



Somatic embryogenesis and plant regeneration capacity from mature and immature durum wheat embryos

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Abstract

Seven Moroccan durum wheat genotypes were tested on callus induction and plantlet regeneration from immature and mature embryos cultures. The genotypes tested were: Karim, Sebou, Isly, Jawhar, Anouar, Ourgh and Massa. For all the cultivars, five parameters were considered: Callogenesis, zygotic germination, Callus growth, percentage of embryogenic calli, percentage of regeneration and Number of plantlets per regenerating callus (NPRC). The results of the study showed that morphogenetic capacity of callus was influenced by the genotype and explant sources. Immature embryos gave much higher levels of embryogenic callus and plant regeneration than mature embryos. The highest regeneration capacity was observed from immature embryos in Anouar, Karim, Ourgh and Sebou. Therefore, they could be used in *in vitro* selection. Among the regenerated plants, albino plants have been observed from immature embryo calluses. Other variations were also observed after transfer of plantlets to soil such as abnormalities in spike morphology and leaf coloration.

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Introduction

Plant regeneration from *in vitro* tissue culture is a critical step in the application of biotechnology techniques in genetic improvement. In grasses, regeneration from cells and tissues is still a limiting factor to the application of these techniques in breeding programs. In wheat, several *in vitro* regeneration studies have been achieved from various explants such as immature embryos (Ahloowalia, 1982; Bennici *et al.*, 1988; Bouiamrine *et al.*, 1999, Hafeez *et al.*, 2012), the leaf segments (Zamora and Scott, 1983; Yu *et al.*, 2012), immature inflorescences (Ozias-Akins and Vasil, 1982; He and Lazzeri, 2001), coleoptiles (Benkirane *et al.*, 2000) and mature embryos (Ozias-Akins and Vasil 1983; Özgen *et al.*, 1998). However, the immature embryos are the explant of choice for induction of somatic embryogenesis and regeneration both in bread wheat (Özgen *et al.*, 1996) and in durum wheat (He and Lazzeri, 2001). The only drawback, however, is their unavailability throughout the year in contrast to mature embryos.

The response of wheat tissue culture to callus induction and plant regeneration also depends on several factors including the composition of culture medium (Mathias and Simpson, 1986; Mzouri and Amssa, 2002b; Ren *et al.*, 2010), the explant used (Ozias-Akins and Vasil, 1982; Redway *et al.*, 1990), physiological status of the source plant (Redway *et al.*, 1990; Hess and Carman, 1998), but mainly the genotype (Mzouri *et al.*, 2001; Vendruscolo *et al.*, 2008). Therefore, the choice of genotypes that may possibly be integrated into a breeding program involving plant biotechnology technique lies mainly in their ability to produce embryogenic calli and to regenerate plants.

The aim of the present work is to study the morphogenic capacities of seven Moroccan genotypes of durum wheat from two types of explants: the immature and mature embryos. This study is a part of a program of *in vitro* selection we have undertaken in our laboratory to improve the tolerance to drought stress in durum wheat.

Material and methods

Plant materials and explant preparation

Seven genotypes of wheat (*Triticum durum* Desf.) were evaluated for their ability to somatic embryogenesis and plant regeneration. The genotypes were: Karim, Sebou, Isly, Jawhar, Anouar, Ourgh and Massa whose seeds were provided by INRA (National Institute for Agricultural Research, Morocco). Callogenesis was induced from two types of explants: mature and immature embryos. The immature embryos were collected from seeds in the milky phase, approximately 14-16 days after anthesis. At this stage, the embryos have a size between 1 and 1.5 mm. The caryopses were disinfected under continuous stirring for 20 to 30 seconds in 70° ethanol and then for 15 minutes in 30% bleach and rinsed three times in sterile distilled water (under laminar flow).

As for the mature embryos, the caryopses were disinfected by soaking in ethanol (95°) for one minute and then passing through a 2.5% formaldehyde solution stirring for 20 minutes followed by rinsing twice by sterile distilled water and finally soaking in 20% bleach for 15 min with stirring. Under laminar flow, the caryopses were rinsed three times in sterile distilled water and were then soaked and incubated on filter paper in sterile Petri dishes for 20 hours.

