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EFFECT OF GENOTYPES AND CULTURE MEDIA ON EMBRYOGENIC CALLUS INDUCTION AND PLANTLET REGENERATION FROM MATURE AND IMMATURE BREAD WHEAT EMBRYOS

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ABSTRACT

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', 'Amal', 'Mehdia' and 'Rajae' were cultivated on four media (M1 to M4). A significant effect of variety, medium and variety x medium interaction were observed for callus induction from both immature and mature embryos. All tested media induced embryogenic callus for all varieties. For plantlet regeneration, the induction media used for callus induction had a significant effect on plantlets regeneration ($p<0.001$). M2 (21.988%) with 1.4168 number of plantlets regenerated per callus and 8 number of plantlets per regenerating callus showed higher plantlets regeneration rate from immature embryos and the same medium (20.223%) with 0.8427 of plantlets regenerated per callus and 7 number of plantlets per regenerating callus for plantlets regeneration rate from mature embryos.

The most favourable medium was M2 for all the varieties from both immature and mature embryos. This medium will be used for genetic transformation by Agrobacterium from immature and mature embryos.

Key words: Bread wheat; somatic embryogenesis; picloram; 2, 4–D; plantlets regeneration.

Abbreviations: 2,4–D: 2,4–dichlorophenoxyacetic acid; IAA: Indole-3-acetic acid; MS: Murashige and Skoog medium; RFWGR: Relative fresh weight growth rate.

INTRODUCTION

Bread wheat (*Triticum aestivum L.*) is one of the major staple food crops grown worldwide (Bhalla et al., 2006) that covers almost 17% of total cultivated area of the world. In Morocco, bread wheat is grown over an area of about 2.18 million hectares annually, and ranks the second after barley and durum wheat, with respect to production (MAP, 2012). A number of environmental factors are responsible for low yield, but the main reasons for the decline were abiotic stresses (Khavarinejad, 2012). Drought and salt stress are the most common environmental limitations that cause significant reduction of growth, development and yield of wheat.

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 studies, mainly due to the lack of explants with high regeneration efficiency (Yu et al., 2008).

In cereals, 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

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(Hafeez et al., 2012), leaf segments (Yu et al., 2012), immature inflorescences (He and Lazzeri 2001), coleoptiles (Benkirane et al., 2000) and mature embryos (Özgen et al., 1998). However, the immature embryos are the explants of choice for induction of somatic embryogenesis and regeneration in both bread wheat (Özgen et al., 1996) and in durum wheat (He and Lazzeri 2001). The 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 depends on several factors including the composition of culture medium (Tinak et al., 2013), the explants used (Redway et al., 1990), physiological status of the source plant (Hess and Carman, 1998), but mainly the genotype (Mzouri et al., 2001). When mature embryos are used as explants source, frequency of plant regeneration is low as compared to immature embryo culture. However, it is usually difficult to obtain immature embryos throughout the year, and the suitable stage for their culture is also strictly limited. The use of mature embryos from dry seeds has several advantages: mature embryos are easy to handle, available throughout the year and in bulk quantities. Therefore, mature embryos as favourable explants source are explored broadly in cereal tissue culture.

The aim of this study was to define the suitable media for callus induction and plant regeneration of four Moroccan varieties of bread wheat from two types of explants: the immature and mature embryos, and to obtain a suitable media for genetic transformation of these varieties. While doing so, we compared the effects of media, varieties and their interaction on callus induction and plant regeneration obtained from mature and immature embryo as explants.

MATERIAL AND METHODS

Four Moroccan bread wheat varieties ‘Achtar’, ‘Amal’,’Mehdia’ and ’Rajaa’ were used as sources of immature and mature embryos. The seeds were procured from the Experimental Research Station of INRA at Marchouch, Rabat, Morocco.

