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Full Length Research Paper

Evaluation of Pretreatment Efficiency in Durum Wheat Androgenesis: A Study on Green Haploid Production in Tunisian Cultivars

O. Slama Ayed[1]*, J. De Buyser[2], E. Picard[2], Y. Trifa[1] and H. Slim Amara[1]

[1]Laboratoire d'amélioration génétique des Céréales, Institut National Agronomique de Tunisie, 43 Avenue Charles Nicolle 1082 -Tunis- Mahrajène Tunisia.

[2]Laboratoire de Morphogenèse Végétale Expérimentale Haploïde, Bat. 360, Université Paris sud XI Orsay- 91405 Cedex, France.

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The production of double haploids through androgenesis is used by breeders to produce homozygous lines in a single generation. Androgenesis can be achieved by isolated microspore culture, which, however, allows the production of green haploids with a very low efficiency. In order to improve the overall green haploid regeneration in durum wheat, we compare the efficiency of eight different pretreatments. These pretreatments were: cold pre-treatment for 5 weeks, 0.3M mannitol for 12 days at 4°C; 0.3M mannitol for 7 days at 4°C; 0.7M mannitol for 5 days at 4°C; PEG_{4000} 1.5% for 5 days at 4°C; PEG_{4000} 1% for 15 days at 4°C; PEG_{4000} 1% for 10 days at 4°C and control without pretreatment. The cold treatment for 5 weeks (4°C) is the most efficient and was used to test the androgenetic ability by microspore culture method of three Tunisian durum wheat cultivars (Razzek, Khiar and Azizi).This pre-treatment improves significantly the embryogenesis induction and green plants regeneration with green/albina ratio 2 for the cultivar "Khiar". The PEG_{4000} 1% for 10 days seems promising pre-treatment and this is important to perform new experiments to advance their response.

Key words: Isolated microspore culture, double haploid, durum wheat, pre-treatment.

INTRODUCTION

Double Haploid lines are commonly used in plant breeding programs for the production of homozygous lines in a single generation. Androgenesis is one of the most efficient methods for doubled haploid production and can be performed using anther or isolated microspores culture. In bread wheat (*Triticum aestivum* L.) and other cereals, androgenesis was used to develop number of cultivars that are widely cultivated in many countries (Cistué et al., 2003). However, durum wheat (*Triticum turgidum* subsp. *Durum* Desf.) remain recalcitrant to this technique with low regeneration rate and very high frequency of albino plants (Jaiti et al., 2000; Labbani et al., 2005; Cistué et al., 2006, 2009). Albino plants can even reach in many cases up to 100% of the regenerated plants (Labbani et al., 2005). Thus, optimiza-tion of isolated microspore protocol aiming at maximizing the production of green plants of durum wheat remains of considerable interest. Many physiological factors can influence the microspores culture response such as growth conditions of donor plants, developmental stage of microspores, types of pre-treatments and composition of induction and regeneration media (Touraev et al., 1996; Hu and Kasha, 1997; Liu et al., 2002; Zheng et al., 2003; Cistué et al., 2009) . Among them, pre-treatment is the most important factor. The stress treatment allows the conversion of microspores into embryos by diverting microspores from their normal gametophytic pathway into sporophytic development(Touarev et al., 1996; Hu and Kasha, 1999). It also plays an important role in the

*Corresponding author. E-mail: olfa.slama@planet.tn, olfayed@yahoo.fr.

Abbreviations: **DH,** Doubled haploid; **PEG,** polyethylene glycol; **2,4-D,** 2,4 dichlorophenoxyacetic acid.

development of the embryos into green seedlings (Shariatpanahi et al., 2006) and consequently improves the yield of green plants regeneration (Kasha et al., 2001; Li and Devaux, 2003). So far, in order to enhance durum wheat microspores reorientation and improve green plants regeneration, various stress pretreatments were applied including cold (Labbani et al., 2005), osmotic shock using mannitol (Cistué et al., 2006; Labbani et al., 2007). It was shown by Labbani et al. (2005) that a long term (five weeks) at 4°C cold pre-treatment of the microspores could be promising for green regeneration in durum wheat. Cistué et al. (2006) reported that anthers pre-treated in 0.7M mannitol for 5 days improved green plants regeneration. Labbani et al. (2007) showed that a pre-treatment consisting in a combined mannitol 0.3 M and cold for 7 days had a strong effect on the number of embryos produced and the regeneration of green plants. In our study, we have tested these different types of stress to compare their efficiency. In order to try to further improve the rate of haploid production through durum wheat microspores culture, we tested also the efficiency of a novel combined pre-treatment cold and PEG for different duration. In solution, PEG is used to induce an osmotic stress. This pre-treatment creates more negative water potential, facilitating a water-restricted environment (Iliié-Grubor et al., 1998). Finally, using the best pre-treatment identified, we have produced haploid plants from three different durum wheat genotypes by isolated microspores culture.