Preparation of media and cultivation

The nutrient medium of Murashige and Skoog (1962) was used as the basic medium modified for the callogenesis (MC), for regeneration (R1) and for rooting (R2) (Table 1). Prepared media were sterilized by autoclaving at 120 °C for 20 minutes. Mature and immature embryos were aseptically dissected from seeds and placed with the scutellum upwards on a solid agar medium in sterile Petri dishes. The cultures were then incubated in the dark in growth chamber maintained at 25±2°C. After 40 days of culture on callus medium (MC), the calli were then subcultured on R1 regeneration medium and placed in a growth chamber under a photoperiod of 16 hours of light/24 hours.

After five weeks of culture on regeneration medium R1, calli with shoots were then transferred to the rooting

medium R2. The regenerated plantlets were then transferred to the soil after having developed a good root system.

Parameters evaluation

The studied parameters were calculated for each genotype, using the following formulae:

- Percentage of callus induction = (number of calli / total number of explants cultured) \times 100;
- Percentage of zygotic germination = (number of explants germinated / total number of explants) \times 100;
- Percentage of embryogenic callus = (number of embryogenic calli / total number of calli induced) \times 100;
- Regeneration percentage = (number of calli with regenerated seedlings / total number of calli) \times 100;
- The number of plantlets per regenerating callus (NPRC) was estimated by counting regenerated plantlets after five weeks of culture on R2. Counting was done during the transfer of plantlets to soil for acclimatization;
- The fresh weight of callus was determined on 40-day calli chosen at random and individually weighed using an electric balance.

Ex vitro transfer and acclimatization

The green seedlings having reached the two-leaf stage with a good root development were transferred to soil in pots covered with plastic in the form of mini-greenhouse to maintain saturated moisture conditions. The pots were then placed in a growth chamber at a temperature of $25 \pm 2^\circ\text{C}$ for 3 weeks. The plantlets were finally transferred into large pots under natural conditions.

Statistical analysis

Statistical analysis of data was carried out using the R statistical environment (R Development Core Team, 2012). Data were analyzed using the analysis of variance technique and comparison of means was done by LSD test. The student's t-test was applied at a probability level of $p = 0.05$ to find significant differences between the means.

Results

Callogenesis and zygotic germination

Observations made during the incubation of cultures showed that the callus is induced after 3-5 days of seeding in immature explants and after 5 to 8 days for mature explants. The calluses observed after 1 month of incubation allowed us to distinguish differences in color and morphology. Thus, it was possible to distinguish compact smooth calli, compact calli wholly or partly nodular (Figure 1a) termed embryogenic and non-embryogenic calli that look translucent and viscous (Fig. 1b). The latter generally have a size larger than the compact calluses. The morphology and appearance of callus induced depend on the nature of the explant used. Indeed, nodular compact calli are rarely induced from mature explants.

Table 1. Media composition of callogenesis (MC) regeneration (R1) and rooting (R2).

Medium	MC	R1	R2
Components			
Macroelements	MS	MS/2	MS/2
2.4-D (mg l^{-1})	2	0.2	-
BAP (μM)	-	10	-
ANA (μM)	-	5	-

2.4-D = 2,4-Dichlorophenoxyacetic Acid

BAP = benzylaminopurine

NAA = naphthalene acetic acid

Table 2 presents the results obtained on percentage of callus formation, zygotic germination percentage and fresh weight of callus obtained from two types of explants for the seven genotypes. The results for the induction of callus showed that callus formation was induced in all immature explants. Mature embryos callus was affected by genotype and varied significantly from 82.05 to 93.85 in Karim and Sebou, respectively. Zygotic germination was also affected by the genotype from the two types of explants. In immature embryos, this germination varied between 19.40 (in Jawhar) and 47.80 (in Sebou). In mature embryos, this variation ranged from 58.14 (Sebou) to 84.20 (Isly). Callus growth was also influenced by genotype in the two types of explants used.