Callogenesis was induced from two types of explants: mature and immature embryos. The immature embryos were collected from seeds grown in greenhouse in the milky phase, approximately 12-16 days post-anthesis. The immature seeds were then surface-sterilized by washing in ethanol 70% (v/v) for 3 minutes, followed by a bath of 2.4% sodium hypochlorite plus a drop of Tween 20 for 15 minutes with agitation. Thereafter, they were rinsed three times in sterile distilled water (under laminar flow). Sterilization of mature seeds was done in the same way as immature seed, except the duration of 2.4% sodium hypochlorite bath that increased to 30 min. After sterilization, immature embryos were aseptically excised and placed scutellum up on the culture medium with the embryo-axis in a contact with the medium, mature seeds were imbibed in sterile distilled water for overnight at room temperature to facilitate the embryos excision, and then the mature embryos were aseptically dissected away from the caryopses and the remaining endosperm and radical removed to prevent early germination, and placed in Petri dishes containing the induction medium (M1 to M4; Table 1) .

Table 1. Composition of the callogenesis media

Components	Medium tested			
	M1	M2	M3	M4
Macroelements	MS	MS	MS	MS
Oligoelements	MS	MS	MS	MS
Vitamins	MS	MS	MS	MS
Fe-EDTA	MS	MS	MS	MS
L-asparagine (mg/L)	150	150	150	150
Myo-Inositol (mg/L)	100	100	100	100
Sucrose (g/L)	20	20	20	20
2,4–D (mg/L)	2	-	2	-
Picloram	-	2	2	-
PH	5.7- 5.8	5.7- 5.8	5.7- 5.8	5.7- 5.8
Phytagel (g/L)	2.5	2.5	2.5	2.5

For callus formation and maintenance media, mature embryo and immature embryo explants were transferred to Petri dishes containing M1 to M4 medium. The basal medium used was MS (Murashige and Skoog,

1962) medium modified for the callogenesis (Table 1). The cultures were incubated on the media in the dark at 25°C and sub-cultured two times onto a fresh medium at 2 weeks intervals. After 40 days the callus weight, were recorded at the end of callogenesis and their relative fresh weight growth rate (RFWGR) of callus were determined (Daud et al., 2012).

RFWGR = [(FWf – FWi) / FWi × 100], where FWf = final fresh weight and FWi = initial fresh weight.

For differentiation of callus into shoots, after five weeks, calli that was initiated from immature and mature embryos were transferred to the regeneration medium (Iraqi et al., 2005) and incubated in the light (16 h per day) and temperature of 25°C. The regeneration rate was calculated eight weeks after transfer of the callus. Percentage of plants regeneration was calculated as follows: [(the number of plantlets regenerated/the number of callus transferred to the regeneration medium) × 100], (Tang et al., 2006).

Experimental design and statistical analysis: A randomised complete block design (RCBD) was used with 4 varieties and 4 media (4×4=16 treatments). The treatments consisted of 5 replications of each medium for each variety; each dish contained 20 explants (immature embryos or mature embryos). For the analysis of weight and RFWGR of callus and percentage of plants regeneration, number of plantlets per regenerating callus and number of plantlet per callus, analysis of variance (ANOVA) was performed using the General Linear Model (GLM) procedure in SAS (SAS Institute, 1985). Mean of treatments were compared using Duncan's Multiple Range test (Steel and Torrie, 1980).

RESULTS

Callus initiation and growth

Four media supplemented with different growth regulator of M1 (2,4–D), M2 (picloram), M3 combinations (2,4–D+ picloram) and M4 (MS without hormone) and two different explants immature and mature embryos sources were tested in order to obtain the best wheat callus formation and regeneration. The callus was induced after 3-5 days of seeding in immature explants and after 5 to 8 days for mature explants. For all genotypes, callus production frequency was more than 95% from the two explants, except in the M4 (MS without hormone) which lead to the regeneration of plants similar to those of seedling and there was no callus formation. All embryos on M4 produced shoots and roots (Data not shown).

