International Scholars Journals

International Journal of Biochemistry and Biotechnology ISSN: 2169-3048 Vol. 5 (5), pp. 702-710, May, 2016. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Effects of media, varieties and their interaction on callus induction and plant regeneration in bread wheat

Ahmed M. Bidouane[1,3], Brahim Rhissassi[2], Fouzia Lahlafi[3], and Simone Ohayon[1]*

[1]Biotechnology Unit, Institut National de la Recherche Agronomique (INRA), Avenue de la Victoire, B.P. 415, Rabat, Morocco.

[2]ICARDA-INRA Cooperative Research Project, International Center for Agricultural Research in the Dry Areas (ICARDA), B.P. 6299, Rabat, Morocco.

[3]Laboratoire d"agrophysiologie et de culture *in vitro* Ibn Tofail University, Faculty of Sciences, BP: 133, 14000 Kenitra, Morocco.

Accepted 27 April, 2016

A study was conducted at Biotechnology Research Unit of National Institute of Agronomic Research (INRA), Morocco during the year 2013. In this study, the effects of media, varieties and their interaction on callus induction and plant regeneration in bread wheat obtained from immature and mature embryos, used as explants, were studied. Four Moroccan bread wheat varieties 'Achtar', 'Arréhane', 'Marchouch' and 'Mehdia' and five induction and maintenance media (M1 to M5) were used to study effects of genotype and medium on embryogenic callus induction and plantlets regeneration. A significant effect of variety, medium and variety × medium interaction was observed for callus induction and regeneration from both immature and mature embryo explants. With respect to plantlets regeneration, the induction and maintenance media used for callus induction had a significant effect on plantlets regeneration ($p<0.001$). M1 (60.44%) and M4 (52.55%) showed higher plantlets regeneration rates from immature embryos and M3 (58.23%) for plantlets regeneration rates from mature embryos. The plantlets regeneration varied significantly depending on the varieties and the induction media used. Using immature embryos, the favorable medium was M1 for the varieties 'Arréhane' and 'Mehdia', whereas, M1 and M3 for 'Achtar' and M4 medium was favorable for 'Marchouch'. For the mature embryos, the favorable medium was M3 for the varieties 'Achtar', 'Arréhane' and 'Mehdia', whereas M4 was the favorable medium for 'Marchouch' variety. These media will be used for embryogenic callus induction from mature and immature embryos and for genetic transformation.

Key words: Bread wheat, immature embryos, mature embryos, regeneration, somatic embryogenesis.

INTRODUCTION

Genetic engineering of wheat is likely to play an increasingly important role in improving agronomic traits such as quality, disease resistance, salinity and drought tolerance. Wheat has remained to be difficult in the transgenic study, mainly due to the lack of explants with high regeneration efficiency (Yu et al., 2008).

In cereals, only cells and immature or young tissues can be induced to somatic embryogenesis, as they respond efficiently to regeneration (Repellin et al., 2001), In wheat, different explant sources were studied for somatic embryogenesis: shoot tips (Viertel and Hess, 1996), seeds (Gosch-Wackerle et al., 1979), nflorescences (Redway et al., 1990), young leaves (Zamora and Scott, 1983), mature embryos (Delporte et al., 2001; Wu et al., 2002), immature embryos (Macchii et al., 1998), and anthers (Brisibe et al., 2000). Immature embryos seem to be the best explant source for callus induction and somatic embryogenesis of cereals (Wu et al., 2002; Pellegrineschi et al., 2004). Therefore , wheat

*Corresponding author. E-mail: ohayon1980@gmail.com

transgenic plants have been obtained frequently using immature embryos as explant (Tang et al., 2006). However, because immature embryos are usually difficult to obtain throughout the year and their suitable stage for culture is strictly limited, recently the focus has shifted towards mature embryos. The use of mature embryos has many advantages. Thus, mature embryos are easy to handle, easy to store and they are always available.

The main inconvenience with mature embryo as explant is the low plant regeneration, which make them difficult to use in practice. It is well known that the frequencies of callus induction and plant regeneration in wheat tissue culture depend completely on medium composition (Fennell et al., 1996; Iraqi et al., 2005) and explant sources (Zhang and Seilleur, 1987; Redway et al., 1990). Zhou and Lee (1983) first reported that mature embryo of wheat could be used to induce callus and obtain successful regenerated plantlets. In order to improve culture efficiency, many factors affecting embryogenesis development and plant regeneration have been studied widely in wheat mature embryos culture. Ozgen et al. (1998) investigated 12 common winter wheat genotypes from mature embryos and found all cultivars had the ability to form callus and regenerate shoots, but there were strong genotype differences. Mendoza and Kaeppler (2002) compared the effects of different auxins and carbon source on callus growth of the wheat cultivar „Bobwhite" and observed that these factors could significantly improve the rates of callus induction and plant regeneration on optimal media. However, the regeneration efficiency of mature embryo-derived callus is still relatively low and generally obtained either by using model cultivar or by using endosperm-supported callus induction. Not only it is complicated to operate, but also not conducive to the application of this technology.

