J Plant Biotechnol (2021) 48:246−254
DOI:https://doi.org/10.5010/JPB.2021.48.4.246

ISSN 1229-2818 (Print)
ISSN 2384-1397 (Online)

Research Article

Biolistic transformation of Moroccan durum wheat varieties by using mature embryo-derived calli

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Received: 20 November 2021 / Revised: 22 December 2021 / Accepted: 23 December 2021
ⓒ Korean Society for Plant Biotechnology

Abstract Environmental stresses are estimated to have reduced global crop yields of wheat by 5.5%. However, traditional approaches for the transfer of resistance to these stresses in wheat plants have yielded limited results. In this regard, genetic transformation has undoubtedly opened up new avenues to overcome crop losses due to various abiotic stresses. Particle bombardment has been successfully employed for obtaining transgenic wheat. However, most of these procedures employ immature embryos, which are not available throughout the year. Therefore, the present investigation utilized mature seeds as the starting material and used the calli raised from three Moroccan durum wheat varieties as the target tissue for genetic transformation by the biolistic approach. The pANIC-5E plasmid containing the *SINA* gene for drought and salinity tolerance was used for genetic transformation. To enhance the regeneration capacity and transformation efficiency of the tested genotypes, the study compared the effect of copper supplementation in the induction medium (up to 5 μM) with the standard MS medium. The results show that the genotypes displayed different sensitivities to $CuSO_4$, indicating that the transformation efficiency was highly genotype-dependent. The integration of transgenes in the T_0 transformants was demonstrated by polymerase chain reaction (PCR) analysis of the obtained resistant plantlets with primers specific to the *SINA* gene. Among the three genotypes studied, 'Isly' showed the highest efficiency of 9.75%, followed by 'Amria' with 1.25% and 'Chaoui' with 1%.

Keywords Biolistic transformation, Copper sulphate, Durum wheat, Matures embryos, Plasmid pANIC-5E, *SINA* gene.

Abbreviations ANalysis Of Variance (ANOVA), Cetyltrimethylammonium bromide (CTAB), General Linear Model (GLM), National Institute of Research for Agriculture (INRA), Least Significant Difference (LSD), Murashige and Skoog (MS), Phosphinothricin (PPT), Seven In Absentia (SINA).

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Introduction

Wheat is one of the most important crops grown worldwide. It is the third largest cereal in terms of production in the world after rice and maize and the second most important in terms of calories after rice. It is the primary source of proteins in low- and middle-income countries (www.wheat.org). Environmental stresses, such as drought, salinity of soil, and extreme temperatures, have decreased its production by 20 to 30%. Moreover, with such an increasing population expected to reach 9 billion by 2050, wheat production has to be increased by about 60 to 70% (www.wheat.org). There has been considerable work done to develop drought tolerant wheat through classical breeding. However, progresses made are slow and still not up to the mark. In this way, genetic engineering proved to be an important method towards improving stresses tolerance in wheat varieties.

The transformation efficiency is determined by two groups of factors: the genetic transformation procedure and the target tissue. First, out of the various DNA delivery methods, biolistic and *Agrobacterium*-mediated methods have played an impor-

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tant role in wheat transformation (Tao et al. 2011). Biolistic bombardment remains however the most widely used method for obtaining transgenic wheat plants since monocotyledons have shown natural resistance to *Agrobacterium* infection (Ziolkowski 2007) and the efficiency of the *Agrobacterium*-mediated method is usually no higher than 1 to 5% (Pukhalskii et al. 1996; Wu et al. 2003). Second, an essential prerequisite for the production of transgenic plants is the availability of a highly regenerable target tissue. Several factors have been described to influence the response of wheat to tissue culture including composition of culture medium for callus induction and plant regeneration (Ekom et al. 2014; Greer et al. 2009), the explant types (Redha and Talaat 2008), and the genotype (Vendruscolo et al. 2008; le Roux et al. 2016).