Table 2. Mean of relative fresh weight growth rate of callus (RFWGR) and callus induction of four immature embryos varieties obtained in three induction and maintenance media after 4 weeks of culturing and their effect on plantlets regeneration (%), number of plantlets per callus

Variety	Callus weight (mg)	RFWGR (%)	Callus Induction (%)	Plantlet regeneration (%)	Number of plantlet regenerated per callus	Number of plantlets per regenerating callus
Achtar	1718.7b*	8061a	96.667a	15.414a	0.5885a	4.870a
Amal	2688.1b	25734a	97.333a	9.813bc	0.3954a	4.244a
Mehdia	6516.0a	33781a	98.000a	7.958c	0.6294a	7.183a
Rajae	2728.5b	18237a	97.000a	11.188ab	0.5354a	5.946a
CD	1579	29394	2.377	2.602	0.2296	3.133
Medium						
M1	3841. a	16841ab	96.750ab	9.047b	0.44387b	5.324a
M2	1668.4b	7446b	97.750a	17.156a	0.7691a	6.737a
M3	4729.0a	40074a	95.500b	5.258c	0.42384b	4.197a
CD	1368	25456	2.058	2.240	0.1976	2.697

For immature embryos, the relative fresh weight growth rate (RFWGR) was calculated after 5 weeks of incubation of the explants, with no significant difference results among varieties across media (Table 2); 'Mehdia' recorded the highest (33781%) and Achtar the lowest (less than 8061%). With respect to the mature embryo derived callus, 'Mehdia' recorded the highest RFWGR (14589%), and 'Achtar' the lowest (6052%) (Table 3). The relative fresh weight growth rate (RFWGR) calculated after 5 weeks the immature embryos explants also differed significantly among varieties across media (Table 2); M3 recorded the highest (40074%) whereas, M2 was the lowest (7446%); with regards to the matures explants, M3 (11035%) provided the highest RFWGR, whereas, M2 (4638%) was the lowest (Table 3).

Callus production from immature embryos was strongly influenced by the media and the variety used (Table 4). A significant ($p<0.001$) interaction between variety and medium was observed (Table 4). RFWGR of callus calculated after 5 weeks of culture on different induction media showed that the highest RFWGR was observed on M3 and M1 for varieties 'Mehdia 'and 'Amal' (Table 5).

Table 3. Mean of relative fresh weight growth rate of callus (RFWGR) and callus induction of four mature embryos varieties obtained in three induction and maintenance media after 4 weeks of culturing and their effect on plantlets regeneration (%), number of plantlets per regenerating callus and number of plantlets regenerated per callus

Variety	Callus weight (mg)	RFWGR (%)	Callus induction (%)	Plantlet regeneration (%)	Number of plantlets regenerated per callus	Number of plantlets per regenerating callus
Achtar	2456.7b*	6052b	96.000a	13.023a	1.0867a	6.858a
Amal	2661.6b	8445b	96.333a	9.857b	0.4676b	5.100a
Mehdia	4742.1a	14589a	96.667a	4.451c	0.3583b	6.400a
Rajae	2763.4b	6355b	95.667a	8.755b	0.4797b	3.900a
CD	1113	3172	2.032	3.819	0.2749	3.455
Medium						
M1	3927.5a	10907a	96.5000a	10.605a	0.69187a	6.154a
M2	1854.4b	4638b	97.0000a	11.955a	0.7566a	7.100a
M3	3686.0a	11035a	96.7500a	4.222b	0.1259b	1.300b
CD	964	2747	1.7597	3.819	0.2749	3.455

M4: No callus formation.
*The values followed by the same letter are not significantly different at alpha =0. 05 according to the Duncan's multiple range test.