Table 2. Performance of seven durum wheat genotypes evaluated in relation to percentage of callus induction, embryo germination and callus weight from immature and mature embryos.

Explants	Genotype	Callus Induction (%)*	Germinated embryos (%)*	Weight of callus (mg)*
Immature embryos	Karim	100	33.03 ± 2,09 b	66.2 ± 1.23 c
	Sebou	100	47.80 ± 2,87 a	82.83 ± 3,38 ab
	Isly	100	46.34 ± 4,00 a	75 ± 3,00 b
	Anouar	100	32.25 ± 1,96 b	63.2 ± 1,68 c
	Ourgh	100	38.76 ± 0,53 b	66.56 ± 2,73 c
	Jawhar	100	19.40 ± 1,66 c	84.53 ± 2,48 a
	Massa	100	22.90 ± 0,58 c	78.83 ± 3,20 ab
Mature embryos	Karim	82.05 ± 1,06 c	70.19 ± 1,40 b	77.83 ± 4,05 bc
	Sebou	93.85 ± 0,38 a	58.14 ± 3,46 c	98.26 ± 1,68 a
	Isly	86.28 ± 1,80 bc	84.20 ± 2,53 a	86.56 ± 4,54 b
	Anouar	82.70 ± 1,73 c	70.41 ± 0,50 b	73.23 ± 2,79 c
	Ourgh	86.23 ± 3,89 bc	73.36 ± 0,81 b	68.23 ± 1,71 c
	Jawhar	90.96 ± 1,18 ab	69.37 ± 1,22 b	101.5 ± 4,45 a
	Massa	86.703 ± 1,05 bc	59.69 ± 2,38 c	97.76 ± 2,74 a

* Means, within the same column and embryo type, lacking a common letter differ at 0.05 according to LSD Test

Table 3. Performance of seven durum wheat genotypes evaluated in relation to percentage of embryogenic callus and regeneration capacity from immature and mature embryo derived calli.

Explants	Genotype	Embryogenic callus (%)*	Regeneration (%)*	NPRC*
Immature embryos	Karim	82.50 ± 1,68 b	89.99 ± 1,92 a	21,08 ± 0,18 a
	Sebou	74.97 ± 1,75 c	88.88 ± 1,11 a	18,35 ± 0,36 ab
	Isly	54,01 ± 1,74 e	73,33 ± 3,85 b	15,66 ± 2,35 b
	Anouar	89,69 ± 1,99 a	93,33 ± 1,92 a	20,41 ± 0,51a
	Ourgh	91,43 ± 0,37 a	92,22 ± 1,11 a	18,95 ± 0,47 ab
	Jawhar	65,51 ± 0,75 d	59,99 ± 3,33 c	15,62 ± 2,69 b
	Massa	57,08 ± 0,83 e	48,88 ± 4,00 d	10,46 ± 1,33 c
Mature embryos	Karim	52,66 ± 1,76 a	57,77 ± 2,93 a	7,97 ± 0,32 b
	Sebou	47,33 ± 5,45 a	38,88 ± 2,93 b	6,24 ± 0,25 c
	Isly	35,33 ± 2,40 b	31,10 ± 2,93 b	5,08 ± 0,21 d
	Anouar	50 ± 5,77 a	62,21 ± 2,93 a	9,17 ± 0,22 a
	Ourgh	44,66 ± 2,40 ab	52,22 ± 4,00 a	6,31 ± 0,19 c
	Jawhar	10 ± 1,15 c	11,10 ± 2,22 c	3,56 ± 0,77 e
	Massa	4,66 ± 0,66 c	6,65 ± 3,83 c	3,54 ± 0,11 e

* Means, within the same column and explant type, lacking a common letter differ at 0.05 according to LSD Test

NPRC = Number of plantlets per regenerating callus

Table 4. Statistical results (t-test) for comparison between mature and immature embryos response in seven durum wheat varieties.