Immature embryos have been the most widely used explants to genetically transform wheat (Shewry and Jones 2005; Vasil 2005; Xia et al. 2012).Yet, immature embryos are not available throughout the year; require controlled conditions for raising explant donor plants, and skilled personnel to isolate them at a suitable stage (Parmar et al. 2012). Unlike their immature counterpart, mature embryos are available in large quantity throughout the year, can easily be stored as dried seeds and showed a regeneration response comparable to that of immature embryos (Maddock 1985; Ozgens et al. 1996; Ozias-Akins and Vasil 1983). Thus, the present study will exclusively focus on mature embryos as starting explants for transformation.

As the culture media constituents influenced growth and morphogenesis of plant tissues, many authors have observed that elevated concentrations of copper increases the effectiveness of the formation of embryos and regeneration in monocotyledonous and dicotyledonous plants, including triticale (Purnhauser and Gyalai 1993), barley (Dahleen 1995) and wheat (Ghaemi et al. 1994; Purnhauser 1991; Purnhauser and Gyulai 1993; Sparks et al. 2014; Tahiliani and Kothari 2004). These results are not surprising given the essential role played by this micro-element in orchestrating a range of physiological and biochemical functions. Copper is a constituent of the protein component of several enzymes, mainly those participating in electron flow, catalyzing redox reactions in plant cells. Also, copper takes part in the processes of photosynthesis, respiration, carbohydrate biosynthesis and nitrogen metabolism (Lolkema 1985; Purnhauser and Gyulai 1993). It is then essential to study the effect of $CuSO4$ on plant regeneration efficiency for Moroccan wheat varieties, especially after bombardment stress.

Genotypes are known to greatly influence the applicability and efficiency of biolistic gene transfer (Ekom et al. 2014; Iser et al. 1999; Rasco-Gaunt et al. 2001; Ye et al. 2001). Efficient transformation systems are usually well developed for a single responsive genotype and are typically not transferrable to alternative genotypes (Hardwood 2012). Hence, it is necessary to develop transformation system for Moroccan wheat cultivars that are well adapted to Moroccan environment.

In the present study, seven in Absentia gene (*SINA*) was selected for the genetic transformation of Moroccan durum wheat varieties, with the objective of developing transgenic plants with increased tolerance to water and salinity stress tolerance. The effects of copper supplementation in callus induction media on regeneration capacity and transformation efficiency of the *SINA* gene using wheat mature embryo as explant were studied.

Materials and Methods

Plant materials and culture conditions

The seeds of 'Amria', 'Chaoui' and 'Isly' varieties were provided by the Experimental Research Station of National Institute of Agronomic Research (INRA) at Marchouch, Morocco. 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 excised from the caryopses, the remaining endosperm and radical to prevent early germination. The embryos were then split and placed with the scutellum upwards on 2 induction media, IM1 or IM2. The medium IM1, as defined by (Murashige and Skoog 1962) and the modified medium IM2 with the same components as IM, supplemented with 1.25 mg/l of $CuSO_4$. 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 phytagel. All media were adjusted to pH 5.8. Cultures were incubated at 25°C in darkness for approximately 3 to 4 days before bombardment.

The induction parameters were defined as follow:

$$PCIBB = \frac{\text{Number of induced calli}}{\text{Total number of explants cultured}} \times 100$$

$$PCIAB = \frac{\text{Number of induced calli after bombardment}}{\text{Total number of bombarded calli}} \times 100$$

Where: PCIBB– Percentage of callus induction before bombardment
PSCAB– Percentage of callus induction after bombardment

Bacteria materials and genetic construction

The plasmid used for bombardment is pANIC –5E (Fig. 1), containing the linked selectable marker/herbicide resistance bar (phosphinothricin acetyl transferase) gene (placed under the transcriptional control of the rice actin 1 gene (OsAct1) promoter and the 35S terminator). The vector was modified by cloning the *SINA* gene into the Gateway_ cassette by placing the *SINA* gene inserted between attR1 and attR2 recombination sites (Kindly provided by Mohamed Fokar).
The *Escherichia coli* strain carrying plasmid pANIC- 5E harboring *SINA* gene construct were grown over night in LB solid medium supplemented with 100 mg/l Ampicillin and 50 mg/l Kanamycin at 37°C. A single colony was subcultured in 5 ml LB liquid medium at 37°C for 2 days in a shaking incubator (set at 150 rpm), followed by the addition of 45 ml of fresh LB liquid medium and a further growth for 5 h. Plasmid DNA was isolated using the Bioline ISOLATE II Plasmid Mini Kit.