Table 4. Analysis of variance for effects of variety, medium and their interaction on callus weight and relative fresh weight growth rate (RFWGR) of immature embryos and mature embryos callus and on plantlets regeneration (%), number of plantlets per regenerating callus and number of plantlets regenerated per callus

Explants	Source	Callus weight (mg)	RFWGR (%)	Plantlet regeneration (%)	Number of plantlets regenerated per callus	Number of plantlets per regenerating callus
Immature embryos	Variety	7.41***	12.64***	13.34***	1.75***	15.75***
	Medium	11.19***	14.34***	19.13***	3.65*	19.80***
	Variety*Medium	5.53***	5.67***	0	0	0
Mature embryos	Variety	14.59***	1.12	5.22**	1.27	1.66
	Medium	10.73***	3.52*	68.12***	2.08	4.63*
	Variety*Medium	7.54***	0.96	4.06**	0.22	0.81

*Significant at $p<0.05$; **Significant at $p<0.01$; ***Significant at $p<0.00$.

*Table 5***.** Effect of different induction media on relative fresh weight growth rate (RFWGR), plantlet regeneration, number of plantlets per regenerating callus and number of plantlets regenerated per callus of bread wheat immature and mature embryos

F-Value													
Explants	Varieties	RFWGR (%)			Plantlet regeneration (%)			Number of plantlets regenerated per callus			Number of plantlets per regenerating callus		
		M1	M2	M3	M1	M2	M3	M1	M2	M3	M1	M2	M3
Immature embryos	Achtar	21709ab*	10858ab	22144ab	11.563bc	21.988a	7.123cde	0.5481ab	1.4168a	0.374b	7.000a	8.000a	4.673a
	Amal	8172ab	7461ab	65707a	8.968bcd	13.347b	5.221d	0.3397b	0.4331ab	0.4133ab	4.300a	5.200a	3.233a
	Mehdia	28873ab	8143ab	64328a	-	12.752b	3.164e	-	0.8373a	0.4215ab	-	6.367a	3.837a
	Rajae	8116ab	3324b	8607ab	6.611de	20.538a	5.517de	0.4438ab	0.6544ab	0.5013ab	6.100a	7.875a	3.350a
Matures embryos	Achtar	7384cd	3098d	7675cd	10.605b	20.223a	-	0.7566bc	0.8427a	-	6.383a	7.000a	-
	Amal	8855bcd	3641d	10747bc	-	9.857b	-	-	0.4676cd	-	-	4.925a	-
	Mehdia	14681b	5114cd	23971a	-	4.451c	-	-	0.3583de	-	-	5.227a	-
	Rajae	4676cd	5663cd	10816bc	-	13.288b	4.222c	-	0.8336b	0.125e	-	6.100a	4.167a

*The values followed by the same letter are not significantly different at alpha =0, 05 according to the Duncan's multiple range test.

Plantlets regeneration

After 5 weeks of culturing on the induction media, callus was transferred to the regeneration media (Figure 1). Observations made on cultures breading the regeneration phase allowed noting that germination of somatic embryos began after 6-10 days of culture on regeneration medium. This germination was sometimes accompanied by rhizogenesis. In some cases we saw calluses producing only roots that were sometimes with chlorophyll. After 8 weeks of the culturing, the plantlets regeneration (Figure 1) was recorded (Table 5). The induction and maintenance media used for callus induction had a significant effect on plantlets regeneration ($p<0.001$). Even though M1 and M3 showed higher RFWGR for callus after 5 weeks of culture of the immature embryo and mature embryo (Tables 2 and 3), the plantlets regeneration rates were lower from those calluses, 9.047% and 5.258%, respectively (Table 2). On the other hand, M2 medium which induced least amount of callus, regenerated the highest percentage of plantlet regeneration (17.156%; Table 2), indicating that M2 medium induced more embryogenic callus than other media. In general, from immature embryos, the variety 'Achtar' produced higher plantlet regeneration (15,414%) across different induction media, and Mehdia (7.958%) produced significantly lower plantlets regeneration (Table 2). From mature embyos, 'Achtar' (13%) produced higher plantlet regeneration and the other genotypes were less than 10% (Table 3).

