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in

Porceddu E. (ed.), Damania A.B. (ed.), Qualset C.O. (ed.).
Proceedings of the International Symposium on Genetics and breeding of durum wheat

Bari : CIHEAM
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 110

2014
pages 235-240

Efficient callus induction, plantlets regeneration and genetic transformation of durum wheat

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Abstract. In this study, we tested matured embryos as explants from eight Moroccan durum wheat varieties (Irden, Marzak, Kyperounda, Isly, Amria, Karim, Marouane, and Tomouh) and five induction media (M1 to M5) based on MS media (macro and oligo-elements) which differed with respect to concentrations of plant hormones (2,4-D and BA), vitamins, sucrose, maltose, L-asparagine, and solidifying agents. All tested media induced embryogenic callus for the varieties and regenerated plantlets. However, a significant effects of variety, medium and variety x medium interaction were observed for callus induction and regeneration. We used embryogenic callus derived from immature embryos for genetic transformation of *HVA1* gene of barley. In this study, we identified for the first time, favorable media for induction and regeneration from mature embryo explants of Moroccan durum wheat varieties. Also, we successfully transformed durum wheat with *HVA1* gene via particle bombardment. Since the transgenic plants developed in this study contained barley *HVA1* gene, further analysis for tolerance to water and salt stresses in subsequent generations needs to be undertaken.

Keywords. Durum wheat – Mature embryos – Plantlets regeneration – Somatic embryogenesis – Genetic transformation – HVA1 gene.

Induction efficace de cal, régénération de plantules et transformation génétique du blé dur

Résumé. *Dans cette étude, nous avons testé des explants d'embryons matures, provenant de huit variétés marocaines de blé dur (Irden, Marzak, Kyperounda, Isly, Amria, Karim, Marouane et Tomouh) et cinq milieux de culture d'induction (M1 à M5) à base de milieu MS (macro et micro-éléments) qui différaient par la concentration d'hormones (4,4-D et BA), vitamines, saccharose, maltose, L-asparagine et agents solidifiants. Tous les milieux testés ont induit un cal embryogène chez les variétés et les plantules régénérées. Cependant, un effet significatif de la variété, du milieu, et de l'interaction milieu x variété a été observé pour l'induction de cal et la régénération. Nous avons utilisé des cals embryogènes issus d'embryons immatures pour la transformation génétique avec le gène HVA1 d'orge. Dans cette étude, nous avons identifié pour la première fois, un milieu favorable pour l'induction et la régénération à partir d'explants d'embryons matures des variétés marocaines de blé dur. Nous avons aussi transformé avec succès le blé dur avec le gène HVA1 d'orge par bombardement de particules. Puisque les plantes transgéniques développées dans cette étude contenaient le gène HVA1 d'orge, des analyses supplémentaires devraient être menées pour évaluer la tolérance au stress hydrique et salin chez les générations suivantes.*

Mots-clés. *Blé dur – Embryon mature – Régénération de plantules – Embryogénèse somatique – Transformation génétique – Gène HVA1.*

I – Introduction

Durum wheat (*Triticum turgidum* L. subsp. *durum*) is the most important cereal crop in the Mediterranean basin. In Morocco, durum wheat is grown over an area ranging from 1 to 1.2 million hectares annually, and ranks the third after bread wheat and barley with respect to production (MAPM 2011). The country's wheat productivity has been affected by various biotic and abiotic stresses (Karrou 2003).

Immature embryos were the most efficient tissue source to regenerate plants *in vitro* (Jones 2005). 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. For this purpose, mature embryos as a favourable source of explants are explored broadly in wheat tissue culture. Though the major hurdle with mature embryos as explants is their low frequency of plant regeneration (Ren *et al*. 2010). Although plant regeneration has been achieved previously from callus cultures derived from mature embryos of durum wheat (Neiverth *et al*. 2010), the regeneration efficiencies were inconsistent and also depended on genotype and medium composition (He *et al*. 1988). Moreover, studies on *in vitro* plantlet regeneration in durum wheat using mature embryos as explants derived from Moroccan varieties are lacking.

In Morocco, drought is a major environmental stress that limits cereal productivity and consequently Morocco is not self-sufficient in cereal production. Genetic transformation of crops is a powerful research tool for gene discovery and function to investigate genetically controlled traits and is fast becoming a key element in the process of varietal improvement. Development of a reliable genetic transformation protocol is necessary to facilitate genetic improvement of wheat for drought tolerance. The development of methodology for the delivery of genes from other species into intact plant tissues by particle bombardment has revolutionized the field of wheat transformation (Bahieldin *et al*., 2005; Matsumoto and Gonsalves, 2012). However, no reports are available regarding genetic transformation of Moroccan durum wheat cultivars.