Parameters	Mat. emb.	Imm. emb.	t-test for Equality of means (Critical Value of t)	p-value*
	Mean	Mean		
Callogenesis	100,00	86,96	12,296	8.829e-11
Ger. emb.	69,33	34,35	-11,5174	2.82e-14
WC	86,20	73,88	-3,4605	0.001445
EC	34,95	73,60	7,3267	1.019e-08
Reg	37,13	78,09	6,808	3.474e-08
NPRC	5,98	17,22	2,021075	5,617e-14

* Significant at $p \leq 0.05$ by a Student t-test

Mat. Emb: Matures embryos. Imm. Emb: Immatures embryos. Ger. Emb: Germinated embryos WC: Weight of callus. EC:

Embryogenic callus. Reg: regeneration. NPRC = Number of plantlets per regenerating callus.

Embryogenic and regeneration capacity

Table 3 illustrates the results obtained for embryogenic competence, regeneration capacity and Number of plantlets per regenerating callus (NPRC) for the seven genotypes tested from both types of explants.

These results show a very marked effect genotype in the production of embryogenic calluses from both types of explants. For all genotypes, calluses from immature embryos are more embryogenic than mature embryos. Ourgh and Anouar, for instance, showed very high embryogenic capacities from immature embryos reaching respectively 91.43% and 89.69%. From the mature embryos, beside Karim (52.66% embryogenic callus), two genotypes also showed embryogenic capacity with the high rates of 44.66% for Ourgh and 50% for Anouar.

Observations made on cultures during the regeneration phase (Figure 1c) allowed to note that germination of somatic embryos begins after 4-6 days of culture on regeneration medium R1. This germination was sometimes accompanied by rhizogenesis. Different calli obtained responded differently to culture conditions of the regeneration phase. In some cases we saw calluses producing only roots that are sometimes with chlorophyll. In some other cases, depending on the genotype, the calli retain their white appearance continuing to grow on regeneration medium or tending to become necrotic. These types of calluses remained non-morphogenic, even after an extended period on the regeneration medium R1 or after transfer to R2 medium. Table 3 shows results on regeneration in seven genotypes. The genotype effect is very marked on regeneration capacity of callus from both types of explants. The four genotypes Anouar, Ourgh, karim and Sebou showed very high rates of regeneration of calluses from immature embryos. These rates were respectively 93.33%, 92.22%, 89.99% and 88.88% for the four genotypes. The same genotypes also showed the highest rates from mature embryos. The low levels of regeneration were obtained in Massa and Jawhar for calluses from both immature and mature embryos.

The average Number of plantlets per regenerating callus (NPRC) was determined after five weeks of culture on

R2, just before the transfer of plantlets to soil (Figure 1d). The results show that the NPRC is also affected by the genotype in both cultures from immature and mature embryos. Karim, Anouar, Sebou and Ourgh cultivars, which showed very high rate of regeneration from immature embryos, also showed high NPRC equaling respectively 21.08, 20.08, 18.95 and 18.35.

Comparison between mature and immature embryos

Table 4 summarizes the comparison of two types of explants for all parameters studied in seven genotypes: callogenesis, zygotic germination, weight of callus, embryogenic competence, regeneration percentage and NPRC. For all parameters, differences between the two explants were significant. The immature embryos showed callogenesis, embryogenesis, regeneration and NPRC much higher than in mature embryos. The latter, however, showed higher zygotic germination and higher callus growth.

Variation in morphological characteristics

The observations made during the regeneration phase allowed to note the presence of callus with only albino plantlets, with both green and albinos seedlings (Figure 1e), with abnormal-leaf seedlings and with chimeric seedlings. Genotypes in which albinism was observed are Karim, Isly and Anouar. The observed rates are however very low and do not exceed 6%. In all three genotypes, albinism was observed only in calli from immature embryos. No albino plants were regenerated from calli coming from mature embryo culture.