Particle bombardment, production and selection of transformants

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 1,100 psi helium pressure, at a target distance of 9 cm and under a vacuum of 28 mmHg with 1 μm gold particles. The gold particles used for the bombardment were coated with the plasmid DNA (pANIC- 5E) containing the *SINA* gene as per the procedure described by Iraqi et al. (2005). The Biolistic PDS-1000/He system (Bio-Rad, USA) was used for the bombardment. Following bombardment, calli were first incubated in high osmotic medium for 16 h, and then incubated in IM1 or IM2 induction medium for 4 weeks in the dark at 25°C. The calli were then transferred to the regeneration medium supplemented with 1 mg/l of Zeatin and 3 mg/l of PPT (Phosphinothricin) for a period of 9 weeks at 25°C, under 16/8 h light/dark cycle with a medium change every 3 weeks. The surviving regenerated plantlets were transferred for rooting to MS half-strength medium lacking hormones. The rooted plants showing resistance to PPT were transplanted into soil filled pots and grown in a green-house until maturity.

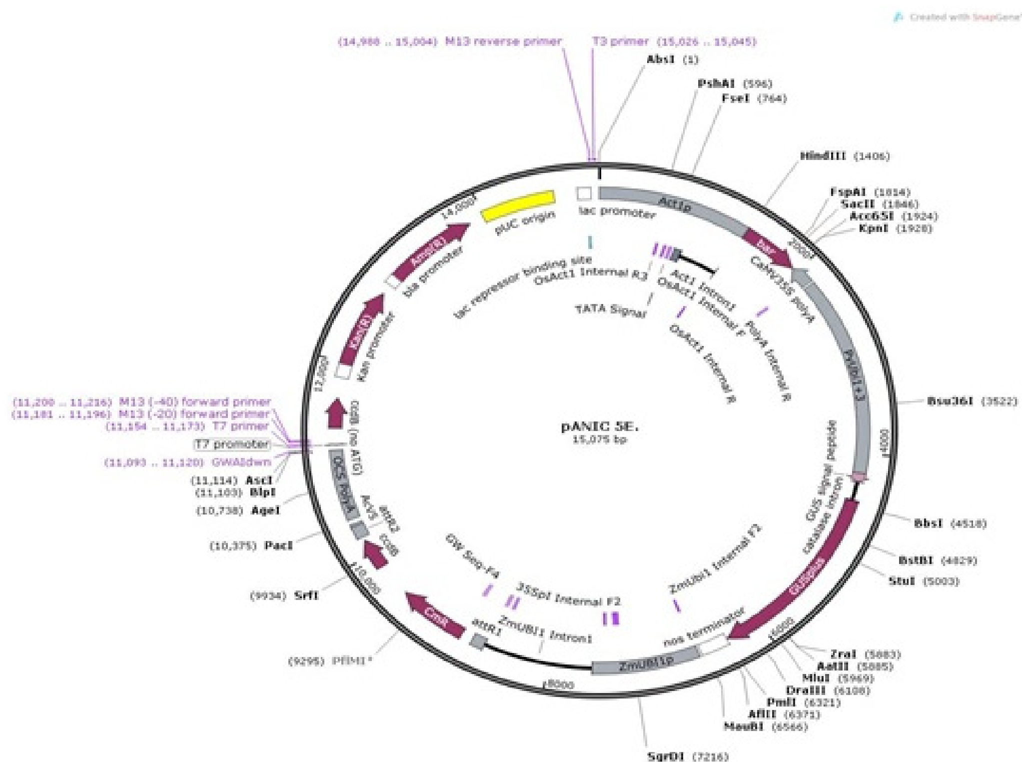

Fig. 1 Vector maps of pANIC-5E (created with snapGene)

The regeneration parameters were defined as follow:

$$PCR = \frac{\text{Number of calli with regenerated seedlings}}{\text{Number of calli transferred to regeneration}} \times 100$$

$$PPR = \frac{\text{Number of plantelets regenerated}}{\text{Number of calli transferred to regeneration}} \times 100$$

$$NPPRC = \frac{\text{Number of regenerated plantelets}}{\text{Number of calli with regenerated seedlings}} \times 100$$

Where: PCR– Percentage of callus regeneration
PPR– Percentage of plantlets regeneration
NPPRC– Number of plantlets per regenerating callus

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

Statistical analysis

The treatments consisted of 10 replications of for every medium and variety; each replication contained 20 explants (mature embryos). For the analysis of callus induction and resistant plantlets to selective agent phosphinothricin (PPT), 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.