The objective of this study was to define suitable media for callus induction and plant regeneration of Moroccan durum wheat varieties using mature embryos as explants. While doing so, we compared the effects of media, varieties and their interactions on callus induction and plant regeneration obtained from mature embryos as explants. Here we also report successful transformation of a Moroccan durum wheat variety by particle bombardment using immature embryos as explants source.

II – Materials and Methods

1. *In vitro* culture

Field grown seeds (matured caryopses) of durum wheat cultivars Irden, Marzak, Kyperounda, Isly, Amria, Karim, Marouane and Tomouh were used as the source for mature embryo culture. The seeds were procured from Experimental Research Station of INRA at Marchouch, Rabat, Morocco.

The seeds were then surface-sterilized (Tinak *et al*. 2013), Mature embryos were aseptically dissected away from the caryopses, and the remaining endosperm and radical were removed to prevent early germination. The embryos were placed in a Petri dish containing the induction medium based on M1 (Iraqi *et al*. 2005), M2 (Karim *et al*. 2005), M3 (Gadaleta *et al*. 2006), M4 (Pellegrineschi *et al*. 2002),or M5 (Przetakiewicz *et al*. 2003) (Table 1). The relative fresh weight growth rates (RFWGR) of callus were determined:

$$RFWGR = (FW_f - FW_i) / FW_i \times 100$$

where FW_f = final fresh weight and FW_i = initial fresh weight.

After five weeks, embryogenic calli from each replication were transferred to the regeneration medium (Iraqi *et al*. 2005). Percentage of plants regenerated was calculated as follows: (the number of plantlets regenerated / the number of callus transferred to the regeneration medium) x 100.

Table 1. Media composition.

	Medium tested				
Component	**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. Genetic transformation

Immature embryos were excised out from immature seeds collected 12-16 days post-anthesis sterilized (Iraqi *et al*., 2005) and cultured on induction and maintenance medium (MS Asp; Iraqi *et al*., 2005). The embryos whose cells started rapid division were selected for subsequent transformation and subculturing. The plasmid used for bombardment pBY520 contained the linked selectable marker/herbicide resistance *bar* (phosphinothricin acetyl transferase) gene (driven by cauliflower mosaic virus 35S promoter and the nopaline synthase nos terminator) and the barley *HVA1* gene (driven by rice *Act1* promoter and terminated by the potato protease inhibitor *pin II*). After the bombardment, embryos were left in the same medium in the dark for 16 hours at 25 °C and subsequently transferred to the MS Asp medium without mannitol for a period of 4-5 days. The resistant calluses were transferred to the regeneration medium (Iraqi *et al*., 2005) supplemented with PPT. For root induction, the regenerated shoots were transferred in MS half-strength medium lacking hormones and supplemented with PPT. Herbicide resistance of the putative transgenic wheat plants was determined by painting leaves of plants at the fifth or sixth leaf stage with basta (0.3% w/v) with 7 days between applications to minimize escapes. Plants were scored as susceptible or resistant according to the degree of leaf desiccation after 7 days (Pellegrineschi *et al*., 2002).

III – Results and discussion

1. *In vitro* culture

Callus production was strongly influenced by the media and the variety used. A significant ($p < 0.001$) interaction between variety and medium was observed. RFWGR of callus calculated after 5 weeks of culture on different induction and maintenance media showed that the highest RFWGR was observed on M1 for varieties Irden (8914%), Marouane (7249%), Marzak (8969%) and Amria (9792%); and for the rest of varieties, M5 gave the highest RFWGR (Table 2). In all these varieties, except Irden, culturing on M3 medium resulted in lowest RFWGR of callus.

Table 2. Effect of medium and variety on relative fresh weight growth rate of callus (RFWGR) and plantlet regeneration in durum wheat.

