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

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ABSTRACT

In wheat, immature embryos are the most widely used explant to initiate cultures, but they are inconvenient due to their temporal availability and production requirements. Mature embryos are easily stored and are readily available as mature seeds. However, plant regeneration frequencies from cultures derived from mature embryos are generally low. This study was undertaken to improve callus induction and plant regeneration from durum wheat (*Triticum turgidum* L. var. *durum*) mature embryos for biolistic and Agrobacterium-mediated transformation. Five wheat genotypes were evaluated for their response to callus induction and regeneration on M3 medium (M3a, M3b, M3c, M3d, M3e and M3f) modified with different concentrations of 2,4 Dichlorophenoxyacetic acid (2,4-D), that is, 1mg/L and 2mg/L, vitamins, Maltose and sucrose, which gives good results both in the callus induction and in the regeneration of plantlets. A significant effect of variety, medium and variety x medium interaction were observed for callus induction and regeneration. Relative fresh weight growth rate (RFWGR), calculated after 4 weeks of incubation of the explants across six media, was highest for Chaoui (8189.2%), and the lowest for Kyperounda and Marouane (4750.3% and 4027.7%). M3e recorded the highest (6923.9%) followed by M3d (6424.2%), M3f (5971.4%) and M3c (5663.3%), and M3a (5275.4%), whereas, M3b was the lowest (3950.4%). M3e, M3c, M3d and M3f showed higher RFWGR for callus after 8 weeks of culture, M3f medium showed the highest percentage of plantlet regeneration (54.80%), and all of the other media they gave similar results. In this study favorable media, which gave good results both in the callus induction and in the regeneration of plantlets, were identified and will be used for callus induction and genetic transformation.

Key words: durum wheat, somatic embryogenesis, mature embryo, genetic transformation.

INTRODUCTION

In Morocco, cereals and their derivatives have undeniable nutritional, social and economic roles. Cereals are of paramount importance and their consumption is one of the highest in the world Cereal demand is almost synonymous with demand for food. Among cereals, durum wheat is grown over an area ranging from 1 to 1.2 million hectares annually, and ranks third after bread wheat and barley (MAPM, 2011). Drought is the most important environmental stress affecting the durum wheat crop, causing a severe decrease in performance. The transfer of resistance to abiotic stresses such as drought, using traditional approaches is limited, because of the complexity of the characteristics of tolerance (Patnaik and Khurana, 2001).

Selection by *in vitro* culture and genetic transformation might allow the acquisition of this tolerance while overcoming the difficulties of classical improvement.

In cereals, plant regeneration has been achieved from callus, immature embryos (Chauhan et al., 2007), mature embryos (Bi et al., 2007), leaf segments (Wang and Wei, 2004), mesocotyl segments (Jelaska et al., 1984), coleoptiles (Sahrawat and Chand,

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2004), apical meristems (Mchugen, 1983), inflorescences (Chen et al., 1985) and also from anthers (Lezin et al., 1996).

Immature embryos were the most efficient tissue source to regenerate plants in vitro (Ozias-Akins and Vasil, 1982). 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 year round and in bulk quantities. Therefore, mature embryos as a favourable explant source are explored broadly in cereal tissue culture. However, the major hurdle with mature embryos as explant is their low frequency of plant regeneration (Chen et al., 2006).

Endosperm-supported (ES) culture has been explored to improve mature embryo culture efficiency (Bartok and Sagi, 1990), but few experiments have been reported since then, and therefore its reliability and universality need to be confirmed.

Studies already done by Hallal et al. (2009) and Tinak et al. (2013) reported that the medium M3 developed by Gadaleta et al. (2006) has not yielded good results in the induction, but the best results in terms of regeneration were observed in callus incubated on this medium.

Based on these considerations, the objective of this study was to assess the ability of the somatic embryogenesis of five varieties of durum wheat on six induction media, to determine the most appropriate for each variety, to be used later for genetic transformation.

MATERIAL AND METHODS

Plant material and preparation of explants. ‘Kyperounda’, ‘Isly’, ‘Chaoui’, ‘Marouane’, and ‘Tomouh’ are the five varieties of Moroccan durum wheat (Abbad-Andaloussi and Chahbar, 2005) used in this study.

The seeds were surface-sterilized by washing in running tap water two times. Then they were rinsed with sterile distilled water 2 times for 2 min; next, we sterilized the washed seeds by immersing them first in 70% (v/v) ethanol for 1 min, and then in 50% (v/v) CLOREX (12% sodium chlorite; Age Company, Casablanca, Morocco) with a few drops of Tween 20, for 20 min. After rinsing the seeds with sterile distilled water three times, we soaked them in sterile distilled water overnight. Mature embryos were aseptically dissected away from the caryopses and the remaining endosperm and radicle removed. Embryo sections (1-2 mm) containing the shoot apex, scutellar node and first internode (mesocotyl), were placed onto the surface of callus induction media. Eight embryos were cultured in each Petri plate, and the plate of embryos comprised one experimental unit.

