

## **Development of an efficient regeneration system for bombarded calli from immature embryos of Moroccan durum wheat varieties**

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**Abstract.** One of the biggest obstacles limiting genetic transformation of durum wheat is the lack of an efficient regeneration system for bombarded tissues. Our study aims to optimize culture conditions for regenerating bombarded calli from immature embryos of four durum wheat varieties ‘Amria’, ‘Chaoui’, ‘Isly’ and ‘Marouane’, through comparing the effects of phytohormones (IAA, zeatin and their interaction) and nitrogen amount and sources on callus induction and plant regeneration. Both tested induction media induced approximately the same rate of induced calli for all the tested varieties. However, the interaction of the induction and the regeneration media showed a highly significant effect on plantlet regeneration for all tested varieties. After bombardment, IM1/RM2 combination proved to be the favourable medium with up to 200% and 120% plantlets regenerated for ‘Chaoui’ and ‘Isly’ varieties respectively. Encouraging results obtained in this study will help to promote the research in genetic transformation and its improvement.

**Key words:** Bombarded calli, callus induction, durum wheat, immature embryos, plantlets regeneration.

### **INTRODUCTION**

Durum wheat (*Triticum turgidum subsp. durum*), also called pasta or macaroni wheat, is a tetraploid species. It is the second most cultivated species of wheat after bread wheat, although it only represents around 5% of global wheat production (Arzani & Ashraf, 2017). It is grown mainly in countries in the Mediterranean basin, North America and Australia (Ranieri, 2015). It is a major staple food crop in North Africa and West Asia, and contributes to food and nutrition security of these countries. Commercially produced dry pasta, or pasta secca, is made almost exclusively from durum semolina. The course semolina is used to produce couscous in Morocco and other North African countries. Several recent reports have revealed the negative impact of climate change on

durum wheat production, especially for African regions which already have low productivity levels (Ouraich & Tyner, 2014). Natural variability for adaptation to climate change and various biotic and abiotic stresses are limited. Genetic transformation is an alternative to conventional breeding for the development of stress tolerant cultivars of durum wheat (Semenov et al., 2014).

Genetic transformation of durum wheat has been challenging due to its recalcitrant nature for *in vitro* regeneration (Moghaieb et al., 2010; Bouiamrine et al., 2012) and its large genome size (approximately 17,000 Mb). The biolistic method has proven to be an effective method of genetic modification for many crop species such as wheat, but it is essential to target cells that are competent for both transformation and regeneration. Yamashita et al. (1991) and Hunold et al. (1994) independently showed that more than 90% of bombarded cells integrate DNA in their nucleus and express the *gus* gene, but the limiting step is cell division and plant regeneration.

Based on many previous reports, there is no unique method to achieve efficient culture conditions after bombardment. The induction of direct shoot regeneration depends on several factors, e. g., genotype (Ozgen et al., 1996; Vendruscolo et al., 2008), type and concentration of auxin (Mendoza & Kaeppler, 2002; Filippov et al., 2006), medium components (Redway et al., 1990; Mendoza & Kaeppler, 2002; Greer et al., 2009), and the nature and the stage of the plant organ from which the explant was derived (Ozias-Akins & Vasil, 1982; Redway et al., 1990; Hess & Carman, 1998). Most transformation protocols are developed for single genotypes and can't be easily extrapolated to other genotypes with different abilities to form embryogenic callus and regenerate plants. Cultivar plays the greatest role in the competency for somatic embryogenesis, outweighing other known factors such as explant source (Li et al., 2003), donor plant conditions (Maës et al., 1996) and even medium composition (Mathias & Simpson, 1986). Although cultivar properties are fixed, other factors can be altered to improve somatic embryogenesis, especially constituents of the media used throughout the process.

Several studies have shown that modification of ammonium nitrate concentration ( $\text{NH}_4\text{NO}_3$ ) is important for induction of somatic embryogenesis in wheat (Greer et al., 2009), rice (Grimes & Hodges, 1990), barley (Mordhorst & Lörz, 1993) and other species (Choi et al., 1998; Kothari et al., 2004). Greer et al. (2009) showed that increasing the nitrogen content by 3- and 6-fold and modifying the nitrogen sources resulted in a two-fold increase in the regeneration of primary embryos and a seven-fold increase in the number of regenerated transgenic wheat plants for the cultivar 'Superb'.

