 replications for each treatment. Each separate culture process represented as a replication. In fact, for each replication of a treatment, the microspores of 4 spikes were isolated and cultured. After embryo production, the number of produced embryos in each separate culture process were counted and then divided into 4 to gain the number of produced embryos per one spike. After that, 100 large embryos (≥ 2 mm) obtained from each replication were transferred to plant regeneration medium, 190 - 2 medium for estimating of total and green plant regeneration. Two to three weeks after transferring embryos into plant regeneration medium, the plant regeneration frequency was calculated as the total number of plantlets regenerated from each 100 transferred embryos and also the percentage of green plants among the total number of regenerated plants was determined in each replication. Generally speaking, data of embryos number per spike, total and green plant regeneration percentage for each replication came from independent culture processes. LSD test was carried out for mean comparisons of the three above mentioned traits.

RESULTS

Microspore derived embryos first emerged in culture after 3 weeks. Significant differences were found between pretreatments and also between media in the number of embryos per spike, the percentage of total plant regeneration and the percentage of green plant regeneration. The effects of pretreatments and media on means of 3 studied traits were presented in Tables 3 and 4, respectively. In the case of embryo production per spike, "3 weeks cold + mannitol" pretreatment was dramatically superior to other pretreatments (Figure 1). 3 weeks cold and "7 days cold + mannitol" pretreatments did not have a significant difference for embryo production per spike. Pretreatment of "cold + (chemical + heat)" produced the lowest embryo production. The number of embryos produced using "3 weeks cold + mannitol", "7 days cold + mannitol" and "3 weeks cold" pretreatments were higher than the control and the number of embryos produced using "cold + (chemical + heat)" pretreatment was dramatically lower than control.

Concerning total plant regeneration, "3 weeks cold", "3 weeks cold + mannitol" and "7 days cold + mannitol" pretreatments produced significantly higher plant regeneration than control (without pretreatment). The number of

Table 4. Effects of different embryo induction media on means of androgenic traits in isolated microspore culture of the cultivar Falat.

Medium	Means		
	EM	TR	GR
NPB-99	190.00a	60.66ab	23.40c
CHB-2	111.33b	65.66a	50.76a
W14	175.41a	55.00ab	34.90b
C17	190.50a	52.33b	23.56c
P2	6.83c	-	-

EM: Produced embryos per spike; TR: Total plant regeneration per 100 cultured embryos; GR: Green plant regeneration percentage.
Means within the same column having a different letter are significantly different at P=0.05 (LSD test).

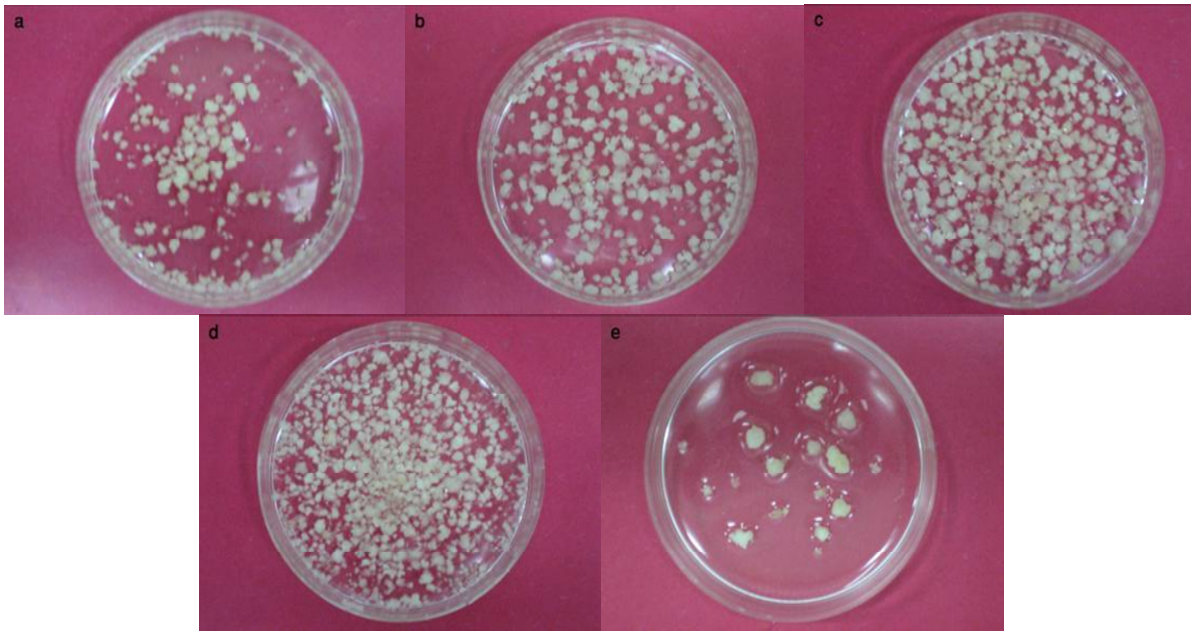

Figure 1. Comparison the effects of different pretreatments on embryogenesis in isolated microspore culture of the cultivar Falat: **(a)** control, **(b)** 3 weeks cold, **(c)** 7 days cold + mannitol, **(d)** 3 weeks cold + mannitol, and **(e)** cold + (chemical + heat)

produced embryos using "cold + (chemical + heat)" pretreatment was very low. Therefore, they were not transfered to plant regeneration medium.