Genomic DNA isolation and PCR analysis

The total genomic DNA was extracted from leaf material using a modified CTAB procedure (Udupa et al. 1998) for all the regenerated PPT resistant plantlets. PCR analysis of genomic DNA was carried out using 50 ng DNA template, 1x Taq DNA polymerase buffer, 200 µM of each dNTP; 0.5 µM of each primer, and 0.6 unit of Taq DNA polymerase (Promega) in a 20-µL reaction volume. Primers pairs used for amplification of the *SINA* gene were F: 5'-ATGGAACCTCGAATCAATG-3' and R: 5'-TCATATCGAAACAGGCTGTTC-3'. The PCR protocol was as follow: an initial denaturation at 95°C for 5 min, 35 cycles of 94°C for 30s, 59°C for 30s, 72°C for 45s, and final extension at 72°C for 5 min. The PCR products were run on a 1% agarose gel stained with ethidium bromide, and visualized with UV light (306 nm). The amplified transgene product size was compared with the positive control.

Results

Callus induction

The callus induction rate was assessed after 4 days of embryos culture before bombardment. Results show an important callus induction rate that varies between 89% and 93%, without significant differences between genotypes (Table 1). There was no significant difference in the callus induction rate resulting from $CuSO_4$ supplementation for 'Amria' and 'Isly'. The 'Chaoui' variety, however, showed significant drop in induction rate under $CuSO_4$ rich medium IM2 (Fig. 2A). The rate of survived calli after bombardment did not differ significantly between genotypes, with a survival rate in excess of 95% for all three varieties (Table 1). Similarly, there was no significant effect of $CuSO_4$ supplementation for all tested genotypes (Fig. 2B).

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

Variety	Callus induction (%)	Callus survival after bombardment (%)	Callus regeneration (%)	Plantlet regeneration (%)	Number of plantlets per regenerating callus
'Amria'	92.29a	95.77a	4.64b	21.26b	2.55ab
'Chaoui'	89.79a	98.76a	4.90b	17.42b	2.10b
'Isly'	93.75a	99.07a	14.48a	53.71a	4.08a
LSD	4.72	3.63	5.25	19.08	1.74
IM1	94.58a	98.17a	8.26a	30.16a	2.85a
IM2	89.30b	97.56a	7.76a	31.43a	2.97a
LSD	3.85	2.96	4.29	15.58	1.42

Use of the same letter "a" or "b" indicates that the results are not significantly different at α=0.05 according to the Least Significant Difference (LSD) test.