Plant growth regulators are also important factors influencing *in vitro* plant regeneration from embryos (Brown et al., 1989). Auxins and cytokinins are the most common and important plant growth regulators for regulating growth and morphogenesis in plant tissue and organ cultures (George et al., 2008). Concentrations of auxins and cytokinins and their combinations play a major role in promoting regeneration in wheat (Malik et al., 2004). The purpose of this study was to optimize culture conditions for regenerating bombarded calli from immature embryos of four Moroccan durum wheat cultivars, through comparing the effects of plant growth regulators and nitrogen source and concentration on callus induction and plant regeneration.

## MATERIAL AND METHODS

### Plant materials

Seeds of Moroccan durum wheat varieties ‘Amria’, ‘Chaoui’, ‘Isly’, and ‘Marouane’ were supplied by INRA Experimental Station, Marchouch, Morocco. Immature embryo explants were collected 12–16 days post-anthesis, from greenhouse grown plants.

### Seed Sterilization and Embryo Culture

Immature seeds were surface-sterilized by washing in 70% ethanol (v/v) for 3 min, followed by 2.4% sodium hypochlorite plus a drop of Tween 20 for 10 min with agitation. Thereafter, they were rinsed three times in sterile distilled water. Immature embryos were aseptically dissected away from the caryopses and the remaining endosperm and radical removed to prevent early germination. The embryos were then placed on two induction media: IM1 (Murashige & Skoog, 1962) with 20.6 mM NH<sub>4</sub>NO<sub>3</sub> and 18.8 mM KNO<sub>3</sub>; and IM2, a modified MS containing 62.5 mM NH<sub>4</sub>NO<sub>3</sub> as the sole nitrogen source (and double total amount of nitrogen as compared to IM1). Both media were supplemented with 20 g L<sup>-1</sup> sucrose, 2 mg L<sup>-1</sup> picloram, 100 mg L<sup>-1</sup> myo-inositol, 150 mg L<sup>-1</sup> L-asparagine and 2.5 g L<sup>-1</sup> Phytigel™. pH was adjusted to 5.8. The immature embryos were cultured for 4 to 5 days in the dark at 25 °C. Following bombardment, embryos were incubated on the same induction medium for 40 days. The studied induction parameters for each genotype were:

$$PCIBB = \frac{NIC}{TNEC} \times 100 \quad (1)$$

$$PSCAB = \frac{NICAB}{TNBC} \times 100 \quad (2)$$

where PCIBB – percentage of callus induction before bombardment; NIC – number of induced calli; TNEC – total number of explants cultured; PSCAB – percentage of survived calli after bombardment; NICAB – number of induced calli after bombardment; TNBC – total number of bombarded calli.

### Rooting and elongation of in vitro regenerated shoots

After 40 days, embryogenic calli from immature embryos were transferred to two different regeneration media: RM1 as described by Iraqi et al. (2005) composed of MS medium supplemented with 100 mg L<sup>-1</sup> myo-inositol, 2 mg L<sup>-1</sup> IAA (indole-3-acetic acid) and 30 g L<sup>-1</sup> sucrose; and RM2, with the same components of RM1 except 1 mg L<sup>-1</sup> zeatin was used instead of IAA; and RM3, with 0.5 mg L<sup>-1</sup> zeatin and 0.1 mg L<sup>-1</sup> IAA. Calli were incubated in 16/8 h light/dark cycle at 25 °C. The media were solidified using 3 g L<sup>-1</sup> Phytigel™. pH was adjusted to 5.7 before sterilization at 120 °C for 20 min. IAA, zeatin and MS vitamins were filter-sterilized and added to the medium after autoclaving. The studied regeneration parameters, calculated eight weeks after transfer of callus to regeneration medium, were:

$$PCR = \frac{NCRP}{NCTR} \times 100 \quad (3)$$

$$PPR = \frac{NPR}{NCTR} \times 100 \quad (4)$$

$$\text{NPPRC} = \frac{\text{NRP}}{\text{NCRP}} \times 100 \quad (5)$$

where PCR – percentage of callus regeneration; NCRP – number of calli with regenerated plantlets; NCTR – number of calli transferred to regeneration; PPR – percentage of plantlets regeneration; NPPRC – number of plantlets per regenerating callus; NPR – number of plantlets regenerated; NPPRC – number of plantlets per regenerating callus.

### Experimental design and statistical analysis

The treatments consisted of 10 replications of each medium for each variety; each replication contained 20 explants (immature embryos). In addition, 3 replications of non-bombarded calli for each medium and each variety were done. Analysis of variance (ANOVA) was performed using the *General Linear Model (GLM)* procedure in SAS (SAS Institute 1985). Means of treatments were compared using the *Least Significant Difference (LSD)* test. *Student's t-test* was applied at a probability level of  $p = 0.05$  to find significant differences between the means.