The highest percentage of green plant regeneration was produced using "7 days cold + mannitol" pre-treatment followed by "3 weeks cold + mannitol" pretreatment. Using these pretreatments the number of regenerated green plants was significantly higher than that of the control.

Also, significant differences between the different embryo induction media were observed for all of the studied androgenic traits. C17, NPB-99 and W14 media produced the highest number of embryos per spike. The lowest number of embryos was produced by P2 medium. Comparing different media, CHB-2 medium produced the highest total plant regeneration and the lowest one obtained by C17 medium. In the case of P2 medium, because of very low number of produced embryos, they were not transferred to plant regeneration medium. The CHB-2 medium significantly produced the highest percentage of regenerated green plant followed by W14 medium (Figure 2). Considerable difference was not observed between NPB-99 and C17 media for this trait. In general, although, embryo production in CHB-2 medium was significantly lower than NPB-99, W14 and C17 media, but the total and green plant regeneration percentages using CHB-2 medium were higher than other tested media.

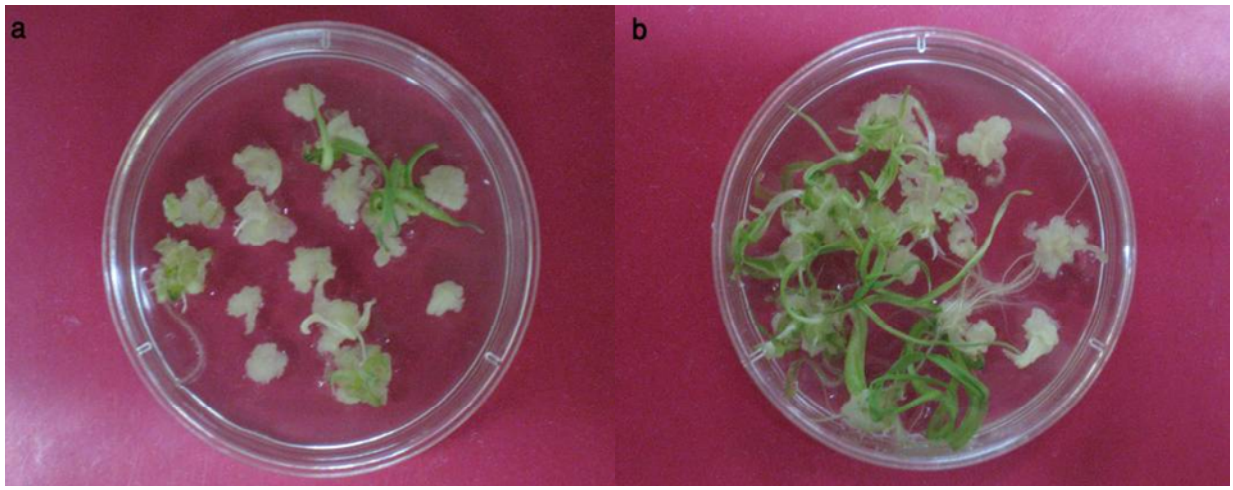

Figure 2. Plant regeneration of embryos produced in NPB-99 **(a)** and CHB-2 **(b)** media in isolated microspore culture of *Triticum aestivum* cv. Falat.

DISCUSSION

Microspore culture is one of the most important methods for producing doubled haploid plants and the efficiency of this method is influenced by several mentioned factors. Hence, different attempts have been done to find out the effects of these factors in isolated microspore culture of wheat, which is an important crop in worldwide (Hu and Kasha, 1997; Hu and Kasha, 1999; Liu et al., 2002; Labbani et al., 2005). However, the number of published reports on the effects of these factors in microspore culture of Iranian hexaploid wheat cultivars is too low. So, in this research the effects of the 2 most important factors including stress pretreatments and embryo induction media in isolated microspore culture of an Iranian hexaploid wheat cultivar “Falat” have been investigated.

There are different reports about the application of stress pretreatments. Some authors have found that it is necessary to use an appropriate stress pretreatment to gain a proper result in microspore culture of wheat (Touraev et al., 1996; Hu and Kasha, 1999; Liu et al., 2002) on the other hand, a recent report shows that it is better to not use stress pretreatments (Shariatpanahi et al., 2006). Therefore, to find out if it is necessary to use stress pretreatments or not, the effects of 4 different stress pretreatments have been studied on androgenic traits in comparison with control in which any apparent stress pretreatment was used. Results showed that embryogenesis can occur in wheat, via direct culture of isolated microspores in embryo induction medium without using an apparent stress pretreatment (control). Although any stress pretreatment was used, the production of embryos could be due to the presence of maltose in embryo induction medium. Maltose which was used as carbon source in induction medium hydrolyzes very slowly and causes starvation stress to microspores and it seems that could be enough for changing the developmental pathway of microspores (Zheng, 2003). Although, the microspore embryogenesis could be occur without using an apparent stress pretreatment, the present results obviously showed that, there was significant differences between the control and the stress pretreatments. So, the results indicated the necessity of using a suitable stress pretreatment to gain a good embryogenesis and green plant regeneration as some previous reports (Touraev et al., 1996; Hu and Kasha, 1999; Liu et al., 2002).