Culture media, induction and regeneration. The callus induction and maintenance media consisted of M3a (Gadaleta et al., 2006) and M3a modified by additions of vitamins, sucrose, maltose, and 2,4-D with different combinations,generating 5 additional media (M3b, M3c, M3d, M3e and M3f) (Table 1).

Cultures were incubated in the dark in an incubator at 25 ° C ± 2 °C for 4 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 determined (Daud et al., 2012):

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

The embryogenic callus were evaluated 8 weeks after culture initiation. For plantlet regeneration, the embryogenic callus was transferred to MS medium with 3mg l^{-1} IAA, 2 mg l^{-1} MS Vitamins, 3% (w/v) sucrose and 3.5 g Phytagel l^{-1}. Cultures were incubated at 25±2°C under a photoperiod of 16/8 h day/night. The regeneration frequency was scored 4 weeks after transfer of callus clumps to regeneration medium.

Percentage of plants regeneration was calculated as follows: (the number of plantlets regenerated / the number of callus transferred to the regeneration medium) x 100.

Experimental design and statistical analysis. A randomized complete block design (RCBD) was used with 8 varieties and 5 media (8x5 = 40 treatments). Each treatment consisted of 6 replications of each medium for each variety, each replication with 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 1985). Mean of treatments were compared using Duncan's Multiple Range test (Steel and Torrie, 1980).

Table 1. Media composition

Components	Medium tested*					
	M3a	M3b	M3c	M3d	M3e	M3f
Macroelements	MS	MS	MS	MS	MS	MS
Oligoelements	MS	MS	MS	MS	MS	MS
Vitamins	-	MS	-	-	MS	MS
Fe-EDTA	MS	MS	MS	MS	MS	MS
L-asparagine (mg/L)	150	150	150	150	150	150
Myo-Inositol (mg/L)	100	100	100	100	100	100
Sucrose (g/L)	-	-	-	30	30	-
Maltose (g/L)	40	40	40	-	-	40
2,4-D (mg/L)	1	1	2	1	1	2
pH	5.7- 5.8	5.7- 5.8	5.7- 5.8	5.7- 5.8	5.7- 5.8	5.7- 5.8
Phytagel (g/L)	3.5	3.5	3.5	3.5	3.5	3.5
Thiamine (mg/L)	0.5	-	1	1	-	-

2,4-D = 2,4 Dichlorophenoxyacetic acid; MS = Murashige and Skoog medium.

RESULTS

Callus initiation and growth

Mature embryos of all five genotypes were cultured in six combinations of callus induction medium containing 2,4-D with different concentrations. Embryos of all five genotypes swelled up after just three days of inoculation and callus emerged afterwards.

Callus growth was influenced not only by variety and medium but also by variety × medium interaction (Table 2). Among the five varieties tested, 'Marouane' (9.64 mm) 'Tomouh' (9.50 mm), and Kypyrounda (9.41 mm), showed the highest mean callus diameter after 4 weeks of incubation of the explants on different media, followed by 'Chaoui' (8.79 mm) and 'Isly' (8.62 mm), (Table 2). On the other hand, callus from M3f, M3c media (more than 9.8 mm) showed greater mean diameters, followed by M3e (9.48 mm) and M3d (9.25 mm), whereas, M3a (7.92 mm) and M3b (8.36 mm) media showed the lowest diameters (Table 3).

All the genotypes showed a good callus growth on all the media, but at different levels. The rate of increase of callus diameter was proportional to incubation period.

The relative fresh weight growth rate (RFWGR) calculated after 4 weeks of incubation of the explants also differed significantly among varieties across media (Table 2); 'Chaoui' recorded the highest (8189.2%), followed by 'Isly' (5835%) and 'Tomouh' (5705%), whereas, 'Kyperounda' and Marouane were the lowest (4750.3 and 4027.7%). RFWGR calculated after 4 weeks of incubation of the explants also differed significantly among media across varieties (Table 3): M3e recorded the highest (6923.9%) followed by M3d (6424.2%), M3f (5971.4%) M3c (5663.3%), and M3a

(5275.4%), whereas, M3b was the lowest (3950.4%).

Callus production was strongly influenced by the media and the variety used (Table 2). A significant (P<0.001) interaction between variety and medium was observed (Table 3). RFWGR of callus calculated after 4 weeks of culture on different induction and maintenance media, was the highest on M3e for variety 'Chaoui' (13995%); M3c for variety 'Isly' (9091%); M3f for Kyperounda (6636%); and M3d for Mourouane and Tomouh varieties, with respectively 5205% and 8103% (Table 4).