MATERIALS AND METHODS

The experiments presented in this article were performed in Laboratory of Morphogenesis Haploid Vegetable Experimental at Paris XI University-Orsay, France.

Donor plants and growth conditions

Three Tunisian durum wheat cultivars were used as donor plants in this study: Azizi, Khiar and Razzek.
Plants were cultivated in pots and grown in a controlled greenhouse, regulated with a photoperiod of 16 h light/ 8 h dark. Light intensity was set at 1150 $\mu E.m^{-2}.s^{-1}$. Temperature was set at 22°C during the day and 15°C in the night. Humidity was kept at 70%.

Spike pre-treatments

Spikes from Razzek variety were collected when most of the microspores were at the late uninucleate stage and stored in the different pre-treatment solutions in the dark at 4°C.

Spikes were subjected to pretreatments before using them for isolated microspores culture. Pre-treatment were based on cold, cold mannitol and cold PEG_{4000} treatments. In solution, mannitol and PEG were used to induce stress.

A total of seven pretreatments and control without pre-treatment were used:

(i) No pre-treatment.
(ii) Cold pre-treatment for 5 weeks.
(iii) 0.3 M mannitol for 12 days.
(iv) 0.3M mannitol for 7 days.
(v) 0.7M mannitol for 5 days.
(vi) PEG_{4000} 1.5% for 5 days.
(vii) PEG_{4000} 1% for 10 days.
(viii) PEG_{4000} 1% for 15 days.

Microspore isolation

Microspores were isolated and cultivated according to the protocol described by De Buyser et al. (2002). After pre- treatment, the spikes were sterilized with calcium hypochlorite 5% during 10 min followed by three washings with sterile water. The extracted microspores were cultivated in CHB3 medium based on Chu et al. (1990) modified by adding 90 g/l of maltose (Table 1). Microspores were adjusted at density of 50 000 microspores ml^{-1} and were cultivated in 35 × 15 mm Petri dishes. Immature ovaries were added to the culture at density of four per milliliter, immediately preceding the incubation. The inclusion of ovaries in these culture systems seemed to enhance embryogenesis and/or improve the quality of the embryos (Zheng et al., 2002). Plates were sealed and incubated in the dark at 27°C.

Embryo induction and plant regeneration

After embryos were generated and had reached a size of 1 to 2 mm of diameter for about 4 weeks, they were aseptically transferred to solid MS medium (Murashige and Skoog, 1962) without growth regulators at a density of 30 embryos per Petri dish. The dishes were incubated in a growth room at 25°C with 16 h light /8 h dark photoperiod, at a light intensity of 80 - 100 $\mu E\ m^{-2}\ s^{-1}$. Approximately 2 weeks after embryos transfer, the numbers of regenerated plantlets (green and albino) were counted.

Data collection

Data were recorded as numbers and as percentages. All percentages were calculated based upon the total number of cultured microspores. Number of induced microspores, dividing cells and proembryos were counted under an inverted microscope.

All data were based on a minimum of 3 replications per experiment for each particular treatment or genotype. Each experiment contains only one variable.

The data were statistically analysed using ANOVA conducted using SAS computer software (1988). Mean comparisons were made with the student test. A difference was considered significant at P=0.05.

RESULTS

Spikes were collected when microspores were at the late uninucleate stage (Figure 1a). Subsequently, tillers containing spikes at the desired development stage could be pre-selected on the basis of their morphology (Figure 1b).

Microspore embryogenesis and haploid plant regeneration

In freshly isolated durum wheat microspores cv. “Razzek” (Figure 2a), the first induction occurred 3 days after culture and were observed in the fraction of enlarged microspores

Table 1. Composition of CHB3 medium used for durum wheat isolated microspores culture based on medium of (Chu et al., 1990) modified.