	RFWGR (%)					Plantlet regeneration (%)				
Variety	**M1**	**M2**	**M3**	**M4**	**M5**	**M1**	**M2**	**M3**	**M4**	**M5**
Irden	8914	1773	2462	5576	6713	30.6	34.58	34	21.2	11.2
Marouane	7249	6832	4281	5458	5634	35.44	35.54	51.32	24.5	15.88
Kyperounda	5438	3520	3290	5558	6039	39.6	23.92	55	21	13.2
Isly	5712	3379	3956	3826	6153	37	47	49.2	23.9	20.22
Marzak	8969	4668	4396	6760	6861	24.6	17.54	52.8	32.42	31
Karim	6832	5118	4335	6788	8137	44.36	17.2	58.58	29.8	47.42
Amria	9792	6090	4930	6764	8500	27	39	36.4	9	20
Tomouh	2231	1975	2020	1594	2620	35.6	31.8	65	32	11.8

After 5 weeks, callus was transferred to the regeneration medium. After 8 weeks of culturing, the number of plantlets regenerated was recorded (Table 2). The induction and maintenance media used for callus induction had a significant effect on plantlet regeneration ($p < 0.001$). Even though M1 and M5 showed higher RFWGR for callus induction after 5 weeks of culture (Table 1), the plantlet regeneration rates were lower from those calluses, 34.27% and 21.34%, respectively (Table 1). On the other hand, M3 medium which induced least amount of callus, regenerated the highest percentage of plantlets (50.29%; Table 1), indicating M3 medium induces more embryogenic callus than other media.

M1 medium yielded the highest RFWGR for the varieties Irden, Marouane, Marzak and Amria, whereas M5 for the rest of the varieties (Table 2). In all these varieties, except 'Irden', culturing on M3 medium resulted in lowest RFWGR of callus. These results indicate 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) with mature seeds of wheat in the subculture media ; or 3 mg/L (as in the case of M5), similar to the results obtained by Munazir *et al*. (2010) with mature seeds culture of wheat. The 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 contrast, Mendoza and Kaeppler (2002) showed in bread wheat cultivar Bobwhite that callus weight tended to decrease when concentration of 2,4-D was increased.

Regeneration of plantlets from mature embryos derived callus was also controlled by their genetic makeup (Bahman *et al*., 2012). In our study, the varieties Karim, Isly, and Tomouh produced higher plantlet regeneration, whereas Irden and Amria produced significantly lower plantlet regeneration. The other genotypes were in between. However, plantlet regeneration varied significantly depending on the varieties and the induction and maintenance media used. For the varieties Marouane, Kyperounda, Marak, Karim, and Tomouh, the favorable medium was M3, whereas, for Isly, Irden and Amria, both M2 and M3 were favorable (Table 2).

2. Genetic transformation

The untransformed callus became yellow, like that of the controls (Fig. 1c). The resistant callus was then transferred to regeneration media (Iraqi *et al*., 2005) supplemented with 3 mg/L of basta. Green and vigorously growing plants survived (Fig. 1d) were transferred to the rooting medium (half-strength MS) also with 3 mg/L of basta (Fig. 1e). The regenerated plantlets were transferred to pots in the greenhouse for acclimation (Fig. 1f). 14 plants, only from 'Irden' variety, survived on the 3 mg/L of basta-selection. Recognizing that the selection system could permit false positive plants, a second selection was done by painting leaves with 0.3% of basta to demonstrate the expression of the basta herbicide-resistance gene *bar* (Pellegrineschi *et al*., 1998). After 7 days, for all the putative transformants, leaves painted stayed green, whereas for the control they became yellow and died. These putative transformants were phenotypically normal and fertile (Fig. 1g).

In this study, we identified for the first time, favorable media for induction and regeneration from mature embryo explants of Moroccan durum wheat varieties. Also, we successfully transformed durum wheat with *HVA1* gene via particle bombardment. Since the transgenic plants developed in this study contained barley *HVA1* gene, further analysis for tolerance to water and salt stresses in subsequent generations needs to be undertaken.

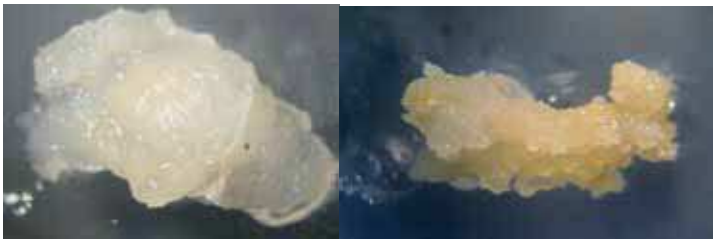

Figure 1. Genetic transformation of variety 'Irden' of durum wheat by particle bombardment using immature embryo-derived calli as the target tissue (Upper a, b, c; middle d, e, f; bottom g). The calli were bombarded with plasmid pBY520. a: basta leaf paint assay for transformant. b, c : callus tissue after selection with 3 mg/L of basta: untransformed calli (yellow) and transformed callus (white). d: putative transformed plantlets in regeneration medium with 3 mg/L of basta. e: putative transformed plantlets in rooting medium with 3 mg/L of basta. f: acclimatation of putative transformed plants. g: T1 progeny plants.