## RESULTS

### Callus induction

Two induction media (IM1 and IM2) were tested. Callus was induced 3 to 5 days after plating of the immature embryos.

Prior to bombardment, no significant differences were observed between varieties, induction media, or their interaction, since callus formation was induced from 100% of the immature embryo explants (Table 1).

**Table 1.** Callus induction, survival, and plant regeneration from immature embryos of four durum wheat varieties on two induction media after 4 weeks of culture

	Callus induction (%)		Callus survival after bombardment (%)		Callus regeneration (%)		Plantlet regeneration (%)		Number of plantlets per regenerating callus	
	IM1	IM2	IM1	IM2	IM1	IM2	IM1	IM2	IM1	IM2
Variety										
Amria	100a	100a	91.4b	87.3b	18.4bc	5.8b	55.0bc	16.1b	2.0bc	0.8bc
Chaoui	100a	100a	99.1a	96.7a	44.7a	22.4a	181.5a	59.1a	3.7a	2.0a
Isly	100a	100a	94.5b	93.0a	27.2b	20.5a	104.4b	53.7a	2.8ab	1.7ab
Marouane	100a	100a	94.0b	88.0b	13.7c	4.9b	29.8c	5.9b	1.3c	0.4c
LSD	0	0	4.2	4.8	9.6	8.4	57.7	31.5	1.0	1.0
IM1	100a		95.1a		28.8a		106.9a		2.7a	
IM2	100a		92.0b		15.1b		39.0b		1.4b	
LSD	0		2.1		4.2		22.0		0.5	