Components	Quantity
Macro elements (gl^{-1})	
KNO_3	1.415
$(NH_4)_2\ SO_4$	0.232
KH_2PO_4	0.200
$CaCl_2$, $2H_2O$	0.083
$MgSO_4$, $7H_2O$	0.093
$FeCl_3$	0.027
Na_2EDTA	0.0373
Micro elements (mgl^{-1})	
$ZnSO_4$, $7H_2O$	5
$MnSO_4$, $7H_2O$	5
H_3BO_3	5
KI	0.4
Na_2MO_4, $2H_2O$	0.0125
$CuSO_4$, $5H_2O$	0.0125
$CoCl_2$, $6H_2O$	0.0125
Vitamins (mgl^{-1})	
Glycine	1
Thiamine HCl	2.5
Pyridoxine HCl	0.5
Nicotinic acid	0.5
Biotine	0.25
Calcuim panthotenate	0.25
Aminoacids (mgl^{-1})	
Glutamine	1000
Myo-Inositol	300
Growth regulators (mgl^{-1})	
2,4-D	0.5
Kinetin	0.5
Maltose gl^{-1}	90
pH	5.4

(Figure 2b). These divisions led to the formation of embryogenic microspores that presented a fibrillar cytoplasmic structure (Figures 2c and 2d) which appeared after 7 days of culture in CHB3 meduim. Only those were able to develop into proembryos. Ten days after culture, a fraction of proembryo emerged from exine wall (Figure 2e) and can develop into mature embryos after 25 days on culture (Figure 2f).

Embryos floating on the surface of the induction medium (Figure 3a) were formed from individual micro-spores by direct embryogenesis. These embryos were transferred to the solid regeneration medium and a fraction of them regenerated directly into green (Figure 3b) or albino plantlets (Figure 3c).

Effect of pre-treatments

Eight pre-treatments were compared in the present study on cv. “Razzek”, in order to select those more efficient to induce embryo formation and plant regeneration in durum wheat by isolated microspores culture (Table 2).

Isolated microspores were induced from all kind of pretreatments and also for the control after 3 days of culture except 15 days PEG_{4000} 1%. This pre-treatment was lethal to microspores. The absence of pre- treatment permits induction of microspores with frequency of 12.16 and 0.002% at 3 and 7 days of culture, respectively.

However, no embryogenesis was obtained without pre-treatment. For all pre-treatments, the viability of micro-spores decreased during the culture and between the third and seventh day of culture ranged from 4 to 17% (Table 1). No strong differences were found in isolated microspores response to mannitol based pre-treatments. Indeed, the first cell divisions could happen but no microspore could develop into an embryo.

The best results were obtained with cold pre-treatment

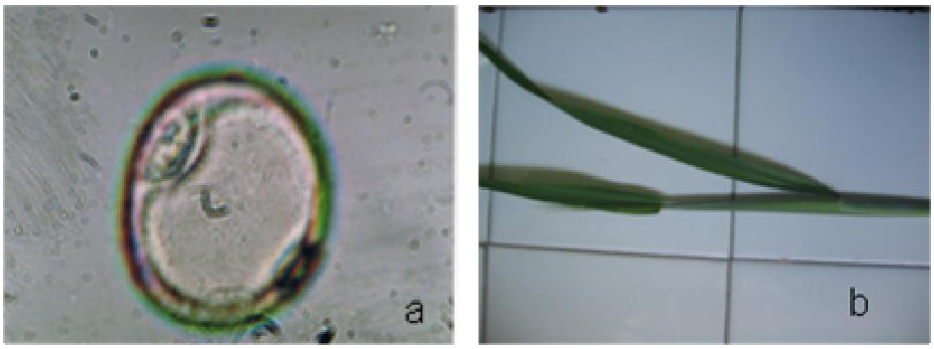

Figure 1. Cytological and morphological stages of isolated microspores culture of durum wheat. a: Optimal cytological stage of culture (late uninucleate stage). b: Optimal morphological stage corresponding to optimal cytological stage.