After a period of acclimatization in plastic pots containing garden soil covered with transparent plastic (Figure 2a), the plantlets were transferred into large pots under natural conditions. The observation of the regenerated plants after their transfer to the ground showed few phenotypic variations, in some somaclones from Karim, Sebou, Isly and Ourgh, pertaining mainly to the morphology of the spike (Figure 2b) and the leaf pigmentation. However the majority of these mature plants were phenotypically normal (Figure 2c).

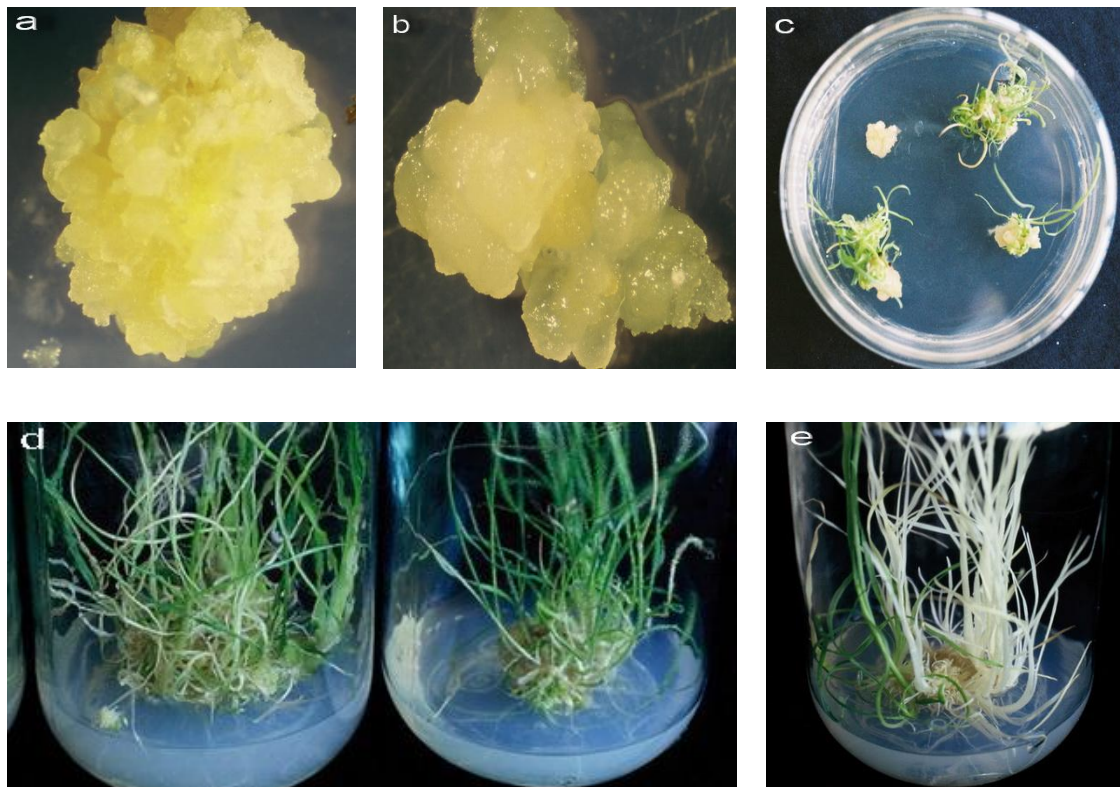


Fig. 1. Callus induction and plant regeneration in durum wheat.

- a. Embryogenic nodular calli induced from immature embryos explant.
- b. Soft and watery non-embryogenic calli from mature embryos explant.
- c. Germination of somatic embryos on R1 medium.
- d. Shoots and Roots Development on MShormone-free medium (R2).
- e. Albino and green shoots development on MS hormone-free medium (R2) from callus induced from immature embryos explant of Karim.

Discussion

Somatic embryogenesis and plant regeneration from *in vitro* tissue culture in wheat are affected by several factors including the composition of culture medium, the nature and age of the explant and especially genotype (Mathias and Simpson, 1986; Redway *et al.*, 1990; Özgen *et al.*, 1998; Mzouri *et al.*, 2001).