An appropriate stress pretreatment could improve significantly androgenic traits yield. On the other hand the results showed that, application of a very strong pretreatment such as "cold + (chemical + heat)", which was a combination of 3 stresses, not only had not a positive effect on embryo production, but also decreased it. In fact, using this pretreatment the majority of microspores lost their viability during 1 week after culture in the embryo induction medium. Cold and cold in combination with mannitol pretreatments presented the positive effect on embryogenesis, total and green plant regeneration in this research. These results are in harmony with the previous reports (Gustafson et al., 1995; Hu and Kasha, 1999). The duration of 3 weeks cold pretreatment was chosen on the basis of our former studies (unpublished data). Here, we found the positive effect of cold pretreatment on embryogenesis and total plant regeneration, but this treatment had not a significant effect on green plant regeneration. Although cold shock was used in different species including bread wheat (Gustafson et al., 1995), durum wheat (Labbani et al., 2005), barley (Kasha et al., 2001), etc, the precise mechanism for reprogramming of microspores towards embryogenesis is still obscure. However, it was proposed that cold shock increases the viability of embryogenic microspores, indu-

ces the formation of 2 equal nuclei, delays the pollen development and represses gametophytic differentiation (Sangwan and Sangwan-Norreel, 1996; Sopory and Munshi, 1996).

Our results showed that the combination of cold with mannitol was superior to other treatments in Falat spring wheat. Two levels of this pretreatment including 7 days and 3 weeks (reported for the first time) were studied here. The 3 weeks cold + mannitol, increased dramatically the embryo production but the highest green plant regeneration rate obtained using 7 days cold + mannitol. It seems that a higher embryo induction could be the reason of the dramatic increase in embryo production of "3 weeks cold + mannitol". The cold + mannitol pretreatments produced significantly higher green plants than cold pretreatment. A recent study in barley showed that the positive effect of osmotic shock (mannitol) on green plant regeneration could be due to its effect on chloroplast biogenesis during androgenesis (Maraschin et al., 2006). However, many factors influence the green plant regeneration rate including genotype, physiological state of the donor plants, the developmental stage of microspores, medium composition and culture temperature (Chen, 1986; Jähne and Lörz, 1995). According to the results presented here, it seems we could add the pretreatment type and its duration to this list.

Embryo induction medium is another factor that must nourish and also redirects microspores developmental pathway towards embryo formation. It has been shown that several media are effective for microspore culture of hexaploid wheat (Mejza et al., 1993; Touraev et al., 1996; Hu and Kasha, 1997; Patel et al., 2004; Lantos et al., 2005). In the present research, 5 different media were compared for their effects on embryo production, total and green plant regeneration. Microspores cultured in C17 medium produced the highest number of embryos but it had no significant differences with NPB-99 and W14 media. The P2 medium has been effectively used in anther culture of hexaploid wheat (Moieni and Sarrafi, 1995, 1996), but the results of this study showed that it is not effective enough in wheat isolated microspore culture. For total and green plant regeneration, CHB-2 medium was superior to others and followed by W14 medium. Owing to the fact that the production of green plants is the final goal, the CHB-2 medium could be recommended for cultivar “Falat”.

Concentration and type of nitrogen source (Olsen, 1988; Morehouse and Lörz, 1993) and plant growth regulators (Patel et al., 2004) in embryo induction medium are 2 important factors influencing the androgenic traits. Observed differences between media for embryo production, total and green plant regeneration might be due to different amount and type of nitrogen sources as well as different plant growth regulators and their concentrations in these media and even their different pH. However, the highest total and green plant regeneration using CHB-2 medium could be due to the high concentration of organic nitrogen source (1000 mg l^{-1} glutamine).

Overall, it is clear from presented study as well as from previous studies (Hu and Kasha, 1999; Patel et al., 2004) that stress pretreatments and induction media are critical factors in the production of large number of embryos and plants from isolated microspore culture. In the presented experiments, we could produce the same or a higher number of embryos, total and green plants reported in the literature of wheat microspore culture (Hu and Kasha, 1999; Patel et al., 2004; Shariatpanahi et al., 2006). According to this research, it seems that "7 days cold + mannitol", "3 weeks cold + mannitol" pretreatments and also CHB-2 medium could be proposed to gain a reasonable result in wheat cv. Falat because they guaranteed a relatively high embryo production with a good green plant regeneration.

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