

## Development of an efficient regeneration system for mature bombarded calli of Moroccan durum wheat varieties

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### Abstract

This study examined the effects of various environmental and genetic factors on callus induction and plant regeneration of bombarded calli from mature embryos of durum wheat using the biolistic method. In this study, three Moroccan durum wheat varieties ('Isly', 'Amria', 'Marouane') were cultivated on two induction media (IM1 and IM2) with different nitrogen sources and contents. After that, each variety cultured on both induction media was transferred in to two regeneration media (RM1 and RM2) with different phytohormones, whereas each variety distributed through four combinations of treatments: IM1RM1, IM1RM2, IM2RM1, and IM2RM2. A completely randomized design with five replications per treatment for each genotype was used. Parameters considered in this study were phytohormones, nitrogen source and its content, plant variety, and their interactions. The study found that variety, medium and variety × medium interactions have a statistically significant effect on callus induction and plantlets regeneration. Prior to bombardment, the maximum percentage of callus induction was obtained under IM1. Conversely, the callus survival rate was not affected by the induction media once bombarded for all three varieties. The induction media had a significant effect on all regeneration parameters ( $p < 0.01$ ). The variety 'Isly' showed the best regeneration efficiency after bombardment, with nearly 80% of plantlets regenerated under IM1 and RM2 combination. These media can be used for genetic transformation of durum wheat.

**Keywords:** Bombarded calli; callus induction; durum wheat; mature embryos; plantlets regeneration.

**Abbreviations:** ANOVA\_Analysis Of Variance, GLM\_General Linear Model, IAA\_Indole-3-acetic acid, INRA\_National Institute of Research for Agriculture, LSD\_Least Significant Difference, MS\_Murashige and Skoog.

### Introduction

Durum wheat ( $2n = 4x = 28$ ; AABB) is an important cereal crop used for human consumption worldwide. It is the second most cultivated species of wheat after common wheat. In Morocco, durum wheat is an essential contributor to the daily protein and food calories intake of population. It is utilized in the production of semolina, bread and pasta. The annual consumption of bread and cereals per capita in Morocco is expected to reach more than \$770 USD in 2023 (Statista Research Department, 2019).

In recent years, wheat production has been declining due to climate change, with a growing intensity of biotic as well as abiotic stresses. With a world population expected to reach 9 billion by 2050, global agriculture production including wheat has to be increased by about 60-70 percent from the current levels to meet the increased food demand in 2050 (Silva, 2018). To meet this demand, wheat producing countries have to increase wheat yield through developing innovative cropping techniques and wheat cultivars that are resilient to biotic and abiotic stresses. The production of genetically modified crops is one of the approaches to

achieve stress tolerant cultivars of durum wheat that may not be easily developed through conventional breeding methodologies.

Gene transfer technique is now a routine procedure deployed on several plant species, including wheat. The biolistic method has proven to be an effective method for the genetic modification of wheat (Tassy and Barret, 2017). Yamashita et al. (1991) and Hunold et al. (1994) independently showed that more than 90% of bombarded wheat cells are likely to be transformed if they proceed towards cell division and plant regeneration. On the other hand, the *in vitro* regeneration system has a poor efficiency and is considered the most limiting step in the genetic improvement process. Hence, establishing a simple, efficient and stable regeneration system, prior to genetic transformation, has become critical for advancement of gene transfer technologies. Also most studies have focused on the development of efficient transformation systems for a single responsive genotype, and are not readily transferrable to alternative genotypes. The lack of genotype-

independent transformation systems is a challenge, especially for wheat cultivars.

Previous studies have shown that the response of wheat tissue culture to callus induction and plant regeneration depends on multiple factors, including the composition of culture medium (Greer et al., 2009; Ekom et al., 2014), the explant culture (Redha and talaat, 2008), the physiological status of the source plant (Hess and Carman, 1998), and the genotype (Aadel et al., 2016).

The constituents of the media can be adjusted to improve somatic embryogenesis. For example, the modification of ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ) concentration was shown to be an important factor for inducing somatic embryogenesis in cereals (Kothari et al., 2004; Greer et al., 2009). Greer et al. (2009) bombarded scutella in 'Superb' cultivar and reported that the modification of ammonium nitrate concentration in the induction medium increased the number of primary embryos by more than two-fold. Furthermore, the increase in the nitrogen content up to 3- and 6-fold resulted in an increase in the regeneration of plantlets by a factor of 8.4 and 13.4, respectively.