In addition, regeneration efficiency, from immature embryos, of different genotypes ranged from 0 to 80% (Ye et al., 2002; Wang and Fan, 2006). Several authors have studied somatic embryogenesis of wheat cultivars in European (Pastori et al., 2001), Asian (Arzani and Mirodjagh, 1999; Wu et al., 2002), Mexican (Fennell et al., 1996), Australian (Witrzens et al., 1998), and Chinese genotypes (Tang et al., 2006). However, few papers were found in the literature dealing with the regeneration performance of Moroccan genotypes. Therefore, it is very important to establish an efficient regeneration system for the Moroccan wheat from immature embryos to promote the research in the genetic transformation and to determine the suitable media for induction from mature embryos.

To improve the regeneration system of Moroccan well adapted bread wheat variety „Tilila", a medium MS + L-Asparagine (MS Asp), which allows efficient, reproducible and fast regeneration, has been developed by Iraqi et al. (2005). However, this medium did not give the desired results with other bread wheat varieties.

Several studies used MS medium as media for immature embryos culture with different combinations of components as sugars and growth regulators (Murashige and Skoog, 1962; Wu et al., 2002; Eudes et al., 2003; Iraqi et al., 2005; Karim et al., 2005; Gadaleta et al., 2006; Pellegrineschi et al., 2002; Przetakiewicz et al., 2003).

The main objective of this study is to compare the effects of media, varieties and their interaction on callus induction and plant regeneration in bread wheat obtained from immature and mature embryo as explants. While doing so, we defined media suitable for callus induction and plant regeneration of Moroccan bread wheat varieties using immature and mature embryos as explants.

MATERIALS AND METHODS

Plant materials and explants preparation

This study was conducted at Biotechnology Research Unit, National Institute of Agronomic Research (INRA), Morocco during 2013. The seeds of the bread wheat cultivars „Achtar", „Arréhane", „Marchouch" and „Mehdia" were procured from Experimental Research Station of INRA at Marchouch, Rabat, Morocco. Field grown seeds of the bread wheat cultivars were used as source of mature embryos for *in vitro* culture, while greenhouse grown bread wheat plants were used as source of immature embryos.

The immature seeds were surface-sterilized by washing in ethanol 70% (v/v) for 3 min, followed by a bath of 2.4% sodium hypochlorite plus a drop of Tween 20 for 15 min with agitation. Thereafter, they were rinsed three times in sterile distilled water.

Sterilization of mature seeds was done in the same way the immature seeds were sterilized except that the duration of sodium hypochlorite bath was increased to 30 min. The disinfected mature seeds were soaked in sterile distilled water overnight to facilitate the embryos excision.

Immature and mature embryos were aseptically dissected away from the caryopses and the remaining endosperm and radical removed to prevent early germination. The embryos were placed in a Petri dish containing the induction medium (M1 to M5; Table 1). The treatments consisted of 6 replications of each medium for each variety, each replication with 8 immature embryos or 8 mature embryos. The embryos were incubated on the media in the dark at 25°C for five weeks and the callus diameter and weight were recorded respectively each week and at the end of callogenesis and their relative fresh weight growth rate (RFWGR) of callus were estimated according to Daud et al. (2012) as follow:

$RFWGR = (FW_f - FW_i) / FW_i \times 100$, where FW_f = final fresh weight and FW_i = initial fresh weight.

Table 1. Composition of the five induction media used.

Variable	Medium tested				
Components	M1	M2	M3	M4	M5
Macroelements	MS	MS	MS	MS	MS
Oligoelements	MS	MS	MS	MS	MS
Vitamins	MS	MS	Thiamin	MS	B5
Fe-EDTA	MS	MS	MS	MS	MS
L-asparagine (mg/l)	150	-	150	-	-
Myo-Inositol (mg/l)	100	100	100	100	100
Sucrose (g/l)	20	20	-	30	20
Maltose (g/l)			40		
2,4-D (mg/l)	2	2.5	1	2.5	3
BA (mg/l)	-	2.5	-	-	-
pH	5.7- 5.8	5.7- 5.8	5.7- 5.8	5.7- 5.8	5.7- 5.8
Phytagel (g/l)	2.5	2.5	3.5	-	2.5
Bacto agar (g/l)	-	-	-	8	-

2,4-D - 2,4 Dichlorophenoxyacetic acid; BA = 6-benzylaminopurine; B5 = B5 medium (Gamborg et al., 1968); MS = Murashige and Skoog medium.