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

Bahieldin A., Mahfouz H.T., Eissa H.F., Saleh O.M., Ramadan A.M., Ahmed I.A., Dyer W.E., El-Itriby H.A., Madkour M.A., 2005. Field evaluation of transgenic wheat plants stably expressing the HVA1 gene for drought tolerance. *Physiol. Plantarum,* 123, pp. 421-427.

Bahman F.N., Omidi M., Amiritokaldani M., 2012. Callus induction and plant regeneration of wheat mature embryos under abscisic acid treatment. *Int. J. Agriculture and Crop Sci*., 4, pp. 17-23.

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, pp. 31-37.

He D.G., Yang Y.M., Scott K.J., 1988. A comparison of scutellum callus and epiblast callus induction in wheat: the effect of genotype, embryo age and medium. *Plant Sci*., 57, pp. 225-233.

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

Jones H.D., 2005. Wheat transformation: current technology and applications to grain development and composition. *J. Cereal Sci*., 41, pp. 137-147.

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*. *Bull. Soc. Pharm. Bordeaux,* 144, pp. 195-210.

Karrou M., 2003. Conduite du blé au Maroc. *National Institute of Agricultural Research* (INRA Editions), Rabat, Maroc, pp. 57.

Malik S.I., Rashid H., Yasmin T., Minhas N.M., 2003. Effect of 2,4-dichlorophenoxyacetic acid on callus induction from mature wheat (*Triticum aestivum* L.) seeds. *Int. J. of Agriculture and Biol*., 6, pp. 156-159.

MAPM, 2011. Situation de l'Agriculture Marocaine. No. 9, Ministry of Agriculture and Fisheries, Rabat, Morocco, 204 pp. Available at: http://www.agriculture.gov.ma/sites/ default/files/SAM9-2011.pdf.

Matsumoto T.K., Gonsalves D., 2012. Biolistic and other non-*Agrobacterium* technologies of plant transformation. In: *Plant Biotechnology and Agriculture: Prospects for the 21st century.* Altman A., Hasegawa P.M. (eds). Academic Press, USA, pp. 117-129.

Mendoza M.G., Kaeppler H.F., 2002. Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (*Triticum aestivum* L.). *In Vitro Cell. Dev. Pl*., 38, pp. 39-45.

Munazir M., Qureshi R., Ali G.M., Rashid U., Noor S., Mehmood K., Ali S., Arshad A., 2010. Primary callus induction, somatic embryogenesis and regeneration studies in selected elite wheat varieties from Pakistan. *Pak. J. Bot*., 42, pp. 3957-3965.

Neiverth A., Silva J.B.D., Schuster I., Santos M.F.D., Vendruscolo E.C.G., 2010. Regeneration of wheat plants from wheat (*Triticum aestivum* L. cv. CD104) mature embryos. *Scientia Agraria,* 11, pp. 101-108.

Pellegrineschi A., Fennell S., McLean S., Brito R.M, Velazquez L., Salgado M., Olivares J.J., Hernandez R., Hoisington D., 1998. Routine transformation system for use with CIMMYT wheat varieties. In: *Application of Biotechnologies to Wheat Breeding.* Kohli M.M., Francis M. (eds). INIA La Estanzuela, Colonia, Uruguay, pp. 111-119.

Pellegrineschi A., Noguera L.M., Skovmand B., Brito R.M., Velazquez L., Salgado M.M., Hernandez R., Warburton M., Hoisington D., 2002. Identification of highly transformable wheat genotypes for mass production of fertile transgenic plants. *Genome,* 45, pp. 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*., 73, pp. 245-256.

Raziuddin J., Bakht Swati Z.A., Shafi M., Farhat U., Akmal M., 2010. Effect of cultivars and culture medium on callus formation and plant regeneration from mature embryos of wheat (*Triticum aestivum* L.). *Pak. J. Bot*., 42, pp. 639-652.

Ren J.P., Wang X.G., Yin J., 2010. Dicamba and sugar effects on callus induction and plant regeneration from mature embryo culture of wheat. *Agri. Sci. in China,* 9, pp. 31-37.

Tinak Ekom D.C., Gaboun F., Udupa S.M., Benchekroun M.N., Ennaji M.M., Iraqi D., 2013. Efficient callus induction and plantlets regeneration in durum wheat using mature embryos. *Cereal Research Communications,* (DOI: 10.1556/CRC.2013.0006).