Media composition, especially the hormonal balance, is another important factor that influences the formation of regenerable calli from embryos (Miroshnichenko et al., 2017). Auxins and cytokinins are the most widely plant growth regulators to control callus induction, plant cell division and organ regeneration (Nic-Can et al., 2016). Concentrations of auxins and cytokinins and their combinations play a key role for enhanced regeneration for a large number of plant species (Wójcik et al., 2020), including wheat (seldimirova et al., 2016). Another factor to consider in the explant is the embryos maturity. Multiple studies of wheat have shown that immature embryos are more efficient in callus induction and shoot regeneration than mature embryos (Alikina et al., 2016; kumar et al., 2017). However, unlike immature embryos, mature embryos have the advantage of being available throughout the year, in bulk quantities and easy to handle.

This study aims to optimize the culture conditions for regenerating bombarded calli from mature embryos of wheat, through comparing the effects of phytohormones and nitrogen sources on callus induction and plants regeneration of three Moroccan durum wheat cultivars.

## Results

### Callus induction

The calli were induced after 4 to 6 days of culturing on induction media IM1 and IM2. These two induction media were evaluated for the best wheat callus induction and regeneration.

Prior to bombardment, the callus induction was significantly affected by the medium ( $p < 0.05$ ) and variety used ( $p < 0.01$ ). The 'Amria' and 'Isly' varieties showed significantly higher percentages of callus induction than 'Marouane' variety (Table 1). Moreover, the rate of callus induction in IM1 was generally better than in the IM2 for all three varieties (Table 2).

### Effect of bombardment

#### Callus survival

The average survival rate of embryonic calli was reduced for all genotypes after bombardment (Fig 1a). There were no statistically significant differences in survival rate between

the genotypes nor between induction medium (Table 1). However, across all cultivars, post-bombardment survival was better for the callus induced on IM2 than on IM1 (Table 1, Fig 2a).

### Regeneration

Similarly the regeneration efficiency was also negatively affected by bombardment. All tested varieties exhibited a lower callus regeneration (Fig 1b), plantlet regeneration frequency (Fig 1c) and number of plantlets per regenerating callus (Fig 1d) post-bombardment compared to non-bombarded calli. However, there were no statistically significant differences in number of plantlets per regenerating callus between the bombarded and non-bombarded group for the varieties 'Amria' and 'Isly' (Fig 1d).

### Effect of media on regeneration after bombardment

After 40 days of culture in the induction media, the embryogenic calli were transferred into two different regeneration media. After 8 weeks of the culturing, the percentage of callus regeneration (Fig 2b), plantlets regeneration (Fig 2c) and the number of plantlets per regenerating callus (Fig 2d) were recorded.

The induction medium had a significant effect ( $p < 0.01$ ) on all regeneration parameters. The regeneration medium had a highly significant effect ( $p < 0.001$ ) on the rate of callus and plantlets regeneration, but not on the number of plantlets per regenerating callus.

Even though, the interaction effect (IM  $\times$  RM) showed a significant effect on all regeneration parameters (Table 3). Therefore, the analysis of the different regeneration parameters will be done through comparing different combinations of IM and RM.

### Callus regeneration

The percentage of regenerated calli was significantly affected by the genotype and the media combination (Table 3). The efficiency of callus regeneration was assessed by counting the number of callus producing plantlets, for all studied genotypes and each combination tested. The highest callus regeneration rate was observed on RM2 combined with either IM1 or IM2. As illustrated in Fig 2b, RM2 combined with IM2 gave the best results for both 'Amria' and 'Isly', whereas RM2 combined with IM1 performed best for 'Marouane'.

### Plantlets regeneration

The percentage of plantlets regenerated and number of plantlets per regenerating callus were determined after eight weeks of culture in regeneration medium. All wheat cultivars showed *in vitro* plantlets regeneration capacity.

Results indicated that there was a highly significant difference ( $p < 0.001$ ) in plantlets regeneration capacity between the different media combinations and cultivars (Table 3). The highest regeneration rate was observed for 'Isly' (78.6%) and the lowest was recorded for 'Amria' (Fig 2c). On the other hand, the IM1/RM2 combination produced the highest percentage of plantlets regeneration for the 'Isly' and 'Marouane' varieties, whereas IM1/RM1 was the optimum combination for the 'Amria' variety (Fig 2c).