Plant regeneration

After five weeks, embryogenic calli from each replication were transferred to the regeneration medium described by Iraqi et al. (2005) and incubated in the light (16 h per day) and temperature of 25°C. The medium of regeneration was composed of MS medium supplemented with 100 mg/L of Myo-inisitol, 2 mg/L of IAA (Indole-3-acetic acid) and 30 g/L of sucrose. The medium was solidified by using 3 g/L phytagel. pH was adjusted to 5.7 before sterilization at 120°C for 20 min. The IAA and vitamins (MS) had been sterilized by filtration and added in the medium after cooling. The regeneration rate was calculated eight weeks after transfer of callus. Percentage of plants regeneration was calculated as follows:

(the number of plantlets regenerated / the number of callus transferred to the regeneration medium) × 100.

Experimental design and statistical analysis

A randomized complete block design (RCBD) was used with 8 varieties and 5 media (8×5 = 40 treatments). The treatments consisted of 6 replications of each medium for each variety, each replication with 8 immature embryos or 8 mature embryos. For the analysis of diameter, weight and RFWGR of callus and percentage of plants regeneration, Analysis of Variance (ANOVA) was performed using the General Linear Model (GLM) procedure in SAS, SAS Institute (1995). Residuals were normally distributed, thus no data transformation was required. Mean of treatments were compared using Duncan‟s Multiple Range Test (Steel and Torrie, 1980).

RESULTS

Callus initiation and growth

We observed two kinds of calli in the cultures: non-embryogenic calli were characterized by cream to brownish color and a soft, loose, watery nature, whereas; embryogenic calli were characterized by more pale in color, smooth and compact (Figure 1). Mixtures of embryogenic calli surrounded by non-embryogenic calli were also observed in most of the explants.

Callus growth was influenced by variety and media (Tables 2 and 3). From the four varieties tested, „Achtar‟ showed the highest mean for immature embryos. Callus diameter (8.39 mm) after 5 weeks of incubation of explants on different media, followed by „Arréhane‟, „Marchouch‟, and „Mehdia‟ where callus diameter was around 6.5 mm (Table 4). For mature embryos, callus diameter of „Achtar‟ and „Marchouch‟ showed the greater means (6.89 mm and 6.49 mm respectively), whereas „Arréhane‟ and „Mehdia‟ showed a mean around 5.5 mm (Table 5). Among the five media, immature embryos callus from M1, M3 and M5 media showed greater mean diameters (more than 7 mm), whereas, M2 and M4 medium showed a mean of 6.51 and 6.37 mm respectively (Table 6). With respect to the mature embryos-derived callus, all media except M4 (5.51 mm) showed a mean greater than 6 mm (Table 7). However, all the genotypes showed increasing callus growth upon culturing the explant (mature and immature embryos) on all the media, but at different levels. For example, in „Mehdia‟ variety, the rate of increase of immature callus diameter was proportional to incubation time and higher callus growth rates were observed for M1 (6.90 mm), M3

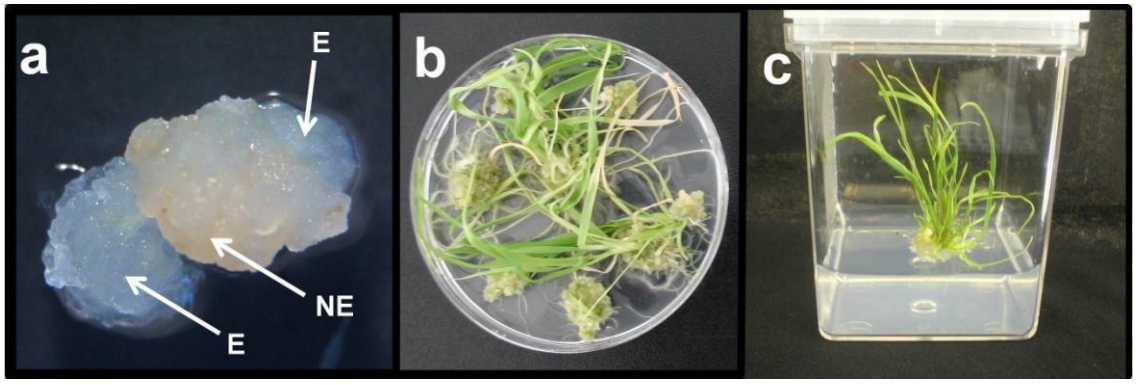

Figure 1. Embryogenesis and plant regeneration from wheat embryos: (a) Embryogenic callus surrounded by non-embryogenic (NE) callus formation after 4 weeks of culture. NE callus was characterized by cream to brownish color and a soft, loose, watery nature, whereas, embryogenic (E) callus was more pale in color, smooth and compact. (b) and (c) Plantlet regeneration on the regeneration medium.

Table 2. Analysis of variance for effects of variety, medium and their interaction on callus diameter, callus weight and relative fresh weight growth rate (RFWGR) of immature embryos callus and on plantlet regeneration (%).