### Effect of bombardment

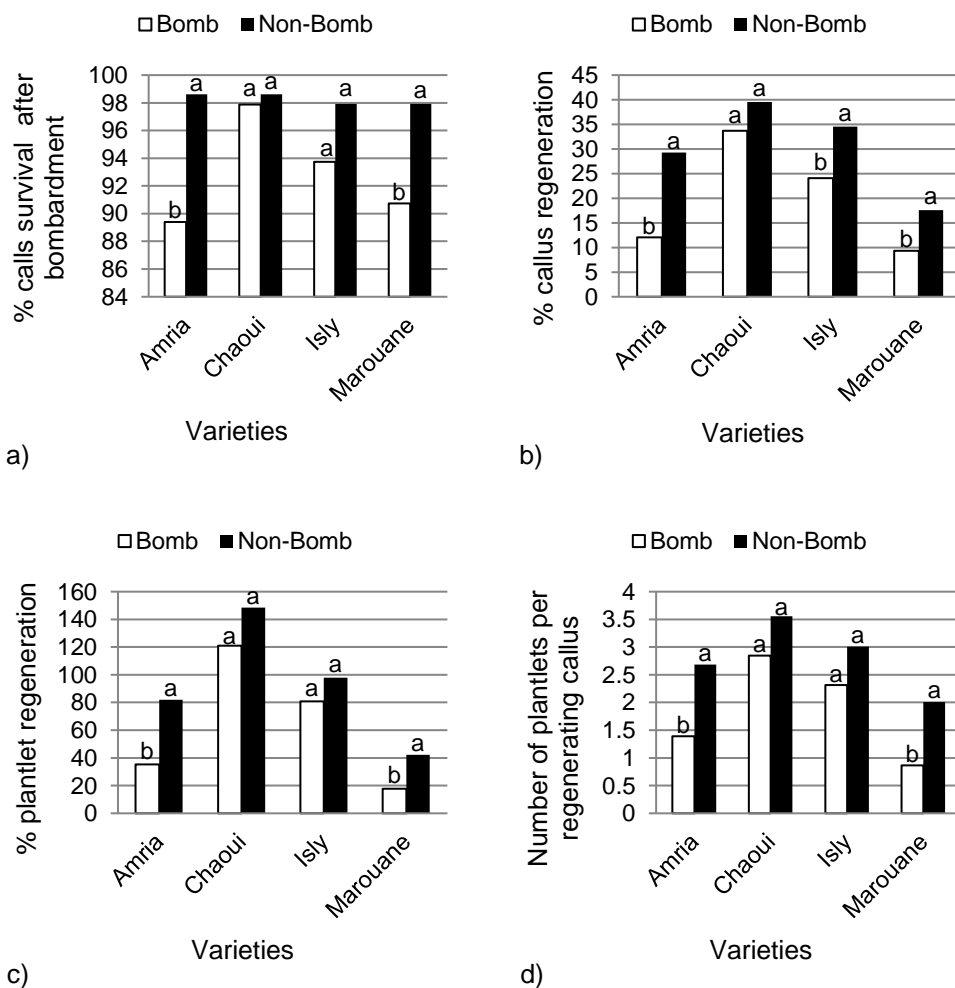
#### a. Callus survival

The cultivars varied in response to bombardment pressure. The highest percentage of survived calli was observed for 'Chaoui' at 99.1% and the lowest was for 'Amria' at

87.3%. After bombardment, the average frequency of callus survival was reduced for all genotypes; however, this reduction wasn't statistically significant for 'Chaoui' and 'Isly' (Fig. 1, a). Across all cultivars, post-bombardment survival was better for callus induced on IM1 than on IM2 (Table 1; Fig. 2, a).

### b. Regeneration

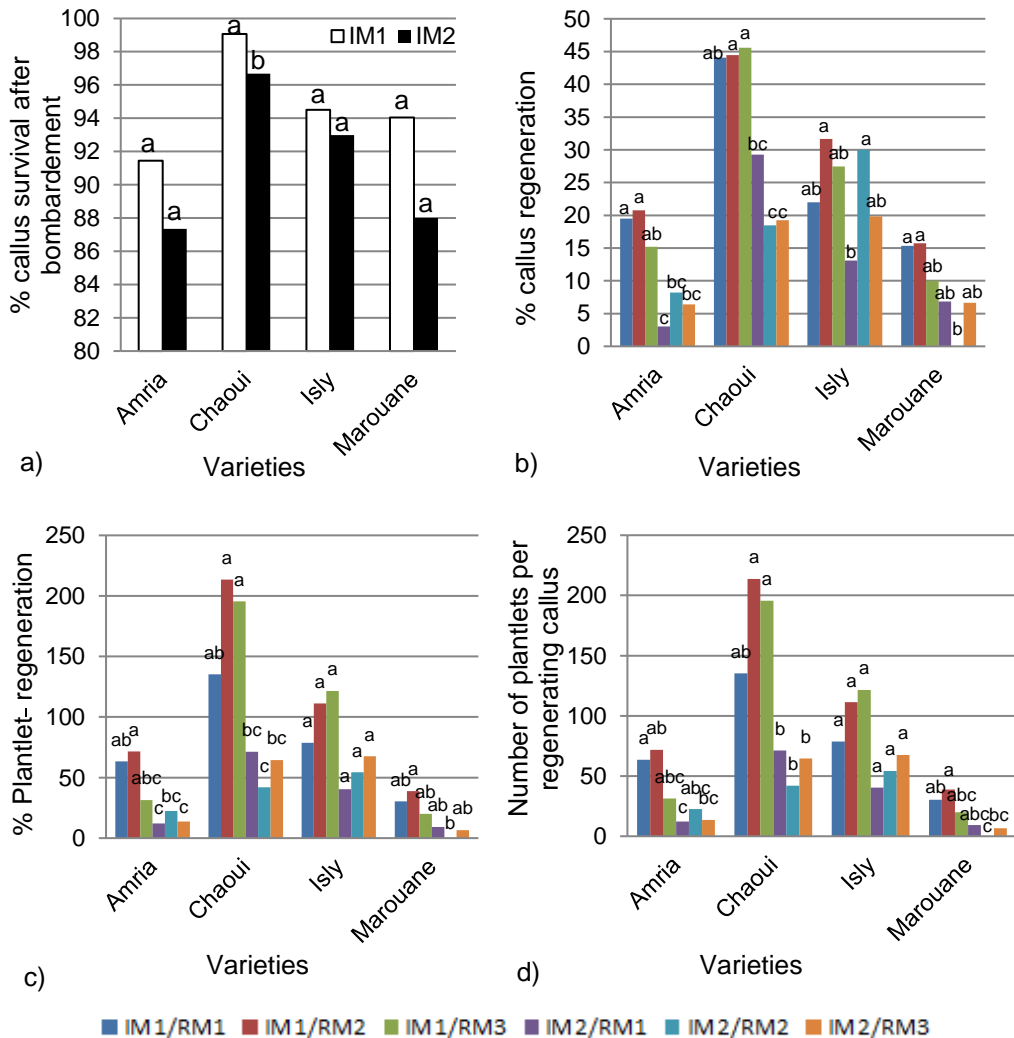
The efficiency of regeneration was also affected by bombardment. 'Amria', and 'Marouane' exhibited lower callus regeneration (Fig. 1, b), plantlet regeneration frequency (Fig. 1, c) and number of plantlets per regenerating callus (Fig. 1, d) following bombardment compared to non-bombarded calli. However, non-significant differences were observed in plantlet regeneration frequency (Fig. 1, c) and number of plantlets per regenerating callus (Fig. 1, d) between bombarded and non-bombarded calli for 'Chaoui' and 'Isly'.