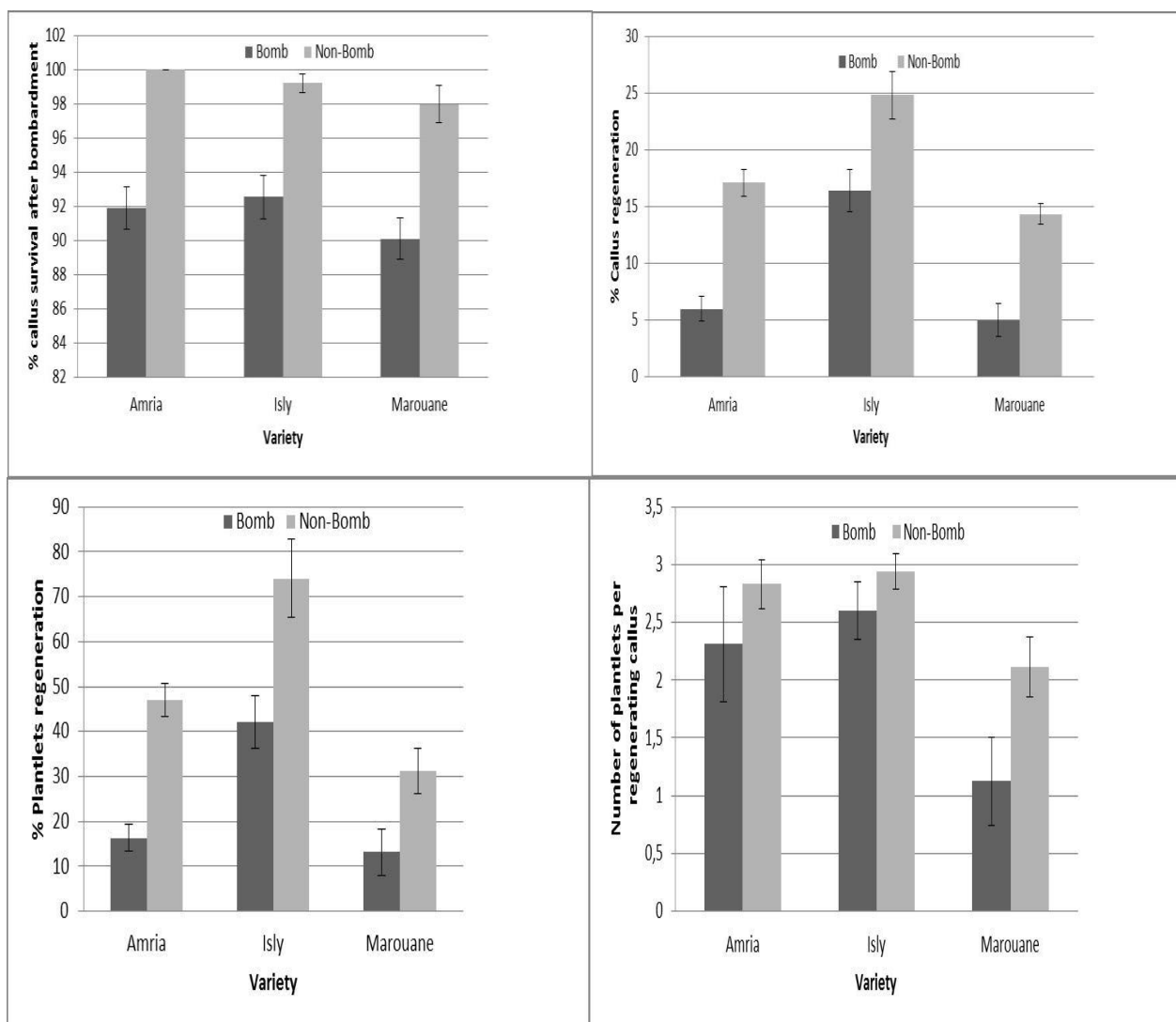
### Number of plantlets per regenerating callus

Similarly, the genotype, the induction medium and the media combination affected the number of plantlets per regenerating callus (Table 3). The IM1/RM2 combination