Variable		F values							
		Callus diameter for 5 weeks					Callus weight	RFWGR	Plantlet regeneration (%)
Source	DF	Week 1	Week 2	Week 3	Week 4	Week 5	After 5 weeks	After 5 weeks	
Variety	3	5.27**	15.16***	23.64***	41.88***	55.46***	36.58***	39.69***	5.33**
Medium	4	4.19***	7.82***	9.79***	11.24***	10.43***	8.57***	7.26***	25.25***
Variety × medium	12	2.09*	2.17*	3.08**	4.85***	6.14***	1.82	2.24*	3.23**

*Significant at $p < 0.05$; **Significant at $p < 0.01$; ***Significant at $p < 0.001$.

Table 3. Analysis of variance for effects of variety, medium and their interaction on callus diameter, callus weight and relative fresh weight growth rate (RFWGR) of mature embryos callus and on plantlet regeneration (%).

Variable		F values							
		Callus diameter for 5 weeks					Callus weight	RFWGR (%)	Plantlet regeneration (%)
Source	DF	Week 1	Week 2	Week 3	Week 4	Week 5	After 5 weeks	After 5 weeks	
Variety	3	23.29***	27.71***	23.97***	32.76***	21.96***	35.07***	14.39***	5.73**
Medium	4	1.6	2.24	3.3*	4.55**	4.51**	3.11*	2.09	9.75***
Variety × medium	12	1.98*	2.7**	2.34*	1.98*	2.26*	2.78**	0.85	3.3***

*Significant at $P < 0.05$; **Significant at $P < 0.01$; ***Significant at $P < 0.001$.

(7.22 mm) and M5 (7.46 mm) and lower growth rates were observed for M4 (4.94 mm). For the same variety, in mature embryos culture, the higher growth rate were observed for M2 (6 mm), M3 (6.68 mm) and M5 (5.98 mm) and lower growth rate were observed for M1 (5 mm) and M4 (4,42 mm) with respect to callus diameter measured after 5 weeks of incubation of the explants.

Relative fresh weight growth rate (RFWGR) calculated after 5 weeks of incubation of immature embryo explants differed significantly among varieties across media (Table 4); „Marchouch‟ recorded the highest rate (4404.7%), followed by „Mehdia‟, „Achtar‟ and „Arréhane‟ (less than 2600%). With respect to the mature embryo derived callus, „Marchouch‟ recorded the highest RFWGR (3087.5%), followed by „Mehdia‟ (2615.1%), whereas, „Achtar‟ (2076.2%) and „Arréhane‟ (1747.9%) were the

Table 4. Mean callus diameter and relative fresh weight growth rate (RFWGR) of immature embryos callus obtained on five induction and maintenance media of the individual four bread wheat varieties and their effect on plantlet regeneration (%).

Variety	Mean			
	Callus diameter (mm)	Callus weight (mg)	RFWGR (%)	Plantlet regeneration (%)
Achtar	8.39^{a}	570.37^{b}	2385.7^{b}	44.42^{ab}
Arréhane	6.33^{b}	502^{b}	2250.1^{b}	39.48^{bc}
Marchouch	6.64^{b}	929.69^{a}	4404.7^{a}	47.71^{a}
Mehdia	6.44^{b}	580.48^{b}	2555.7^{b}	32.67^{c}

Note: The values followed by the same alphabet are not significantly different at alpha = 0.05 according to the Duncan"s Multiple Range Test.

Table 5. Mean callus diameter and relative fresh weight growth rate (RFWGR) of mature embryos callus obtained on five induction and maintenance media of the individual four bread wheat varieties and their effect on plantlet regeneration (%).

Variety	Mean			
	Callus diameter (mm)	Callus weight (mg)	RFWGR (%)	Plantlet regeneration (%)
Achtar	6.89^{a}	506.78^{b}	2076.2^{c}	49.57^{a}
Arréhane	5.39^{b}	319.27^{c}	1747.9^{c}	32.83^{b}
Marchouch	6.49^{a}	699.84^{a}	3087.5^{a}	39.91^{b}
Mehdia	5.62^{b}	559.43^{b}	2615.1^{b}	36.68^{b}

Note: The values followed by the same alphabet are not significantly different at alpha = 0.05 according to the Duncan"s Multiple Range test.

Table 6. Mean callus diameter and relative fresh weight growth rate (RFWGR) of callus from immature embryos of four bread wheat varieties obtained after 5 weeks of culturing on individual induction media followed by 8 weeks culture on regeneration medium and their effect on plantlet regeneration (%).