The study we undertook clearly shows the effect of genotype and explant on *in vitro* morphogenic capacity in the studied durum wheat varieties. Observation of calluses induced from the two types of explants allowed us to distinguish two types of calluses: the compact and nodular embryogenic calluses, which give birth to somatic embryos, and non-embryogenic calli, which look translucent and viscous. These types of callus were

observed frequently and described from different types of explants both in wheat (Nabors *et al.*, 1983; Chowdhury *et al.*, 1991; Mzouri *et al.*, 2001) and other cereal species (Vasil and Vasil, 1981; Šerhantová *et al.*, 2004). The results we obtained also show that callus growth is affected by the genotype. These results are consistent with those reported by several authors (Nasircilar *et al.*, 2006; Malik *et al.*, 2003). Malik *et al.* (2003) also reported that growth of the matter is rapid in non-embryogenic callus and is positively correlated with the weight of dry matter.

Analysis of results showed clearly the importance of genotype in most of the studied parameters. The callogenesis from immature embryos was the only

parameter where the genotype effect was not observed. Indeed, the induction of this type of explant was 100% in the seven varieties and depended on the genotype only when the explants were mature embryos. Results of 100% callus from immature embryos in wheat were reported by several studies (Gonzalez *et al.*, 2001; Malik *et al.*, 2004). The response of mature embryos to callogenesis is generally lower than in the immature embryos in grasses (Özen *et al.*, 1996; Rahman *et al.*, 2008).

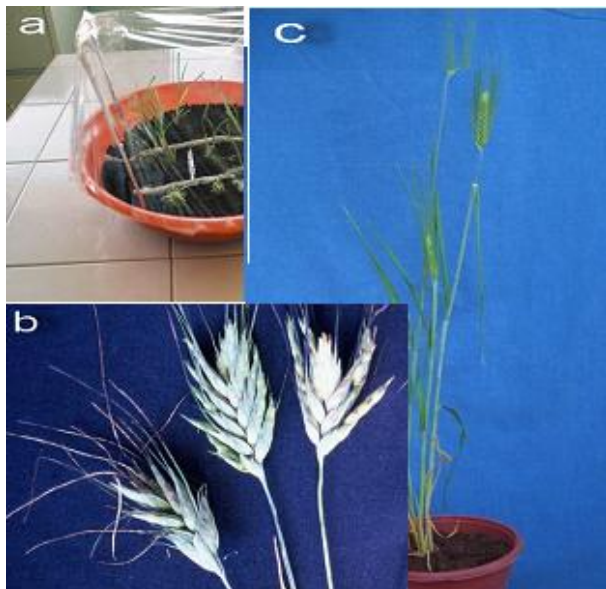


Fig. 2. Vitroplants after transfer to ex vitro conditions
 a. Plantlets in pots covered with clear plastic as a mini-greenhouse
 b. Abnormalities in spike morphology
 c. Morphologically normal plants reaching maturity

Zygotic germination is also a parameter affected by the genotype. Our results agree with those reported in wheat (Bapat *et al.*, 1988; Mzouri *et al.*, 2001; Chevrier *et al.*, 1990) and in other grass species (Abumhadi *et al.*, 2005). The highest frequencies of this germination were observed in mature embryos. This finding was also reported in barley (Abumhadi *et al.*, 2005). Mzouri and Amssa (2002a) studied the effect of the developmental stage on morphogenesis and germination in wheat and concluded that the embryos cultivated at late stages (larger than 2 mm) have higher germination rate than those cultivated at less advanced stages. A correlation between the age of the embryo and the endogenous content of growth regulators, particularly abscisic acid (ABA) has also been reported (Qureshi *et al.*, 1989).

