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Enhancement of *in vitro* regeneration *via* somatic embryogenesis in two bread wheat cultivars using different combinations of plant growth regulators

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Abstract

The present study was conducted to evaluate the effect of different plant growth regulators on regeneration capacity of two bread wheat cultivars Sids 12 and Misr 3 using immature embryos. Thus, different concentrations of 2,4-D or dicamba (2, 2.5 and 3 mg/l) either alone or in combination with 0.1 mg/l BA + 0.1 mg/l TDZ were used for callus induction. Afterwards, the embryogenic calli of each treatment were transferred to the regeneration medium containing 1 mg/l BA + 0.1 mg/l TDZ. The results showed that the combination of 2,4-D with 0.1 mg/l BA + 0.1 mg/l TDZ surpassed significantly all treatments in both cultivars, hence, recorded the highest percentage of somatic embryogenesis 42.85 % and 39.96 for Misr 3 and Sids 12, respectively. While, the combination of Dicamba with 0.1 mg/l BA + 0.1 mg/l TDZ recorded 41.28 % and 37.29 % for Misr 3 and Sids 12, respectively. Also, the combination of 2,4-D with 0.1 mg/l BA + 0.1 mg/l TDZ recorded the highest percentage of regeneration 47.33 % and 46.00 % for Misr 3 and Sids 12, respectively. Whereas, the combination of Dicamba with 0.1 mg/l BA + 0.1 mg/l TDZ recorded 45.66 % and 43.66 % for Misr 3 and Sids 12, respectively. Moreover, the highest significant number of regenerated plantlets/callus was recorded using the combination of 2,4-D with 0.1 mg/l BA + 0.1 mg/l TDZ 11.41 plantlets/callus and 9.03 plantlets/callus for Misr 3 and Sids 12, respectively. In addition, the combination of Dicamba with 0.1 mg/l BA + 0.1 mg/l TDZ recorded 10.93 plantlets/callus 8.75 plantlets/callus for Misr 3 and Sids 12, respectively. Accordingly, an efficient regeneration protocol *via* somatic embryogenesis for these two elite cultivars of bread wheat was established and this efficient protocol will facilitate further improvement of these cultivars through genetic engineering.

Keywords: Wheat, *Triticum aestivum* L., immature embryo, Dicamba, 2,4-D, BA, TDZ, callus induction, somatic embryogenesis.

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1. Introduction

Wheat is an important cereal crop ranks the second after rice with an amount about one-fourth of the total cereal production worldwide. Wheat grains used for several purposes, such as food and feed as well as processing industries. In addition, Wheat is the main food source for the majority of the world's population to meet their calorie requirements [1]. Therefore, wheat demand is raising progressively due to the high increase of world population. Wheat production in Egypt is about 9.8 million metric tons per year, and there is a gap between the production and the consumption reached to 12.8 million tons in 2019/ 2020 [2]. Regarding to the highest increasing number of Egypt population with the limitation of cultivated area and water resources so, there is a great need to increase the productivity of Egyptian wheat cultivars. Great efforts were succeeded to develop elite cultivars of bread wheat in Egypt using classical breeding methods, in spite of the existed technical limitations and longer time requirements in addition to the narrow gene pool in wheat [3,5]. However, due to the vital importance of wheat there is a necessary need to further develop novel high yield and stress tolerant wheat cultivars to fill the gap between the production and consumption. Thus, the use of biotechnological methods including plant tissue culture and

genetic engineering for improving commercial wheat cultivars is ultimate for achieving this goal. Although there are high potentialities of the biotechnological methods, the developing of transformation protocol for wheat commercial cultivars is still limited due to the recalcitrance of these cultivars to *in vitro* regeneration via embryogenesis [6]. Establishment of an efficient protocol for *in vitro* regeneration in wheat is still difficult due to the lack of a robust protocol for different genotypes [7]. Therefore, establishment of an efficient regeneration protocol for *in vitro* regeneration *via* somatic embryogenesis for wheat cultivars remains the bottle neck for the success of developing new transgenic wheat plants [8,10]. Different factors are involved in determining the efficiency of the regeneration protocol; such as genotype [11,12], explant tissue [13], media composition [12,14,15]. Several studies determined the effect of these factors on *in vitro* regeneration via somatic embryogenesis in wheat [16]. The effect of plant genotype on plant regeneration in wheat as all important cereal species have been documented, thus, a high variation among the genotypes is existed [12,17,18]. Immature embryos proved to be the most reliable explant used for efficient regeneration *via* somatic embryogenesis in wheat as reported in many studies [12,17,19,28]. In addition, the influence of medium composition particularly plant growth regulators on *in vitro* cultured tissues is well documented [8, 12,22,24,29,39].

Therefore, the aim of the present study was to establish an efficient regeneration protocol *via* somatic embryogenesis for two Egyptian bread wheat cultivars (Misr 3 and Sids 12) using immature embryos and different concentration of plant growth regulators.