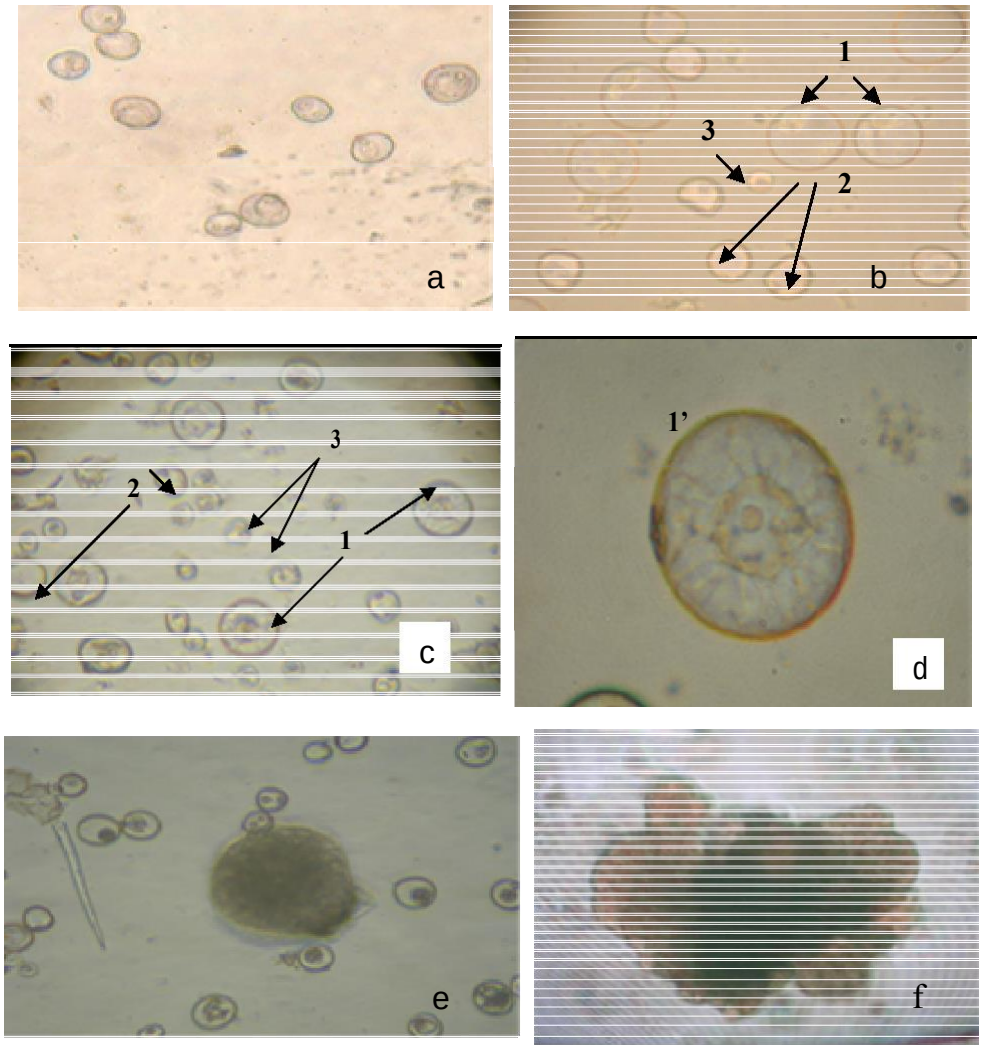

Figure 2. Durum wheat microspore embryogenesis main stages: a. Freshly isolated durum wheat microspores (Gx200); b. Microspores suspension after 3 days in culture : enlarged microspores (1); microspores arrested in the initial stage (2); degenerated microspores (3) (Gx200); c- Dividing microspores after 7 days in culture with fibrillar structure (1)(Gx200) and (1')(Gx400) ; d-microspores with delayed induction (2); degenerated microspores (3)(Gx200); e. Proembryo began to emerge from the exine 10 days after culture initiation (Gx200); f. Embryos formed after 20 - 25 days after culture in medium CHB3 observed with stereoscope.

Table 2. The effect of pre-treatments on isolated microspore culture response of durum wheat cv. " Razzek".

Pre-treatment	Total number of extracted and cultivated microspores	Number and (%) of enlarged microspores		Number and (%) of pro-embryos	Number and (%) of transferred embryos	Number and (%) of regenerated plantlets	
		3 days of culture	7 days of culture			Albina	Green
No pre-treatement	375000	45600 (12.16) abc	8 (0.002) b	-	-	-	-
5 weeks cold at 4°C	225000	31635(14.06) ab	23805(10.58) a	6345 (2.82) a	270 (0.12)	8 (2.96)	-
0.3M Mannitol for 7 days	450000	58680 (13.40) abc	15345 (3.41) b	11 (0.024) b	-	-	-
0.3M Mannitol for 7 days	450000	58680(13.40) ab	15345 (3.41) b	11 (0.024) b	-	-	-
0.7M Mannitol for 5 days	300000	57810 (19.27) a	7440 (2.48) b	-	-	-	-
PEG_{4000} 1.5% for 5 days	150000	7320 (4.88) bc	-	-	-	-	-
PEG_{4000} 1% for 10 days	150000	26085(17.39) ab	6165 (4.11) b	79(0.053) b	46(0.03)	8 (17.4)	-
PEG_{4000} 1% for 15 days	150000	-	-	-	-	-	-

PEG: Polyéthylène glycol; M : Molarity. Number and <% in a same column followed by different letter are significantly different at P=0.05.