**Figure 1.** Effect of bombardment on % callus survival after bombardment (a), % callus regeneration (b), % plantlet regeneration (c) and number of plantlets per regenerating callus (d).

## Effect of media on regeneration after bombardment

After 40 days of culturing on the induction media, embryogenic calli were transferred to the regeneration media. After an additional 8 weeks, the percentage callus regeneration (Fig. 2, b), plantlet regeneration (Fig. 2, c) and the number of plantlets per regenerating callus (Fig. 2, d) were recorded. The medium used for callus induction (IM) had a significant effect ( $p < 0.001$ ) on all regeneration parameters. The use of different regeneration media (RM) did not have any significant effect. However, the interaction effect (IM\*RM) was highly significant ( $p < 0.001$ ) for all regeneration parameters (Table 2). Therefore, the analysis of the different regeneration parameters will be done through comparing different combinations of IM and RM.



**Figure 2.** Effect of induction medium on % callus survival after bombardment (a) for the tested varieties. Effect of induction and regeneration medium combinations on: % callus regeneration (b), % plantlet regeneration (c) and number of plantlets per regenerating callus (d), for the tested varieties.

**Table 2.** Analysis of variance for the effects of variety, induction medium, and regeneration medium, and their interactions on callus and plantlet regeneration (%) and number of plantlets per regenerating callus

	Callus regeneration (%)	Plantlet regeneration (%)	Number of plantlets per regenerating callus
Variety	26.64***	16.10***	11.85***
IM	39.76***	35.91***	28.74***
Variety*IM	3.00*	3.98**	0.42
RM	0.65	0.88	0.03
Var*RM	1.35	0.45	0.52
Combination (IM/RM)	8.21***	8.01***	5.88***
Var* Combination	1.4	1.20	0.61

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

### a. Percentage of callus regeneration

Percentage of regenerated calli was significantly affected by the genotype and the media combination used (Table 2). The efficiency of callus regeneration was assessed by counting the number of callus producing plantlets for each combination tested and all studied genotypes. The highest callus regeneration rates were observed on IM1 combined with the different regeneration media (Table 1). As illustrated in ‘Fig. 2, b’, IM1 combined with either RM1 or RM2 was best for both ‘Amria’ and ‘Marouane’. IM1 combined with either RM2 or RM3 gave the best results for ‘Chaoui’. IM1 or IM2 combined with RM2 performed best for ‘Isly’. Based on these observations, the IM1/RM2 combination is the best common media regime for all tested varieties.

### b. Plantlets regeneration

There was a significant difference ( $p < 0.001$ ) in plantlet regeneration capacity between different media combinations and cultivars (Table 2). The highest rate (213.6%) was observed for ‘Chaoui’, and the lowest was recorded for ‘Marouane’ (Fig. 2, c). The IM1/RM2 combination produced the highest percentage plantlet regeneration for all varieties except ‘Isly’, which showed a non-significant improvement with IM1/RM3 (Fig. 2, c). For the varieties less affected by bombardment stress (‘Chaoui’ and ‘Isly’), the IM1/RM2 combination enhanced regeneration by 28% compared to unbombarded controls with the IM1/RM1 combination used previously in our research laboratory (data not shown).

### c. Number of plantlets per regenerating callus

Number of plantlets per regenerating callus was also affected by genotype, the induction medium, and the combination (Table 2). The IM1/RM2 combination showed the highest number of plantlets per regenerating callus for ‘Chaoui’ and ‘Marouane’ varieties. The best combination for ‘Amria’ was IM1/RM1. No significant differences among all combinations were observed for ‘Isly’ (Table 1; Fig. 2, d).

## DISCUSSION

The first key requirement for a successful transformation system is a highly regenerable target tissue. It is well known that the production of embryogenic calli and regeneration capacities are genotype dependent in many cereal species such as maize (Manivannan et al., 2010), barley (Sharma et al., 2005), rice (Khanna & Raina, 1998)

and wheat (Bennici et al., 1988; Ozgen et al., 1996; Mzouri et al., 2001; Filippov et al., 2006; Vendruscolo et al., 2008).