Plantlets regeneration

After 4 weeks, callus was transferred to the regeneration medium. After 8 weeks of the culturing, the plantlets regeneration was recorded (Table 2). The induction and maintenance media used for callus induction had a significant effect on plantlets regeneration (P<0.001). Even though M3e, M3c, M3d and M3f showed higher RFWGR for callus after 4 weeks of culture (Table 2), M3f medium, regenerated the highest percentage of plantlet regeneration (54.80%; Table 2), and for all of the other media, the percentages were not significantly different.

Table 2. Mean of callus diameter and relative fresh weight growth rate of callus (RFWGR) of five durum wheat varieties, obtained on six induction and maintenance media after 4 weeks of culturing and their effect on plantlets regeneration (%)

Variety	Callus diameter (mm)	Callus weight (mg)	RFWGR (%)	Plantlet regeneration (%)
Marouane	9.64a	939.4c	4027.7c	20.94b
Kyperounda	9.41a	1473.4a	4750.3bc	39.00a
Isly	8.62b	1189.7b	5835.0b	33.83ab
Chaoui	8.79b	1489.6 a	8189.2a	43.33a
Tomouh	9.50a	1099.1bc	5705.0b	32.33ab
CD	0.5675	232.0	1267	13.49
Medium				
M3a	7.92c	1120.0bc	5275.4bc	32.20b
M3b	8.36c	876.2c	3950.4c	22.40b
M3c	9.87ab	1166.4b	5663.3ab	32.13b
M3d	9.25b	1357.3ab	6424.2ab	26.40b
M3e	9.48b	1555.0a	6923.9a	35.40b
M3f	10.28a	1354.6ab	5971.4ab	54.80a
CD	0.62	254.1	1388	14.78

CD (0.05) according to the Duncan's Multiple Range test; M3a to M3f are the induction and maintenance media used. For composition of media, please refer to Table 3.

DISCUSSION

In this study, we reported some valuable clues such as the impact of 2,4-D, maltose, sucrose and vitamins resulting in improved induction and regeneration from mature embryo of five Moroccan durum wheat varieties (Kyperounda, Isly, Chaoui, Marouane, and Tomouh).

As far as explant is concerned, these results are similar to those obtained by many researchers, such as Zale et al. (2004), who also proved that mature embryos can produce sufficient number of regenerated plants. These results are also in agreement with Özgen et al. (1998), according to whom mature embryos have a high frequency of callus induction and regeneration capacity. Therefore, being available through out the year, they can be used in wheat tissue culture. Delporte et al. (2001) used mature embryos successfully and produced calli, which is in agreement with

this research, but instead of using the whole seed or embryo, they have used fragments of embryos. Yu et al. (2008) also used mature embryos in wheat tissue culture.

In plant tissue culture, a desirable genotype is expected to possess high callus induction and plant regeneration capacity. However, numerous studies have shown the absence of relationship between callus induction and plant regeneration capacity. Cai et al. (1989) and Chowdhury et al. (1991) found that there is no significant relationship between callus induction and plant regeneration. It is known that callus induction and regeneration capacity may be controlled independently of each other (Sears and Deckard, 1982; Chowdhury et al., 1991 and Ozgen et al., 1996). Good embryoid production was seen during third and fourth weeks of sub-culture.

Our results showed that callus production was strongly influenced by the medium, the variety and the interaction medium x variety (Table 3) and confirm many others studies which showed that callus induction was influenced by the medium components and the genotype (Gadaleta et al., 2006; Ayolié et al., 2007; Monostori et al., 2008; Ren et al., 2010). M3e medium yielded the highest RFWGR for the variety 'Chaoui', followed by M3c for variety 'Isly' followed by M3f for 'Chaoui', whereas M3f was best for Kyperouada. For the rest of varieties, M3d gave the highest RFWGR (Table 4). In three varieties, 'Marouane', 'Chaoui', and 'Tomouh' culturing on M3b medium, resulted in lowest RFWGR of callus.

These results indicate that callus weight tended to increased when concentration of 2,4-D was increased (2 mg/l). A beneficial effect of 2 and 3 mg/L of 2,4-D on callus induction of wheat mature embryos was also found by Raziuddin et al. (2010). In most of the studies involving wheat mature embryo culture, 2,4-D has been the only auxin used (McHughen, 1983; Heyser et al., 1985; Bartok and Sagi, 1990; Ozgen et al., 1998; Varshney et al., 1996).