First, stress treatment is essential to induce formation of embryogenic microspores. Many authors reported the importance of pre-treatment for successful isolated microspores culture in cereals and suggest a necessity of specific stress treatment to trigger microspore development from gametophytic to sporophytic pathways (Jähne and Lörz, 1995; Hu and Kasha, 1999; Touarev et al., 2000; Liu et al., 2002 and Zheng et al., 2003). Ramirez et al. (2001) showed that in barley, the osmotic regulation was favourable to switch the microspore program regardless of the nature of the osmoticum.

In durum wheat, different results were obtained with cold pre-treatments and cold mannitol by (Labbani et al., 2005; Cistué et al., 2006; Labbani et al., 2007). In our study, we compare these pre-treatments: cold or cold with osmotic pre-treatments including mannitol and other osmotic pre-treatments using PEG at different concentrations and at different time intervals.

Considering our results obtained from isolated microspores culture of durum wheat cv. "Razzek", the best result was achieved with cold pre-treatment (4°C) for 5 weeks that appeared to be the most efficient treatment for embryogenesis induction and embryos development of durum wheat. These results are in agreement with previous studies in bread wheat (Ohnoutkovà et al., 2000; De Buyser et al., 2002) and in durum wheat (Labbani et al., 2005). It was proposed by Duncan and Heberle (1976) that cold pre-treatment slows down degradation processes in the anther tissues thus protecting microspores from toxic compounds released in the decaying anthers. Furthermore, the use of cold pre-treatment improved the capacity of microspores of cv. "Khiar" to regenerate green plants. Hoekstra et al. (1993) showed also the importance of the osmotic pressure during the pre-treatment in order to obtain green plantlets. Our results allow also confirming that embryogenesis and regeneration were strongly dependent on the type of osmotic-cum used. In barley, the combination of cold (4°C) and osmotic (mannitol) regulation during pre-treatment was recently found to be more appropriate using isolated microspores culture (Hoekstra et al., 1993). Mannitol has been recognized to penetrate cells passively and slowly (Cram, 1984) because the plasmalemma is poorly permeable to it (Rains, 1989). Wojnarowiez et al. (2004) showed that mannitol induces both a stable osmotic pressure in the pre-treatment medium and a starvation for microspores during 3 days at 4°C. When mannitol is used at high concentration, water is unable to penetrate into the microspores, probably leading to their alteration. In contrast, in durum wheat, Cistué et al. (2006) improved the rate of regeneration with 0.7M mannitol for 5 to 6 days at 24°C. In our work, the absence of embryogenesis that we noted with cold mannitol pre-treatments at different concen-trations and at different times may be related to the recalcitrance of the genotype used.

Some genotypes naturally respond better than others to pre-treatment applied (Liu et al., 2002).

Table 3. Comparison of induction, embryogenesis and plant regeneration in isolated wheat microspores cultures of 3 genotypes of durum wheat pretreated with cold (4°C) for 5 weeks.

Genotype	Total number of microspores extracted and cultured	Percentage of induction (enlarged microspores)		Percentage of pro-embryos	Number of embryos transferred	Number of regenerated plantlets	
		3 daysculture	7 days culture			Green	Albina
Razzek	600000	14.12 a	9.08 a	2.25 ab	769	-	23
Khiar	450000	17.96 a	7.04 ab	4 a	414	43	23
Azizi	300000	10.18 a	- b	- b	-	-	-

Numbers in the same column followed by different letters are significantly different at 0.05 level by ANOVA test based on a minimum of 3 replications per experiments. The number of regenerated plants was based on the number of transferred embryos.

According to Li and Devaux (2001), the high and low responding genotypes have different requirements for optimal pre-treatment conditions in the same species. Labbani et al. (2005) used two varieties of durum wheat and the quantity and quality of embryos obtained depended on the variety used for the same pre-treatment. Similarly, Li and Devaux (2001) showed that mannitol (0.3M), which is used frequently in barley and which has improved the embryogenesis and regeneration of green plants, proved ineffective in certain recalcitrant barley genotypes. One main problem with some geno-types and some pre-treatments (like mannitol in our case) appeared between one and two weeks of culture. The majority of pro-embryos cannot burst out the exine of microspores (Hu and Kasha, 1999).