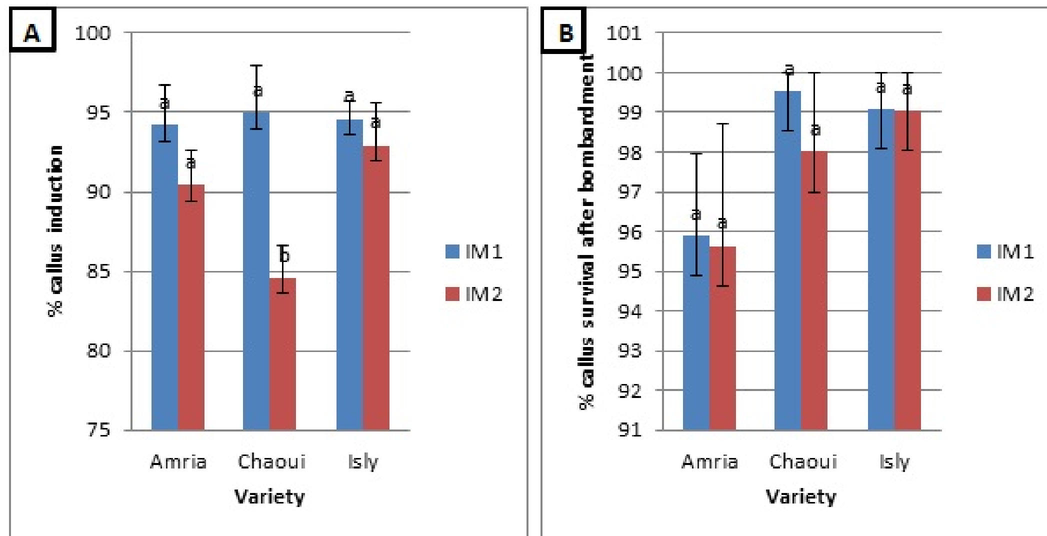

Fig. 2 Effect of the induction medium on callus induction (A) and percentage of surviving calli after bombardment (B) in mature embryos of the three durum wheat varieties after 4 weeks of culture. Use of the same letter "a" or "b" above the bar indicates that the results are not significantly different at α = 0. 05 according to the LSD test

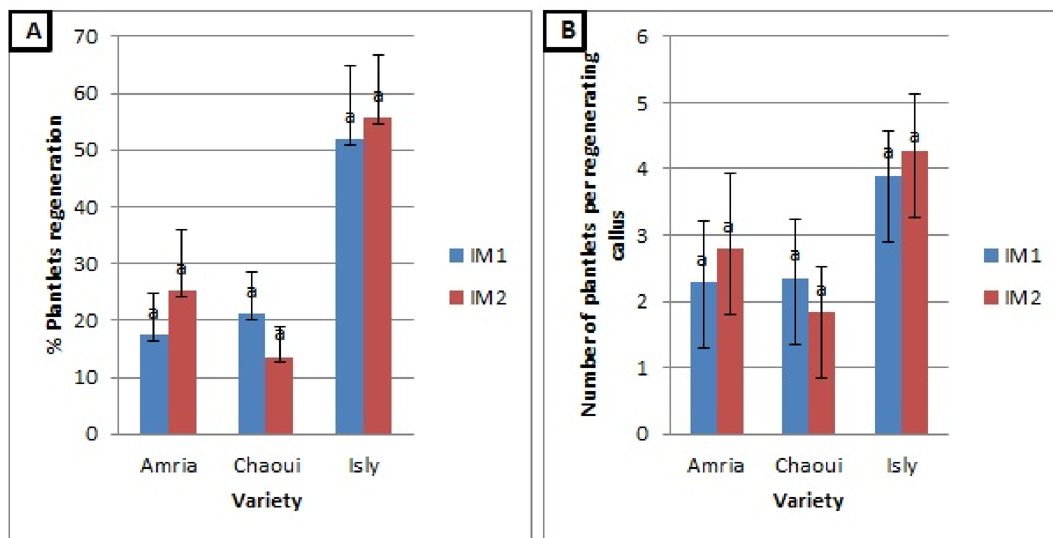

Fig. 3 Effect of induction medium on the plantlet regeneration percentage (A) and the number of plantlets per regenerating callus (B) for mature embryos of the three durum wheat varieties. Use of the same letter "a" or "b" above the bar indicates that the results are not significantly different at α = 0. 05 according to the LSD test

Plant regeneration

For the 'Amria' and 'Isly' varieties, higher concentration of $CuSO_4$ in the induction medium enhanced the rate of plantlets regeneration by about 8% and 4% respectively. For the 'Chaoui' variety, higher concentration of $CuSO_4$ in the medium IM2 reduced the rate of plantlets regenerated by about 8%. Although the results were not statistically significant between induction media in terms of plantlets regeneration parameters (Fig. 3), the differences were significant between genotypes in all studied regeneration parameters. The 'Isly' variety showed the highest regeneration capacity (Table 1).