Medium	Mean			
	Callus diameter (mm)	Callus weight (mg)	RFWGR (%)	Plantlet regeneration (%)
M1	7.25^{a}	684.48^{b}	3094.8^{ab}	60.44^{a}
M2	6.51^{b}	557.08^{c}	2251.8^{c}	22.39^{c}
M3	7.29^{a}	790.6^{a}	3508.5^{a}	45.55^{b}
M4	6.37^{b}	532.63^{c}	2602.2^{bc}	52.55^{ab}
M5	7.33^{a}	663.39^{b}	3038.1^{ab}	26.16^{c}

Note: The values followed by the same alphabet are not significantly different at alpha = 0.05 according to the Duncan"s Multiple Range Test; M1 to M5 are the induction and maintenance media used. For composition of media, please refer to Table 1.

Table 7. Mean callus diameter and relative fresh weight growth rate (RFWGR) of callus from mature embryos of four bread wheat varieties obtained after 5 weeks of culturing on individual induction media followed by 8 weeks culture on regeneration medium and their effect on plantlet regeneration (%).

Medium	Mean			
	Callus diameter (mm)	Callus weight (mg)	RFWGR (%)	Plantlet regeneration (%)
M1	6.06^{a}	465^{b}	2405.1^{ab}	35.83^{b}
M2	6.18^{a}	553.95^{a}	2213.9^{ab}	35.61^{b}
M3	6.26^{a}	584.07^{a}	2651.5^{a}	58.23^{a}
M4	5.51^{b}	469.89^{b}	2050.4^{b}	36.53^{b}
M5	6.47^{a}	533.73^{ab}	2587.4^{a}	32.47^{b}

Note: The values followed by the same alphabet are not significantly different at alpha = 0.05 according to the Duncan"s Multiple Range test; M1 to M5 are the induction and maintenance media used. For composition of media, please refer to Table 1.

Table 8. Effect of medium and variety on relative fresh weight growth rate (RFWGR) of callus from immature embryos.

	RFWGR (%)				
Variable	**M1**	**M2**	**M3**	**M4**	**M5**
Achtar	2597.4d^{efg}*	1999.8^{efg}	2804.4^{cdef}	1914.1^{efg}	2612.9^{cdefg}
Arréhane	2028.8^{efg}	2188^{defg}	2248.3^{defg}	1740.1^{fg}	3045.5^{cde}
Marchouch	5191.1^{a}	3273.3^{cd}	5227^{a}	4562.8^{ab}	3769.5^{bc}
Mehdia	2561.8^{defg}	1546.2^{g}	3754.2^{bc}	2191.7^{defg}	2724.4^{cdefg}

Note: The values followed by the same alphabet are not significantly different at alpha = 0.05 according to the Duncan"s Multiple Range Test; M1 to M5 are the induction and maintenance media used. For composition of media, please refer to Table 1.

Table 9. Effect of medium and variety on relative fresh weight growth rate (RFWGR) of callus from mature embryos.

	RFWGR (%)				
Variable	**M1**	**M2**	**M3**	**M4**	**M5**
Achtar	2264.6^{abcde}	1678.2^{de}	2106.6^{bcde}	2022.5^{cde}	2309.3^{abcde}
Arréhane	1421.9^{e}	1470.6^{e}	2065.2^{cde}	1421.9^{e}	2374.3^{abcde}
Marchouch	3358.4^{a}	3257.9^{ab}	3026.4^{abc}	2711.2^{abcd}	3083.5^{abc}
Mehdia	2575.6^{abcde}	2429^{abcde}	3407.7^{a}	2060.4^{cde}	2582.6^{abcde}

Note: The values followed by the same alphabet are not significantly different at alpha = 0.05 according to the Duncan"s Multiple Range test; M1 to M5 are the induction and maintenance media used. For composition of media, please refer to Table 1.

Table 10. Effect of different induction media on plantlets regeneration of bread wheat immature embryos.

	Plantlet regeneration (%)				
Variable	**M1**	**M2**	**M3**	**M4**	**M5**
Achtar	60^{abc}	17.6^{h}	64^{ab}	50^{bcdef}	26.6^{gh}
Arréhane	67^{ab}	17^{h}	39.6^{defg}	41^{cdefg}	23.8^{gh}
Marchouch	59.25^{abcd}	30.75^{fgh}	33.25^{fgh}	76.2^{a}	32^{fgh}
Mehdia	54^{bcde}	22.75^{gh}	30.5^{fgh}	35^{efgh}	23.4^{gh}

Note: The values followed by the same alphabet are not significantly different at alpha = 0.05 according to the Duncan"s Multiple Range Test; M1 to M5 are the induction and maintenance media used. For composition of media, please refer to Table 1.

lowest (Table 5). RFWGR calculated after 5 weeks of incubation of the immature embryo explants also differed significantly among media across varieties (Table 6): M3 recorded the highest (3508.5%) followed by M1 (3094.8%), and M5 (3038.1%), whereas M2 was the lowest (2251.8%). With regards to the mature embryo explants, M3 (2651.5%) and M5 (2587.4%) provided the highest RFWGR, followed by M1 (2405.1%) and M2 (2213.9%), whereas M4 (2050.4%) was the lowest (Table 7). Callus production from immature embryos was strongly influenced by the media and the variety used (Table 2). A significant ($p<0.05$) interaction between variety and medium was observed (Table 2). RFWGR of callus calculated after 5 weeks of culture on different induction media showed that the highest RFWGR was observed on M3 for varieties „Achtar" and „Mehdia", M1 and M3 for „Marchouch" and M5 for „Arréhane" (Tables 8 and 9).