Indeed, embryos at early stages synthesize enough of this dormancy hormone, which explains the lower germination frequency (Gaspar *et al.*, 1996). The embryos at advanced stages, contain less ABA, which promotes early germination. Germination of embryos in culture is considered undesirable because it prevents the proper development of calluses. To overcome this problem, Chlyah *et al.* (1990) demonstrated that zygotic germination can be avoided by fragmented embryos. The use of small reduced explants has also allowed to obtain high embryogenic capacity cultures.

The genotype effect was also observed for parameters related to embryogenic and regeneration capacities of cultures from both mature and immature embryos. These results agree with several studies conducted on wheat (Bennici *et al.*, 1988; Özgen *et al.*, 1996; Bouiamrine *et al.*, 1999) and many cereal species such as maize (Ombori *et al.*, 2008, Manivannan *et al.*, 2010), barley (Sharma *et al.*, 2005), rice (Kahnna *et al.*, 1998). This genetic variability in the production of somatic embryogenesis and plant regeneration appears to be due to the endogenous content of growth regulators and more specifically the cytokine/auxin ratio (Carman *et al.*, 1987). Genetic control of the ability to *in vitro* culture in wheat has been the subject of several studies (Higging and Mathias 1987; De Buyser *et al.*, 1992; Tyankova *et al.*, 2006). However, information on genetic mechanisms and the number of genes involved in this genetic control remain largely unknown (Bregitzer and Campbell 2001; Tyankova *et al.*, 2006). Studies have reported however that the *in vitro* regeneration in wheat appears to be a quantitative effect character, (Bregitzer and Campbell, 2001) and can be transmitted by heredity (Chevrier *et al.*, 1990).

The choice of using mature embryos as explants for callus induction and regeneration is based mainly on their availability throughout the year. In the culture conditions reported by Özgen *et al.* (1998), mature embryos showed embryogenesis and regeneration capacities higher than immature embryos. The mature embryos were even used as explants in several searches for genetic improvement of certain genotypes (Galovic *et*

al., 2010; Farshadfar *et al.*, 2012; Benderradji *et al.*, 2012).

The nature of the explants has also an effect on callus formation, somatic embryogenesis and regeneration. In fact, the immature embryos showed much higher morphogenetic capacities than mature embryos in all studied genotypes. In durum wheat, the importance of choice of explant for callus induction and regeneration has been the subject of several studies. Best results are, however, obtained from immature embryos (Redway *et al.*, 1990, He and Lazzeri, 2001). It was also reported that the developmental stage of immature embryo also affects the yield of somatic embryogenesis and regeneration (Mzouri and Amssa, 2002b). A correlation between the age of the embryo and the endogenous content of growth regulators, particularly abscisic acid (ABA) has been reported by Qureshi *et al.* (1989). Indeed, their work showed that embryos at early stages synthesize enough acid to ABA, which in turn stimulates somatic embryogenesis and decreases the ability of early germination of embryos. Contrarily, mature embryos contain less ABA, which promotes germination in the culture medium.

The observation of the regenerated plants before transfer to the ground showed morphological changes, including the presence of plantlets with abnormal leaves, albino and chimerical plants.

The number of albino plantlets regenerated remained very low. Indeed, Chlorophyll deficiency or albinism is common in androgenesis in durum wheat and is a major problem for the application of haplodiploidisation in breeding programs of this species (Ghaemi and Sarrafi, 1994). In somatic embryogenesis, conversely, few reports have mentioned regeneration of albinos (Maddock *et al.*, 1983; Bouiamrine *et al.*, 1999).

After transfer to ground, other changes were also observed, concerning especially plant size, spike morphology and leaf pigmentation. Indeed, *in vitro* culture is a source of variability, called somaclonal variation (Larkin and Scowcroft, 1981). These variations can be genetic and therefore persist over generations

and are transmitted by the crossing of epigenetic type. The next work will be devoted to the study of the observed somaclonal variation.

Conclusion

The regenerative potential is affected by the genotype and the nature of explant used. Anouar, Ourgh, Karim and Sebou varieties showed high frequencies of regeneration from mature and immature embryos. These genotypes showed suitable regeneration capacities for use in subsequent programs of *in vitro* selection.

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