In the present study, we have compared four Moroccan durum wheat varieties for their ability to produce embryogenic calli and regenerate plantlets after bombardment. Our results show that the 'Chaoui' and 'Isly' varieties have higher regeneration rates than 'Amria' and 'Marouane'. Mathias & Simpson (1986) and Li et al. (2003) also showed that genotype was the most important factor controlling callus formation and plant regeneration in wheat. Those results can be explained by the variability of genetic components between genotypes and specifically due to the endogenous auxins/cytokinins balance (Carman et al., 1987).

Non-bombarded callus gave higher percentages of regeneration than bombarded callus for all tested varieties. These results are in accordance with several studies which stated that the regeneration frequency of bombarded calluses was always lower in comparison to non-bombarded calluses in rice (Alfonso-rubi et al., 1999), in the eight best 'Bobwhite' accessions (Pellegrineschi et al., 2002) and in Moroccan bread wheat varieties (Ekoum et al., 2014). The 'Isly' and 'Chaoui' varieties were only slightly affected by bombardment compared to 'Amria' and 'Marouane'. The comparison of bombarded and non-bombarded explants is important; the results of this study will be helpful for wheat genetic transformation.

According to many previous reports, callus induction and regeneration capacity of wheat are not only influenced by genotype, but also by other factors such as explant source, culture medium, physiological status of the donor plants, and the interactions between these factors (Ozgen et al., 1996).

In this study, two callus induction media (IM1 and IM2, with different sources and concentrations of nitrogen and different plant growth regulators in the regeneration medium (IAA, zeatin and their combination) were tested for their effects on callus formation and plant regeneration from immature embryo explants of four Moroccan durum wheat varieties ('Amria', 'Chaoui', 'Isly' and 'Marouane').

No significant differences were observed for callus and plantlet regeneration rates between the three regeneration media tested for the same induction medium (Table 2), although a slight improvement using zeatin in RM2 regeneration medium was seen for most varieties. However, the interaction of induction and regeneration media showed a highly significant effect on all regeneration parameters due to the prevailing role of the induction medium on embryogenic callus formation and the ultimate relationship between the two stages.

IM2 induction medium decreased the average percentage of survived calli and plantlet regeneration for both bombarded and non-bombarded calli. Our results are in accordance with the study of Abdollah et al. (2014) who reported that for wheat somatic embryogenesis, when the concentration of ammonium nitrate is more than the normal concentration used in MS medium, the formation of green nodules decreased from  $3.4 \pm 0.2$  to  $1.9 \pm 0.2$  and percentage of nodulated callus from 80% to 50%. It was suggested that the high concentration of  $\text{NH}_4^+$  might be toxic (Mengel & Kirkby, 1982). Indeed, Greer et al. (2009) found that increasing the nitrogen content was ideal for regenerating the wheat cultivar 'Superb', but was minimal for regenerating other cultivars. It was suggested that each species, cultivar and even tissue has its own unique preference for different salt concentrations (He et al., 1989; Maës et al., 1996). In our study, the reduction of plantlet regeneration rates with higher nitrogen was more



significant for bombarded than for non-bombarded callus (from 106% to 38% and from 124% to 59% respectively). This could be explained by the suggestion that after the physical stress of bombardment and the high osmotic treatment of mannitol, cells already plasmolyzed were more sensitive to the high concentration of ammonium nitrate and were unable to regulate their turgor pressure to survive.

## CONCLUSION

An efficient and reliable plant regeneration protocol for immature embryos of four Moroccan durum wheat varieties after bombardment has been established. The present study demonstrated significant effects of genotype, medium and bombardment on embryogenic callus formation and plant regeneration. An induction medium with a high concentration of nitrogen proved to be toxic for the tested Moroccan durum wheat varieties. Bombardment decreased the regenerative capacity of all tested varieties, with less damage for 'Chaoui' and 'Isly' than for 'Amria' and 'Marouane'. The interaction of IM1 induction medium with RM2 regeneration medium enhanced the plantlet regeneration rate for most tested varieties from both bombarded and non-bombarded calli. The results of our study may be beneficial to future applications of durum wheat immature embryo culture for transformation and other biotechnological objectives.

ACKNOWLEDGEMENTS. We are grateful to ICGEB, Italy for generous funding.

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