Plantlet regeneration

The plantlet regeneration (Figure 1) recorded after 8 weeks culturing of callus (5 weeks old) on regeneration medium is presented in Tables 10 and 11. The induction and maintenance media used for callus induction had a significant effect on plantlets regeneration ($p<0.001$). M1 (60.44%) and M4 (52.55%) showed higher plantlets regeneration rates from immature embryos (Table 6) and M3 (58.23%) for plantlets regeneration rates from mature embryos (Table 7).

The varieties of bread wheat used had a significant effect on plantlets regeneration ($p<0.01$). In general, from immature embryos, the varieties „Achtar" and „Marchouch" produced higher plantlets regeneration (more than 44%) across different induction media, and „Mehdia" (32.67%) produced significantly lower plantlets regeneration (Table 4). From mature embryos, „Achtar" (49.57%) produced higher plantlet regeneration and the other genotypes were less than 40% (Table 5).

The plantlets regeneration also varied significantly depending on the varieties × the induction media used (Tables 10 and 11). Using immature embryos, the favorable medium is M1 for the varieties „Arréhane" and „Mehdia", whereas M1 and M3 for „Achtar" and M4 medium is favorable for „Marchouch" variety. For the mature embryos, the favorable medium is M3 for the

Table 11. Effect of different induction media on plantlets regeneration of bread wheat mature embryos.

Plantlet regeneration (%)					
Variable	**M1**	**M2**	**M3**	**M4**	**M5**
Achtar	42.2^{bcdefg}	44.25^{bcdefg}	80.25^{a}	37.5^{cdefg}	45.5^{bcdef}
Arréhane	22.2^{g}	23.6^{fg}	62^{ab}	25.25^{efg}	29.6^{efg}
Marchouch	46.75^{bcde}	32^{efg}	33^{defg}	54.4^{bcd}	31.8^{efg}
Mehdia	34^{defg}	43.6^{bcdefg}	56.75^{bc}	24.5^{efg}	25.6^{efg}

Note: The values followed by the same alphabet are not significantly different at alpha = 0.05 according to the Duncan"s Multiple Range Test; M1 to M5 are the induction and maintenance media used. For composition of media, please refer to Table 1.

varieties „Achtar", „Arréhane" and „Mehdia", whereas M4 is the favorable medium for „Marchouch" variety.

DISCUSSION

Callus production from mature embryos was influenced only by the variety used (Table 3). The effect of the presence of 2,4-D (2,4- Dichlorophenoxyacetic acid) and its concentration depended on the genotype used. For Achtar, Mehdia and Marchouch varieties, M3 medium which contain 1 mg/l of 2,4-D was the best. These results are in accordance with those of Mendoza and Kaeppler (2002), in which the authors found that a decrease in 2,4-D from 2 to 1 mg/l had increased the callus fresh weight by 60%. However, when 2,4-D was increased to 3 mg/l (as in the case of M5), Arréhane variety responded favorably. Similar observations were made by Munazir et al. (2010), who also found that increasing 2,4 D from 2 to 3 mg/l increased the percentage of callus induction and callus growth.

The M3 medium, which contains maltose, seems to have enhanced plantlets regeneration. It has been reported that inclusion of maltose in the induction medium gives rise to higher quality of embryos and causes a higher rate (57%) of conversion of embryos into plantlets (Indrianto et al., 1999). Maltose also maintains viability of isolated wheat (Indrianto et al., 1999). The positive effect of maltose on regeneration were explained by its capacity on maintaining osmolarity in the medium over the whole culture period due to its low hydrolysis to glucose (Orshinky et al., 1990; Indrianto et al., 1999). The results of this work are in agreement with the work of Mendoza and Kaeppler (2002) which indicated that substitution of sucrose by maltose enhanced the regeneration ability of callus from embryos of wheat. The results also confirm the work of Gadaleta et al. (2006), which showed that inclusion of 40 g/L of maltose as unique carbon source had resulted in germination of wheat embryos and developed into plants. Moreover, M3 medium differ with respect to auxin 2,4-D (1 mg/l). Generally, decreasing concentrations of 2,4-D, from 3 or 2 mg/l to 1 mg/l increased rate of plantlets production(Tables 10 and 11).