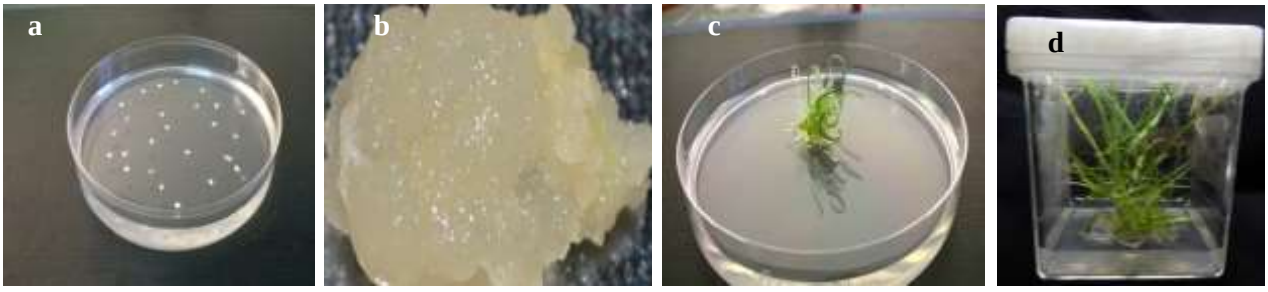

Figure 1. Embryos, callus induction and plant regeneration in bread wheat. (a) immature embryos explant; (b) callus induction on MS medium supplemented with 2.0 mg/L picloram after 4 weeks of culture. (c) Germination of somatic embryos on regeneration medium. (d) Plantlet regeneration on the regeneration medium (Iraqi et al., 2005) after 8 weeks from transfer

The plantlets regeneration also varied significantly depending on the varieties x the induction medium used (Table 4). Using immature embryos, the favourable medium is M2 for the varieties 'Achtar' and 'Rajae' whereas, M1 for Achtar variety. For the mature embryos the favourable medium is also M2 for the varieties 'Achtar' and 'Rajae'.

Number of plantlets regenerated per callus

The number of plantlets regenerated per callus and number of plantlets per regenerating callus was determined after eight weeks of culture on regeneration medium. The four varieties and media used had a significant effect on number of plantlets regenerated per callus from immature embryos (Table 4). For immature embryos (Table 5) 'Achtar' had the highest number plantlets regenerated per callus (1.416) and 'Amal' had the lowest (0.3397). The number of plantlets regenerated per callus were only influenced by media for mature embryos (Table 4). For mature embryos 'Achtar' produced higher number of plantlets regenerated per callus (0.8427) and 'Mehdia' produced significantly lower of plantlets regenerated per callus (Table 5). On the other hand form immature embryos M2 produced higher number of plantlets regenerated per callus (0.7691); M1 and M3 had the lower (0.44387); (0.42384) respectively also for mature embryos M2 was the favourable for production of more plantlets, (0.7566) (Tables 2 and 3).

Number of plantlets per regenerating callus

Number of plantlets per regenerating callus was also a parameter affected by the variety and media for immature embryos (Table 4). 'Achtar' showed the higheest (8.00) and 'Amal' had the lowest (3.233) (Table 5). Among the 3 media used M2 had the higher number of plantlet regenerating callus and M3 (4.197) had a significantly lower number of plantlets per regenerated callus (Table 2). For mature embryos the number of plantlets regenerating callus was influenced by media (Table 4). 'Achtar' showed the highest (7.000) followed by 'Rajae' (6.10), and 'Amal' (4.925) was the lowest (Table 5).