**Table 1.** Induction parameters for three durum wheat varieties obtained on two induction media (IM1 and IM2) after 4 weeks of culture and their effect on callus regeneration (%), plantlets regeneration (%) and number of plantlets per regenerating callus.

| Variety    | Callus induction (%) | Callus survival after bombardment (%) | Callus regeneration (%) | Plantlets regeneration (%) | Number of plantlets per regenerating callus |
|------------|----------------------|---------------------------------------|-------------------------|----------------------------|---|
| 'Amria'    | 92.18 a              | 91.90 a                               | 5.99 b                  | 16.30 b                    | 2.31 a                                      |
| 'Isly'     | 93.18 a              | 92.53 a                               | 16.38 a                 | 42.10 a                    | 2.60 a                                      |
| 'Marouane' | 88.06 b              | 90.10 a                               | 5.00 b                  | 13.12 b                    | 1.12 b                                      |
| LSD        | 2.73                 | 3.55                                  | 2.47                    | 5.64                       | 0.80  |
| IM1        | 92.48a               | 90.61a                                | 8.38b                   | 29.46a                     | 2.54 a                                      |
| IM2        | 90.12b               | 92.58a                                | 10.98a                  | 21.04b                     | 1.58b                                       |
| LSD        | 2.22                 | 2.89                                  | 2.01                    | 4.58                       | 0.65  |

\*The values followed by the same alphabet are not significantly different at  $\alpha = 0.05$  according to the LSD test; LSD: Least Significant Difference.