It has been shown previously that the increasing concentration of 2,4-D had a detrimental effect on callus (increased appearance of brownish and necrotic callus) derived from mature bread wheat embryo, resulted in poor plantlet regeneration (Mendoza and Kaeppler, 2002). Therefore, M3 medium promoted the higher production of embryogenic callus. Solidifying agent used in our study (3.5 g/L of phytagel in M3 compared to 2.5 g/L used in others media) could also had played a role in the conversion of embryos to plantlets. Our results are in agreement with those of Gugsa and Kumlehn (2011). These authors reported that the concentration of phytagel between 3 and 5 g/L is suitable for shoot regeneration of cereals such as 'Ethiopian millet'.

On the other hand, M4 induction medium, which contained 30 g/l of sucrose and 8 g/l of solidifying agent agar, gave the best results with respect to regeneration of Marchouch variety. Bommineni and Juhar (1996) found that callus from induction medium containing 3% of sucrose produced more plantlets on regeneration medium than callus from induction medium containing 2% of sucrose. They found also that 0.8% of agar was optimal for callus development and plantlets regeneration of wheat.

Conclusion

The present study tested and identified the favourable media for induction and regeneration *in vitro* from mature and immature embryos of Moroccan bread wheat varieties. These media will be used for embryogenic callus induction from mature and immature embryos and for genetic transformation. In addition, we found that it is possible to use mature embryos for future experiments of genetic transformation. The use of mature embryos has many advantages: mature embryos are easy to handle, easy to store and they are always available.

ACKNOWLEDGMENTS

DI and SMU are grateful to ICGEB, Italy for generous

funding. SMU is grateful to the ICARDA/Morocco Collaborative Grants Program for the support.

REFERENCES

Arzani A, Mirodjagh S (1999). Response of durum wheat cultivars to immature embryo culture callus induction and in vitro salt stress. Plant Cell Tiss. Org. Cult. 58: 67-72.

Bommineni VR, Jauhar PP (1996). Regeneration of plantlets through isolated scutellum culture of durum wheat. Plant Sci. 116: 197-203.

Brisibe EA, Gajdosava A, Olesen A, Andersen S (2000). Cytodifferentiation and transformation of embryogenic callus lines derived from anther culture of wheat. J. Exp. Bot. 51: 187-196.

Daud MK, Shafaqat A, Variath MT, Zhu SJ (2012). Antioxidative enzymes status in upland cotton callus culture under osmotic stresses. International Conference on Computational Techniques and Artificial Intelligence (ICCTAI'2012), Penang, Malaysia, available at http://psrcentre.org/images/extraimages/212160.pdf

Delporte F, Mostade O, Jacquemin JM (2001). Plant regeneration through callus initiation from thin mature embryo fragments of wheat. Plant Cell Tiss. Org. Cult. 67: 73-80.

Eudes F, Acharya S, Laroche A, Selinger LB et Cheng KJ (2003). A novel method to induce direct somatic embryogenesis, secondary embryogenesis and regeneration of fertile green cereal plants. Plant Cell Tiss. Org. Cult. 73: 147-157.

Fennell S, Bohorova N, Ginkel M, Crossa J, Hoisington D (1996). Plant regeneration from immature embryos of 48 elite CIMMYT bread wheats. Theor. Appl. Genet. 92: 163-169.

Gadaleta A, Giancaspro A, Belchl A, Blanco A (2006). Phosphomannose isomerase, *pmi*, as a selectable marker gene for durum wheat transformation. J. Cereal Sci. 43:31-37.

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.

Gosch-Wackerle G, Avivi L, Galun E (1979). Induction, culture and differentiation of callus from immature rachis, seeds and embryos of Triticum. Z. Pflanzenphysiol. 91: 267-278.

Gugsa L, Kumlehn J (2011). Somatic embryogenesis and massive shoot regeneration from immature embryo explants of Tef. Biotechnol. Res. Int. 2011: 1-7

Indrianto A, Heberle-Bors E, Touraev A (1999). Assessment of various stresses and carbohydrates for their effect on the induction of embryogenesis in isolated wheat microspores. Plant Sci. 143: 71-79.

Iraqi D, Hakam N, Labhilili M (2005). Transformation génétique des embryons immatures du blé tendre (*Triticum aestivum*) et du blé dur (*Triticum durum*). (Genetic transformation of immature embryos of bread wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*). Al Awamia Moroccan J. Agric. Res. 115(2): 3-16. (in French)

Karim R, Chlyah H, Badoc A, Douira A (2005). Obtention de pieds néoformés suite à l‟induction de cals embryogènes d‟embryons zygotiques de blés par le borate de sodium et un extrait de fusarium graminearum. (Obtaining neogenic foot following the induction of embryogenic callus of zygotic embryos of wheat by sodium borate and an extract of Fusarium graminearum). Bull. Soc. Pharm. Bordeaux 144:195-210. (in French)

Machii H, Mizuno H, Hirabayashi T, Li H, Hagio T (1998). Screening wheat genotypes for high callus induction and regeneration capability and immature embryo cultures. Plant Cell Tiss. Org. Cult. 53: 67-74.