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 explants and especially genotype (Mzouri et al., 2001). In this study, we explored three callus induction and maintenance media (referred here after as M1, M2, M3, Table 1) for their effect on callus formation and plants regeneration from mature and immature embryos explants of four Moroccan bread wheat varieties ('Achtar', 'Amal', 'Mehdia', 'Rajae'). Such studies allowed us to determine the medium favourable for each variety, which would help us for the use of mature and immature embryos as explants on *in vitro* plant regeneration and genetic transformation. The study we undertook clearly showed the effect of genotype and explants on *in vitro* morphogenic capacity in the studied bread wheat varieties. The 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 gave birth to somatic embryos, and non-embryogenic calli, which looked translucent and viscous. These types of callus were observed frequently and described from different types of explants in wheat (Mzouri et al., 2001). The results we obtained also showed that callus growth was affected by the genotype×medium for immature embryos (Table 4). These results are consistent with those reported by several authors (Nasircilar et al., 2006). The effect of the presence of 2,4-D (2,4–Dichlorophenoxyacetic acid) at 2 mg/l, picloram at 2 mg/l and their combination (2,4-D + picloram at 2 mg/l) depended on the genotype used. M3 medium supplemented with 2 mg/l of (2,4–D + picloram) yielded the highest RFWGR for the varieties 'Amal', 'Mehdia', whereas M1 for the rest of the varieties (Table 5). These results indicated that callus weight improved by increasing 2,4–D (auxin) to 2 mg/l (as in the case of M1) in agreement with the finding of Malik et al.

(2003). On the other hand, the plantlets regeneration rates were lower when M1 and M3 showed higher RFWGR for callus (Tables 2 and 3); these results are also in agreement with Tinak et al. (2013). Even though M2 medium, which contains 2.0 mg/l of picloram resulted in lowest RFWGR of callus from immature embryos and mature embryos was not proportional to the changes of plantlet regeneration (Table 5). It is known that auxins play a role in the activation of genes involved in cell de-differentiation and division (Dudits et al., 1991).

Regenerable calli obtained on two auxins and their combination varied in their response for regeneration from immature embryos (Table 5). Overall, picloram at 2.0 mg/l produced the highest plantlet regeneration (21.988%) and the number of plantlets per regenerating callus (8.000) followed by 2,4–D at 2 mg/l (7.000 number of plantlets per regenerating callus in 11.563% plantlet regeneration) and then their combination (picloram + 2,4–D) at 2.0 mg/l (4.673 number of plantlets per regenerating callus in 7.123% plantlets regeneration). This result is in agreement with the works of He et al. (2001). This indicated that for scutellum cultures of durum wheat cv Desf, picloram can significantly increase the frequency of regeneration compared with 2,4–D, with the optimised concentration of 2.0 mg /1 in the induction medium. Our results are consistent with others, that 2,4-D at 2.0 mgl/1 alone is the best for callus production and regeneration (Yasmin et al., 2001) while at concentrations higher than 2.0 mgl/1 proved detrimental for regeneration (Khurana et al., 2002).

The two explants differed significantly in their ability to plantlet regeneration, number of plantlets per regenerating callus and number of plantlets regenerated per callus (Table 4). The immature embryos showed the highest plantlet regeneration (19.13%) and the number of plantlets per regenerating callus (19.80%). The regeneration response obtained in the present study is much better than most of the earlier reports, where 0.2-0.3 shoots (Delporte et al., 2001), 8 shoots (Filippov et al., 2006) and 13 shoots per explants (Chauhan et al., 2007) were obtained.

For all parameters, the differences between the two explants were significant. The immature embryos showed RFWGR, callogenesis, regeneration and number of plantlets per regenerating callus much higher than in mature embryos. However, 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), the mature embryos were even used as explants in several searches for genetic improvement of certain genotypes (Benderradji et al., 2012).

The nature of the explants had 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 bread wheat, the importance of choice of explants for callus induction and regeneration has been the subject of several studies. The best results were, however, obtained from immature embryos (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, 2002).

CONCLUSION

The regeneration of plants from somatic embryos of wheat, like other grasses and monocotyledonous species, has long been thought to be very difficult; if not impossible (Vasil, 2007). 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. The various varieties used in this study responded differently to medium type for callus induction and plant regeneration. MS medium containing M1, M2 and M3 were similar for the induction of callus. The regenerative potential was affected by the genotype, media and the nature of explant

used. ‘Achtar’ and ‘Rajae’ varieties showed higher frequencies of regeneration from mature and immature embryos with medium M2. This medium and these genotypes showed suitable regeneration capacities for use in subsequent genetic transformation.

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