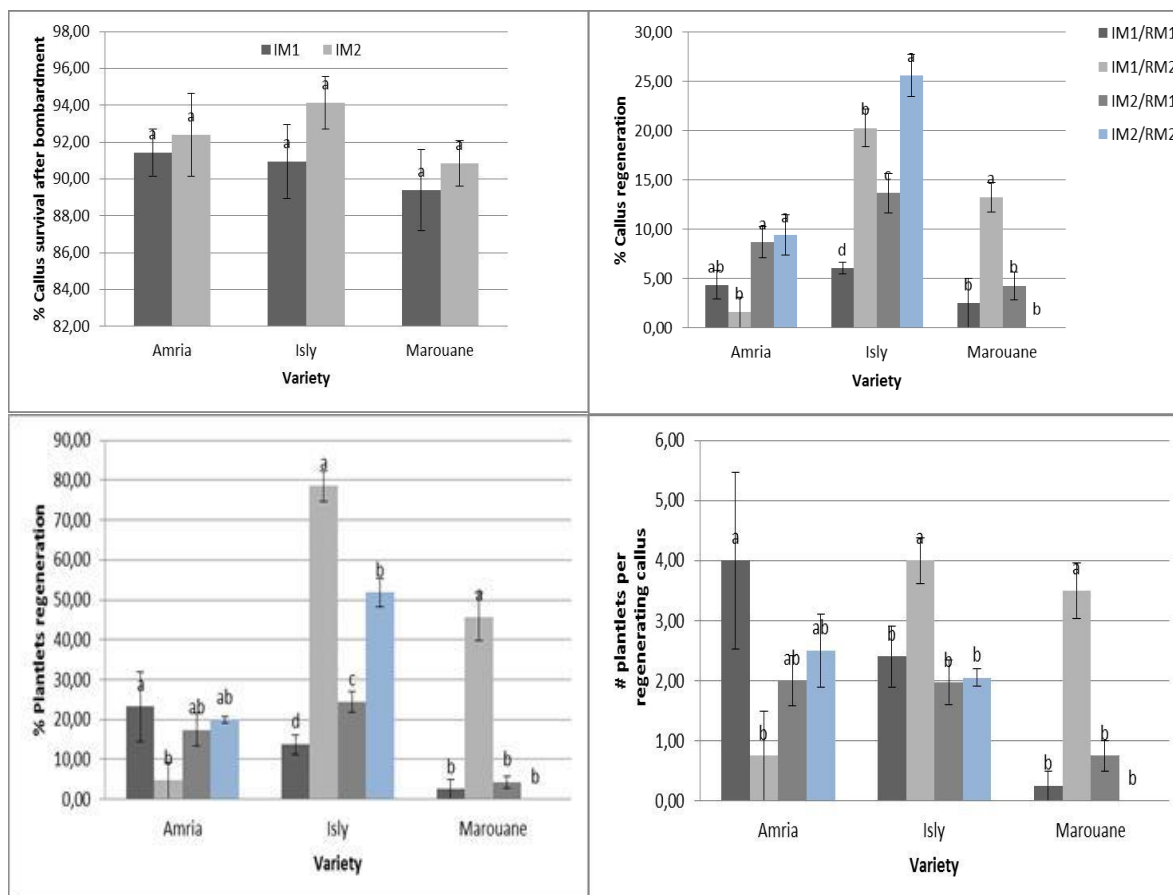


**Fig 1.** Effect of bombardment on (a) callus survival, (b) callus regeneration, (c) plantlet regeneration, and (d) number of plantlets per regenerating callus. The same alphabet on the bar are not significantly different at  $\alpha = 0.05$  according to the LSD test.

**Table 2.** Callus induction and callus survival after bombardment from mature embryos of three durum wheat varieties on two induction media after 4 weeks of culture.

| Variety    | Callus induction (%) |         | Callus survival after bombardment (%) |         |
|------------|----------------------|---------|---------------------------------------|---------|
|            | IM1                  | IM2     | IM1                                   | IM2     |
| 'Amria'    | 93.75a               | 90.62 a | 91.42 a                               | 92.39 a |
| 'Isly'     | 93.64 a              | 92.73 a | 90.94 a                               | 94.14 a |
| 'Marouane' | 89.77 a              | 86.36 b | 89.37 a                               | 90.84 a |
| LSD        | 4.12                 | 4.10    | 5.62                                  | 4.90    |

\*The values followed by the same alphabet are not significantly different at  $\alpha = 0.05$  according to the LSD test; LSD: Least Significant Difference.



**Fig 2.** Effect of induction medium on (a) % callus survival after bombardment. Effect of induction and regeneration medium combination on (b) % of callus regeneration, (c) % of plantlet regeneration, and (d) number of plantlets per regenerating callus. The same alphabet on the bar are not significantly different at  $\alpha = 0.05$  according to the LSD test.

**Table 3.** Analysis of variance associated with factors impacting the regeneration.

| Factors               | Variance in Callus regeneration (%) | Variance in Plantlet regeneration (%) | Variance in number of plantlets per regenerating callus |
|-----------------------|-------------------------------------|---------------------------------------|---|
| Variety               | 57.26***                            | 69.60***                              | 7.70**  |
| IM                    | 6.84**                              | 13.76***                              | 8.79**  |
| Variety x IM          | 15.73***                            | 10.69***                              | 1.55  |
| RM                    | 33.09***                            | 88.01***                              | 0.78  |
| Variety x RM          | 18.51***                            | 48.79***                              | 6.00**  |
| Combination (IM/RM)   | 14.95***                            | 42.16***                              | 3.54*   |
| Variety x Combination | 13.77***                            | 26.80***                              | 6.38***   |

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

showed the highest number of plantlets per regenerating callus for the 'Isly' and 'Marouane' varieties. The best combination for 'Amria' was IM1/RM1, with approximately the same number of plantlets per regenerating callus (4.00) as 'Isly'. The variety 'Marouane' scored the lowest number of plantlets compared with genotypes tested (Fig 2d).

## Discussion

Durum wheat is well-known for being a recalcitrant plant for genetic transformation. Hence, the development of an efficient and high potent *in vitro* regeneration system prior to transformation is a prerequisite for efficient genetic transformation. One of the key factors controlling regeneration is genotype. Various studies identified genotype as a major factor, which contributes to the production of embryogenic calli and regenerated plantlets in many cereal species, including maize (Muppala et al., 2020), barley (Al-ajlouni et al., 2012), rice (Barman et al., 2016) and bread wheat (Ahmadpour et al., 2018). Bouiamrine et al. (2012) stated that callus induction and plant regeneration in durum wheat depends greatly on genotype. Likewise, Mathias and Simpson (1986) reported that the genotype was more important than the medium in terms of regeneration. Those results are partly explained by the genetic variability of the endogenous content of growth regulators between genotypes, more specifically the ratio of cytokinins and auxins (Jiménez and Bangerth, 2001). However, genotype is not the only factor impacting callus induction and regeneration capacity of wheat. It is a multifactorial process influenced by other variables such as explant source, geographical origin and physiological status of the donor plant, culture medium, and their interactions.