$CuSO_4$ supplementation and transformation efficiencies

To confirm the presence of *SINA* gene in the transgenic plants T0, all the independent putative transformants generated were analyzed by PCR amplification of genomic DNA using primers specific to *SINA* gene. Transformation efficiency was calculated as the number of transgenic plants obtained (plants surviving PPT-selection and PCR-positive for the *SINA* gene) per number of bombarded embryos × 100 (Pastori et al. 2001;

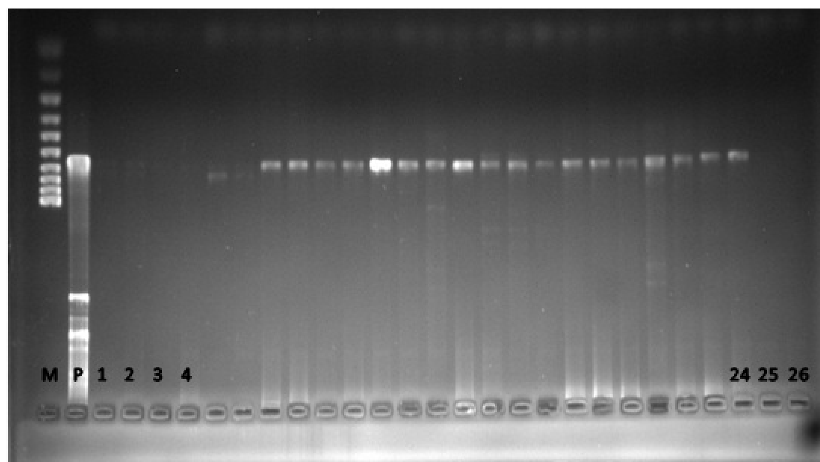

Fig. 4 PCR analysis of T0 transgenic plants with the 680 bp *SINA* primers. P: plasmid pANIC-5E; M: 100-bp marker; 1: water negative control; 2: Plant negative control; 3 to 6, 25, 26: non-transformed plants; 7 to 20: 'Isly' transgenic plants; 21 to 24: 'Amria' transgenic plants

Table 2 Experimental results for plantlet transformation in three durum wheat varieties under two induction media (IM1 and IM2)

Variety	Induction medium	Number of regenerated plantlets	Number of PPT resistant plantlets	Plantlets containing *SINA* gene	Transformation efficiency (%)
'Isly'	IM1	103	67	18	4.50
	IM2	122	68	21	5.25
'Amria'	IM1	37	12	5	1.25
	IM2	55	5	0	0.00
'Chaoui'	IM1	42	4	0	0.00
	IM2	26	14	4	1.00

Pellegrineschi et al. 2002). PCR analysis amplified the expected size for *SINA* gene (680 pb) in transgenic plants and positive controls (pANIC- 5E). Whereas, the negative controls (non-transformed plants, distilled water) and non-transformed T0 plants did not amplify the desired bands (Fig. 4). Transformation efficiencies were 9.75%, 1.25% and 1% for 'Isly', 'Amria' and 'Chaoui' varieties respectively. The 'Isly' variety has a significantly ($p < 0.05$) higher transformation efficiency in both media compared to the other cultivars (Table 2).

The number of PPT resistant plantlets and transformation efficiency obtained were statistically unaffected by the additional $CuSO_4$ in IM2 for three varieties.

Discussion

Several studies have shown that the use of $CuSO_4$ in the basal medium has a stimulatory effect on callus induction, proliferation, somatic embryogenesis and regeneration of monocotyledonous plants such as barley (Dahleen 1995; Makowska et al. 2017; Wojnarowiez et al. 2002); rice (He and Deng-tang 2014; Sahrawat and Chand 1999) and wheat (Cai et al. 2014; Kumar et al. 2017; Purnhauser 1991; Tahiliani and Kothari 2004). Purnhauser (1991) and Cai et al. (2014) showed independently a positive correlation between the rate of wheat callus regeneration and concentration of Cu^{2+} in the culture medium. In the same study, Purnhauser reported that the regeneration of wheat callus is eight times higher on a medium containing a 100 times higher $CuSO_4$ concentration than in the original MS medium. Two mechanisms have been proposed to explain this regeneration improvement: the inhibition of an ethylene precursor (Lidon et al. 1995) and the stimulation of Cu-containing enzymes (Purnhauser and Gyulai 1993).