We showed that PEG_{4000} 1% for 10 days pre- treatment has a beneficial effect on isolated microspores response with high frequency of induction at 3 days of culture and regenerated plantlets. The beneficial effect of PEG is in agreement with previous report of Wojnarowiez et al. (2004) that tested mannitol and PEG at different steps of anther culture in barley to elucidate their influence on both, the overall yield of androgenesis and the structure of plastids in relation to albinism. These authors showed that the use of PEG during pre-treatment was more favourable than mannitol for inducing anther response. The high viscosity of the PEG solution and the restricted water supply led to a high number of plantlets. This sugar is not metabolized by plant cells and on their presence, plastids did not accumulate starch at any stage of the protocol but they started to differentiate into chloroplasts in the microspore-derived embryos.

Considering the rate of embryogenesis obtained with cold PEG_{4000} 1% for 10 days, it is important to perform new experiments with PEG in the aim to confirm their beneficial influence in durum wheat microspores culture and improve their response.

Second important point for the success of microspores culture in durum wheat is obtaining embryogenic microspores with fibrillar structure at 7 days in culture. Only this multicellular structure was embryogenic. In the first week of culture the viability of microspores decreased between 4 and 17% depending on the evolution of microspores. The microspores were capable of following one of three pathways. A fraction of microspores underwent plasmolysis, another fraction of microspores remained blocked in the initial stage. Another fraction of them increased in size with fibrillar structure. Only the latter types of microspores can be embryogenic. The same types of cells were observed in bread wheat by Zheng et al. (2001). This result is in accordance with previous work. Ramirez et al. (2001) showed in barley that most of the cells which responded to the stress treatment were vacuolate microspores indicating that this developmental stage is responsive for embryogenesis induction in barley. Shariatpanahi et al. (2006) proposed that in some species like wheat, that we still do not know exactly what feature of the microspores can be introduced as universal sign of the embryogenic (totipotent) state. Whereas, microspores do not have a starlike cytoplasmic structure but show an increase in the size of the cell and symmetrical divisions can be embryogenic. Our results suggest also that rates of induced microspores at one week from 7% indicated a possible embryogenic evolution.

Third important factor in durum wheat microspores culture is the genotypic effect during the production of haploid plants. This factor has been reported by several research groups (Labbani et al., 2005; Cistué et al., 2006; Slama et al., 2006; Labbani et al., 2007) . As the experiment described in our work, it is possible that only albino plants obtained for cv. “Razzek” and the high frequency of green plants for cv. “Khiar” regenerated with the same method were a characteristic of the vegetal materiel used. Zamani et al. (2000) reported that embryos formation and green plants regeneration were strongly affected by the genotype of donor plants and the induction medium, while albino production was affected only by the genotype. These two genotypes can be exploited for studies about understanding the mechanism of albinism.

In this work, among the three genotypes tested two were responsive and produced embryos and regenerated plantlets. For cv. “Khiar”, from a total of 414 embryos which were transferred on to the regeneration medium, 66 plants were regenerated and 43 of them were green.

Thus, in this cultivar pretreated with cold 4°C for 5 weeks, up to 10% of the transferred embryos were regenerated in green plants and more than 65% of regenerated plantlets were green. The frequency of green regenera-tion derived from isolated microspores cultured of cv. “Khiar” reported here gave the best frequency of microspore green plant regeneration published until now in durum wheat were albinism seemed to be the major problem (Cistué et al., 2006). In fact, Jaiti et al. (2000) obtained 15 green plants from 1097 plants regenerated from 2824 embryos of durum wheat microspores culture cultivated that represented a rate of 1.31%. In another experience performed by Labbani et al. (2007), 85 green plants were regenerated from 3673 embryos that represented a frequency of 2.31%.

These results indicated that the use of cold pre-treatment improves frequency of green plant regene-ration. Yet the number of regenerated plantlets still stay low, some progress has been obtained with durum wheat green regeneration with 43 green plants regenerated and only 23 albina (green /albino ratio 2). Green plant regeneration rates obtained in this study were satisfactory with durum wheat where albinism seemed to be the major problem and that considered as a recalcitrant cereal.

The present protocol of microspore culture, in durum wheat, may be widely applicable for increasing yield of green plant regeneration. This result encouraged the potential for using isolated microspores of durum wheat for breeding and biotechnological applications. However, further studies on the optimization of pre-treatments and protocols are recommended.

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