Three Moroccan durum wheat varieties were compared for their ability to produce embryogenic calli and regenerate plantlets after bombardment. Our results showed that the 'Isly' variety has higher regeneration rates than 'Amria' and 'Marouane'. These results corroborate the outcome from previous studies on Moroccan durum wheat varieties (Ekom et al., 2013; Hakam et al., 2014).

Modification of ammonium nitrate concentration in media has been shown to promote callus induction (Constabel and Shyluk, 1994), somatic embryogenesis in cereals (Kothari et al., 2004; Greer et al., 2009) and genetic transformation (Boyko et al., 2009; Greer et al., 2009). This study experimented two callus induction media (IM1 and IM2), with different sources and concentration of nitrogen, and different plant growth regulators in the regeneration medium (IAA and Zeatin). The effects of these factors on callus formation and plants regeneration from mature embryos explants of three Moroccan durum wheat varieties ('Amria', 'Isly' and 'Marouane') were evaluated. The induction medium IM2 increased the average survival percentage of calli and callus regeneration while the percentage of plantlets regeneration was decreased from 29% to 21% (Table 1). This result could be explained by the fact that the ammonium nitrate acts on the regenerative ability of calli, and not on their capacity to produce primary embryos. These results confirm the study of Abdollah et al. (2014) who reported that for wheat somatic embryogenesis, the formation of green nodules decreased when the concentration of ammonium nitrate was above the standard concentration used in MS medium.

However, published experimental results regarding the impact of ammonium nitrate are not unanimous. Menke-

Milczarek and Zimny (2001) failed to establish a strong link between ammonium nitrate ratio and the efficiency of somatic embryogenesis in wheat. Grimes and Hodges (1990) found that the nitrogen level did not significantly affect the number of regenerated plants in rice. Greer et al. (2009) found that the ideal nitrogen content for regenerating the cultivar 'Superb' was suboptimal for other cultivars. This discrepancy could be explained by the fact that each species, cultivar, or even tissue, has its own preferences in terms of salt concentration (He et al., 1989).

The results reported in this paper showed a highly significant effect ( $p < 0.001$ ) of regeneration medium on callus and plantlets regeneration rate (Table 3). The addition of Zeatin at 1 mg/l concentration in the regeneration medium significantly improved the calli regeneration capacity from 14% in medium with IAA to 35% (data not shown). This observation is consistent with the findings of many researchers. Malik et al. (2017) reported that Zeatin at 1 mg/l concentration greatly increased shoot regeneration (98%) and gave the maximum of mean number of shoots (8-9 shoots per calli). He and Lazzeri (2001) reported higher number of plantlet regeneration (34 plantlets per scutellum) with high regeneration rate (97 to 100%) in durum wheat after one or two passages through a regeneration medium containing Zeatin. The study of Fahmy et al. (2004) concluded that the highest average of regenerated plantlets across the studied wheat cultivars was obtained with a concentration of 1 mg/l of Zeatin. The IAA gave a better result in the case of the 'Amria' variety. The choice of growth regulator for optimum callus growth, development and regeneration depends largely on the genotype of the plant species and the explant (Kumar et al., 2017). It was suggested that the generally low efficiency of exogenous IAA compared to Zeatin could be related to the main roles of auxins. Auxins are thought to regulate or influence diverse responses on a whole-plant level, such as plant organogenesis, flower initiation, formation of meristems, and responses on cellular level, such as cell elongation, division, and differentiation (Hu et al., 2017). This study showed that bombarded calli have a lower regeneration rate than non-bombarded calli for all tested varieties. The adverse effect of bombardment can be explained by the fact that the calli were sensitive to the damage caused by gold particles, resulting in reduced ability to regenerate shoots. These results are aligned with several studies such as the study of Hakam et al. (2014) where bombarded calli were found to have a lower regeneration rate than non-bombarded calli.

## Materials and methods

### Plant materials

The seeds of 'Amria', 'Isly' and 'Marouane' variety were provided by the Experimental Research Station of INRA (Marchouch, Morocco).

### Seed sterilization and embryo culture

Mature seeds were surface-sterilized by washing with 70% (v/v) ethanol solution for 3 min, followed by a bath in 2.4% sodium hypochlorite solution with a drop of TWEEN® 20 detergent for 15 min with agitation. Seeds were then rinsed three times with sterilized distilled water (under laminar flow). The disinfected seeds were soaked in sterilized distilled water overnight until seeds got fully turgid, and embryos swelled and increased in size. Mature embryos

were aseptically dissected out from the caryopses. The remaining endosperm and radical were removed to prevent early germination.