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 callus and on plantlets regeneration (%) in durum wheat

F-Value								
Callus diameter						Callus weight	RFWGR	Plantlet regeneration (%)
Source	Df	Week 1	Week 2	Week 3	Week 4	(after 4 weeks)	(after 4 weeks)	
Variety	4	7.31***	0.42	6.06***	5.07***	8.36***	12.10***	170.58***
Medium	5	5.11***	10.49***	15.61***	16.39***	6.76***	4.35***	252.55***
Variety x Medium	20	2.64***	2.98***	2.31**	2.58***	3.09***	2.86 ***	389.59***

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

The type of sugar used in the media had a significant effect on the production of embryogenic callus from mature embryos in the M3f induction medium; maltose could have promoted the somatic embryos and increased efficiency of conversion of embryos to plant. Replacement of sucrose by maltose increased the mean callus fresh weight (Mendoza et al., 2002).

The beneficial effect of maltose compared to sucrose on callus-promoting activity from wheat mature embryos, is consistent with previous observations reported by Last and Brettel (1990) and Orsinky et al. (1990) using anther culture from a range of genotypes in hexaploid wheat. This result is in agreement with the works of Mendoza and Kaeppler (2002) which indicated that

substitution of sucrose by maltose enhanced the regeneration ability of callus from embryos of wheat. Our results confirm also the works of Gadaleta et al. (2006) which showed that inclusion 40 g/L of maltose as sole carbon source had resulted in germination of wheat embryos and development into plants.

Table 4. Effect of medium and variety on relative fresh weight growth rate of callus (RFWGR) and plantlets regeneration in durum wheat

Variety	RFWGR (%)*						Plantlets regeneration (%)**					
	M3a	M3b	M3c	M3d	M3e	M3f	M3a	M3b	M3c	M3d	M3e	M3f
Marouane	4205 efgh	2406h	3968efgh	5205 cdefgh	3889efgh	4493 defgh	0q	0q	66.7c	0q	15p	44g
Kyperounda	3353fgh	3175fgh	2711gh	6158 bcdefgh	6469 bcdefg	6636bcdef	100a	45f	20n	17o	0q	52d
Isly	5973bcdefgh	5340cdefgh	9091b	6269bcdefg	4069efgh	4268efgh	26k	0q	50e	50e	37i	40h
Chaoui	7744bcde	4790defgh	7525bcde	6385bcdefg	13995a	8696bc	0q	17o	24m	25l	100a	94b
Tomouh	5103cdefgh	4041efgh	5022cdefgh	8103bcd	6198bcdefgh	5763bcdefgh	35j	50e	0q	40h	25l	44g

*CD = 3104 at 0.05 according to the Duncan's Multiple Range test; M3a to M3f are the induction and maintenance media used. For composition of media, please refer to Table 1.
**CD = 4.435 at 0.05 according to the Duncan's Multiple Range test; M3a to M3f are the induction and maintenance media used. For composition of media, please refer to Table 1.

Different studies showed that thiamine is associated with cytokinin and has a role in inducing callus growth and rooting (Peter et al., 2011). Moreover, thiamine was essential in facilitating the production of more secondary metabolites such as proteases in pineapple. Vitamins, in combination with other media constituents, have been shown to have direct and indirect effects on callus growth, somatic growth, rooting, and embryonic development (Peter et al., 2011).

Asano et al. (1996) showed that enhancing embryonic callus of *Zoysia japonica* Steud., a warm season turf grass native to Japan, was obtained by adding thiamine and riboflavin to the media. Thiamine and nicotinic acid have been shown to affect embryogenesis. Barwale et al. (1986) studied the effect of different concentration of both vitamins on immature soybean embryos cultured to a modified MS medium. Thiamine at 1.0 μM, or more, induced 68% embryogenesis compared to 0.2 μM, the level of salts in MS medium, at 33% of the immature embryos. Also, a concentration of 32.4 μM nicotinic acid induced 76% embryogenesis. In this study, plantlets regeneration varied significantly depending on the varieties and induction media used. For Kyperounda, the best medium was M3a, for Chaoui the best media were both M3e and M3f, for Marouane M3c was the best, for Isly both M3c and M3d were the best, and for Tomouh, it was M3b. These results are also in agreement with Bahman et al. (2012), who showed that regeneration of plantlet from mature embryos derived callus was controlled by their genetic makeup. The varieties Kyperounda, Isly, Chaoui and Tomouh produced higher plantlets regeneration, whereas Marouane produced lower regeneration. While the regeneration rate obtained in this study is still low relative to cultures initiated from immature embryos, we identified favourable media for induction and enhancing regeneration efficiencies, from mature embryo of Moroccan durum wheat, so we can consider these results acceptable to be used for genetic transformation.

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