Mathias RJ, Simpson ES (1986). The interaction of genotype and culture medium on the tissue culture responses of wheat (*Triticum aestivum* L. em. thell) callus. Plant Cell Tiss. Org. Cult. 7(1): 31-37.

Mendoza MG, Kaeppler HF (2002). Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (*Triticum aestivum* L.). In Vitro Cell. Dev. B. 38: 39-45.

Munazir M, Qureshi R, Ghulam MA, Umer R, Sabahat N, Khalid M, Shoukat A, Andmuhammad A (2010). Primary callus induction, somatic embryogenesis and regeneration studies in selected elite wheat varieties from Pakistan. Pakistan J. Bot. 42(6): 3957-3965.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum 15: 473-497.

Orshinsky BR, McGregor LJ, Johnson GI, Hucl P, Kartha KK (1990). Improved embryoid induction and green shoot regeneration from wheat anthers cultured in medium with maltose. Plant Cell Rep. 9: 365-369.

Ozgen M, Turet M, Altinok S, Sancak C (1998). Efficient callus induction and plant regeneration from mature embryo culture of winter wheat genotypes. Plant Cell Rep. 18: 331-335.

Pastori GM, Wilkinson MD, Steele SH, Sparks CA, Jones HD, Parry MAJ (2001). Age-dependent transformation frequency in elite wheat varieties. J. Exp. Bot. 52: 857-863.

Pellegrineschi A, Brito RM, McLean S, Hoisington D (2004). Effect of 2,4 dichlorophenoxyacetic acid and NaCl on the establishment of callus and plant regeneration in durum and bread wheat. Plant Cell Tiss. Org. Cult. 77: 245-250.

Pellegrineschi A, Noguera LM, Skovmand B, Brito RM, Velazquez L, Salgado MM, Hernandez R, Warburton M, Hoisington D (2002). Identification of highly transformable wheat genotypes for mass production of fertile transgenic plants. Genome 45: 421-430.

Przetakiewicz A, Orczyk W, Nadolska-Orczyk A (2003). The effect of auxin on plant regeneration of wheat,

barley and triticale. Plant Cell Tiss. Org. Cult. 73: 245-256.

Redway FA, Vasil V, Lu D, Vasil IK (1990). Identification of callus types for long term maintenance and regeneration from commercial cultivars of wheat (*Triticum aestivum* L.). Theor. Appl. Genet. 79: 609-617.

Repellin A, Baga M, Jauhar PP, Chibbar RN (2001). Genetic enrichment of cereal crops via alien gene transfer: New challenges. Plant Cell Tiss. Org. Cult. 64: 159-183.

SAS Institute (1985). SAS/STAT guide for personal computers, Ver. 6. SAS Institute, Cary, NC, USA.

Steel RGD, Torrie JH (1980). Principles and procedures of statistics: a biometrical approach, 2nd edn. McGraw-Hill Kogakusha, New York.

Tang ZX, Ren ZL, Wu F, Fu SI, Wang XX, Zhang HQ (2006). The selection of transgenic recipients from new elite wheat cultivars and study on its plant regeneration system. Agr. Sci.China 5: 417-424.

Viertel K, Hess D (1996). Shoot tips of wheat as an alternative source for regenerable embryogenic callus cultures. Plant Cell Tiss. Org. Cult. 44: 183-18.

Wang HB, Fan YL (2006). Researching the mechanism in plant in vitro culture via “communication” experiment and to establish the methods widely used for tissue culture of wheat. Acta Agron. Sin., 32: 964-971.

Witrzens B, Brettell RIS, Murray FR, McElroy D, Li Z, Dennis ES (1998). Comparison of three selectable markers gene for transformation of wheat by microprojectile bombardment. Aust. J. Plant Physiol., 25: 39-44.

Wu BH, Zheng YL, Liu DC, Zhou YH (2002). Trait correlation of immature embryo culture in bread wheat. Plant Breed., 121: 1-5.

Ye XG, Xu HJ, Du LP, Xin ZY (2002). Study on the factors influencing the efficiency of wheat transformation. Acta Agron. Sin. 35: 30-35.

Yu, Y.J. Wang, M.L. Zhu and Z.M. Wei. 2008. Optimization of mature embryo-based high frequency callus induction and plant regeneration from elite wheat cultivars grown in China. Plant Breed, 1-7.

Zamora AB, Scoot KJ (1983). Callus formation and plant regeneration from wheat leaves. Plant Sci. Lett., 29: 183-189.

Zhang LJ, Seilleur P (1987). A simple and fast method to obtain high frequency of plant regeneration from mature and immature wheat embryos. Bull. Rech. Agron. Gembloux, 22: 187-197.

Zhou MD, Lee TT (1983). Selectivity of auxin for induction and growth of callus from excised embryo of spring and winter wheat. Can. J. Bot., 62: 1393-1397.