The embryos were then split and placed in one of the two induction media, IM1 or IM2. The medium IM1, as defined by Murashige and Skoog (1962), consists of 20.6 mM of  $\text{NH}_4\text{NO}_3$  and 18.79 mM of  $\text{KNO}_3$ . The modified medium IM2 consists exclusively of 62.5 mM of  $\text{NH}_4\text{NO}_3$ . In IM2, the amount of nitrogen is doubled compared with IM1.  $\text{NH}_4\text{NO}_3$  is the only source of nitrogen for wheat scutella. Both media were supplemented with 20 g/l sucrose, 2 mg/l picloram, 100 mg/l Myo-inositol, 150 mg/l L-Asparagine and 2.5 g/l phytigel. All media were adjusted to pH 5.8. The cultures were kept in the dark at 25 °C for 3 to 5 days. The induced embryos were bombarded (see section 3.3). Following bombardment, the calli were incubated in the both induction media for a period of 40 days.

The induction parameters were defined as follow:

- Percentage of callus induction before bombardment = number of induced calli / total number of explants cultured;
  - Percentage of callus induction after bombardment = number of induced calli / total number of bombarded calli.
- The induction parameters were calculated to each genotype.

#### Particle bombardment

Three to five days after placing the mature embryos on callus induction medium, induced embryos were placed in the center of a petri dish for 4 hours in the osmotic MS medium supplemented with 15% mannitol, and bombarded at 1100 psi helium pressure, at a target distance of 9 cm and under a vacuum of 28 mmHg with 1 $\mu\text{m}$  gold particles by employing the Biolistic PDS-1000/He particle delivering system (Bio-Rad, USA).

#### Rooting and elongation of *in vitro* regenerated shoots

After 40 days, the embryogenic calli derived from mature embryos were transferred in to two different regeneration media: RM1 and RM2. RM1, described by Iraqi et al. (2005), composed of MS medium supplemented with 100 mg/l of Myo-inositol, 2 mg/l of IAA and 30 g/l of sucrose. RM2 is identical to RM1 except that the IAA was substituted with 1 mg/l of Zeatin.

Calli were incubated in a 16/8 h light/dark cycle and a temperature of 25 °C. The media of regeneration were solidified by using 3 g/l phytigel. The pH was adjusted to 5.7 before sterilization at 120 °C for 20 min. The IAA, Zeatin and MS vitamins were sterilized by filtration and added to the medium before cooling.

The regeneration parameters were defined as follow:

- Percentage of callus regeneration = number of calli with regenerated seedlings / total number of calli transferred to the regeneration medium;
- Percentage of plantlets regeneration = number of regenerated plantlets / the number of callus transferred to the regeneration medium;
- Number of plantlets per regenerating callus = number of regenerated plantlets / number of calli with regenerated seedlings.

The regeneration parameters were calculated for each genotype eight weeks after the transfer of callus into the regeneration medium.

#### Experimental design and statistical analysis

A completely randomized design with five replications per treatment of each genotype was used in all experiments. In

addition, three replicates of non-bombarded calli were conducted for each medium and variety. Dishes contained 20 explants per treatment group. The statistical significance was evaluated using the ANOVA available in the GLM procedure in SAS® (SAS Institute, 1985). The mean of the different treatments were compared using the LSD test with a significance level  $\alpha$  set at 0.05.

#### Conclusion

This study established an efficient and reliable plant regeneration protocol through somatic embryogenesis post-bombardment for mature embryos from three Moroccan durum wheat varieties. This study demonstrated the significant effects of bombardment, genotype, medium, and genotype/medium interaction on embryogenic callus formation and plant regeneration. The bombardment reduced the regenerative capacity of all tested varieties. Similarly, high concentration of nitrogen in the induction medium significantly decreased the regenerative potential. The use of Zeatin in the regeneration media enhanced the plantlet regeneration rate for most varieties. The variety 'Isly' had the highest regeneration efficiency with IM1/RM2 combination and proved to be the least sensitive to bombardment. These promising results will help drive forward progress in the field of wheat genetic transformation.

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