Our results on the 'Amria' and 'Isly' durum wheat varieties corroborate these earlier studies. The presence of micronutrient copper in the induction medium at a concentration of 1.25 mg/L (~5 μM) increases the regeneration rate of wheat calli for these two varieties, although the enhancement is not statistically significant. The opposite effect occurred for the 'Chaoui' variety: copper supplement at the same concentration decreases the rate of callus induction and regeneration; again the impact remains not statistically significant.

In their study, Xiao-Min et al. (2012) tested three wheat

genotypes ‘CB037’, ‘Xinchun9’, and ‘Bobwhite’. Their results showed that 6.1 μM of $CuSO_4$ was toxic for mature embryos of all three wheat genotypes. Similarly, Khurana-Kaul et al. (2010) observed that the use of 5 μM of $CuSO_4$ inhibited shoot bud formation in monocotyledonous plants. This discrepancy in somatic embryogenesis response to increased Cu2+ can be explained by the fact that every species has its own nutrient requirement. Sahrawat and Chand (1999) and Ghaemi et al. (1994) postulated that the requirement of copper may be cultivar dependent and that the reaction to increased Cu2+ is genotype dependent. This might explain the variety dependent impact of copper on induction for durum wheat.

Allison et al. (2002) concluded in their study that transformation efficiency was consistently 1.3 to 4 times higher in Cu-treated callus cultures (16.6% on average in twelve experiments) than in control Cu-untreated ones (8.4% on average in twelve experiments). Other studies reached a similar conclusion on the positive impact of $CuSO_4$ on transformation efficiency in other monocots (Cho et al. 1998; Ishida et al. 2003).

Our investigation, which is the first one of its kind studying the effect of copper supplementation on transformation of mature wheat embryos using biolistic method, concluded that copper supplement at a concentration up to 5 μM does not have much impact on callus induction, regeneration and transformation efficiency. The disparity of results reported in the literature could be explained by the difference in the transformation method and the nature of the starting explant.

In our study, the significant difference in transformation efficiency reflects the effect of genotype. The ‘Isly’ variety proved to be the most responsive to biolistic transformation using mature embryos. The rate of transformation efficiency of 9,75% is far superior to the typical range of 0.1 to 2.5 % reported for mature and immature wheat embryos in other publications (Greer et al. 2009; Kasirajan et al. 2013; Quin et al. 2014; Rasco-Gaunt et al. 2003; Vendruscolo et al. 2007). A few studies have, however, reported higher efficiencies (Cai et al. 2014; Patnaik and Khurana 2003).

Furthermore, our observations indicate that there was no parallelism between regeneration capacity and transformation frequencies. For ‘Amria’ and ‘Chaoui’ varieties, we did not obtain any transgenic plants from the media recording the highest number of regenerated plantlets (Table 2). This supports similar results showing that the variability in transformation is more correlated to the genotypic and physiological status of the donor plants than to the efficiency of the biolistic procedure or the medium composition (Iser et al. 1999; Miroshnichenko et al. 2011; Rasco-Gaunt 2001; Ye et al. 2001). This finding confirms the works of Galovic et al. (2010) and Ekom et al. (2014) on the bombardment of wheat embryos. They observed that varieties with the highest induction and regeneration capacities exhibited the lowest transformation efficiency.

Conclusion

In the present study, we have been able to successfully introduce *SINA* gene via particle bombardment into Moroccan durum wheat varieties. Results obtained show that the response to $CuSO_4$ supplementation in the induction medium is genotype dependent and did not confirm the suggestion made by previous studies that additional copper supplementation enhances transformation efficiency. The impact of copper on transformation efficiency may depend on genotype, transformation method and type of explant.

Furthermore, the present investigation concludes as many earlier reports that transformation efficiency is highly genotype dependent. The ‘Isly’ variety gave very promising results, suggesting that it could be used in the future for further genetic transformation experiments in wheat. The high transformation efficiency obtained using mature embryos will facilitate studies on functional genomics and crop improvement. Since the transgenic plants developed in this study contained the *SINA* gene, further analysis for tolerance to water and salt in subsequent generations will be studied.

Acknowledgements

The authors would like to thank the International Centre for Genetic Engineering and Biotechnology (ICGEB) for generous funding.

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