2. Materials and Methods

2.1. Plant material and sterilization of immature grains and isolation of explants

Two Egyptian bread wheat cultivars Misr 3 and Sids 12 were obtained from the Department of Wheat, Field Crops Institute, Agricultural Research Center (ARC), Ministry of Agriculture and Land Reclamation, Egypt. Immature grains of wheat cultivars were collected after 12 to 15 days post anthesis to provide Semi-transluscent immature embryos in size 1-1.5 mm. Wheat grains were surface sterilized with commercial Clorox® 20% (Sodium hypochlorite 5.25%) with few drops of Tween 20 for 20 min, followed by washing three times with sterile distilled water, then soaking for 5 min in sterile distilled water (pH 3.0) to equilibrate the high alkalinity of Clorox®. Finally, washing three times with sterile distilled water. Wheat immature embryos in size 1-1.5 mm were aseptically dissected from immature grains of each cultivar under a stereo binocular microscope and cultured on callus induction medium (CIM).

2.2. Callus induction

Wheat immature embryos were cultured with the embryo axis side (up-side of the scutellum) placed in contact to the medium (CIM) to inhibit the germination of immature embryos. Then after three days the embryo axis was removed from the grove of the scutellum using fine-tip forcepses, then scutellum explants were cultured on 12 different media for callus induction. Cultures were incubated in the dark for two weeks at 24 °C in controlled growth chamber. Subsequently, the explants were subcultured for two more weeks onto the same fresh medium. The callus induction medium (CIM) contains MS salts [40] supplemented by1mg/l thiamine-HCl, 0.5 mg/l pyridoxine-HCl, 0.5 mg/l nicotinic acid, 100 mg/l myo-inositol, 0.15 g/l Asparagine, 5 µM $CuSO_4$. $5H_2O$, 20 g/l sucrose and 2.5 g/l Gelrite as a solidifying agent and the pH was adjusted at 5.8. All media used in this study were filter-sterilized using Durapore PVDF 0.22 µm, WHPL 47 mm (Millipore Cat. No. GVWP04700), i.e. double concentrated (2x) CIM medium 500 ml was filter-sterilized then mixed with (2x) 500 ml of (2x) Gelrite 70°C (Gelrite 2.5 g/500 ml was autoclaved for 20 min at 121°C and 15 psi) and poured into Petri dishes 50 ml aliquots (120 X 20 mm). Different concentrations of 2,4-D or dicamba (2, 2.5 and 3 mg/l) either alone or in combination with 0.1 mg/l BA + 0.1 mg/l TDZ were used for callus induction medium.

2.3. Plant Regeneration

Four-week old embryogenic calli were cultured on plant regeneration medium (PRM) in Petri dishes (120 x 20mm) containing 50 ml of the same composition of MS medium used in (CIM) and supplemented with 1 mg/l BA and 0.1 mg/l TDZ, and 30 g/l sucrose. The Petri dishes were incubated under 16/8 hr light/ dark cycle using cool white fluorescent light (10000 lux) and kept at 24°C. Calli of different treatments were subcultured every two weeks. Then after 6 weeks the germinated somatic embryos developed small plantlets and transferred to root development medium (RDM) contains MS salts and vitamins, 30 g/l sucrose and 2 g/l Gelrite) supplemented with 1 mg/l IBA and divided into sterile jars 400 ml in volume (50 ml/jar) and kept for four weeks in the growth chamber under the same light conditions at 24°C.

2.4. Acclimatization

Wheat plants with well-developed roots were transferred to small pots containing a soil mixture of peat moss and sand (3:1) respectively, then covered with plastic pages to keep high humidity 80 %, and then placed in a growth chamber at 20 °C for 3 weeks, then transferred to big pots in the greenhouse until maturity.

2.5. Statistical analysis

Each treatment had 3 replicates with 50 explants per replicate. Each experiment was repeated thrice. Analyses of variance (ANOVA) of the completely randomized design (CRD) were performed on the collected data using SPSS software. The least significant difference test; L.S.D. was used at the level ($p \leq 0.05$) to compare the mean values of the treatments; mean of 3 replicates; $n = 3$, [41].

3. Results and Discussion

3.1. Callus induction and formation of somatic embryos

The aim of the present study was to establish an efficient regeneration protocol *via* somatic embryogenesis for two elite bread wheat cultivars Misr 3 and Sids 12. Thus, twelve callus induction media were used for inducing embryogenic calli with somatic embryos like structures. The embryogenic calli derived from the scutellum explant of immature embryos in size 1-1.5 mm were obtained and data were collected and tabulated. The results in Table 1 and 2 showed that, all tested media produced 100 % of callus

induction for both cultivars. While, the percentage of somatic embryogenesis (No. of calli with somatic embryos/ total No. of explants) recorded the highest level when the combination of 2,4-D (2.5 mg/l) or Dicamba (2.5 mg/l) with BA and TDZ was used for both cultivars. In addition, combination of 2,4-D (2.5 mg/l) with BA and TDZ reached the highest percentage of somatic embryogenesis (42.85 % and 39.96) for Misr 3 and Sids 12, respectively, while, Dicamba (2.5 mg/l) with BA and TDZ recorded (41.28 % and 37.29%) for Misr 3 and Sids 12, respectively. Thus, significant differences were recorded using 2,4-D (2.5 mg/l) with BA and TDZ compared to Dicamba (2.5 mg/l) with BA and TDZ in both cultivars as shown in Table 1 and 2. These results indicated that the presence of the cytokinins (BA and TDZ) significantly enhanced the percentage of somatic embryogenesis when compared with the treatments of auxin as individual treatment and the obtained results are in accordance with some reports used 2,4-D and Dicamba either alone or in combination with low concentrations of cytokinins [29,30]. The influence of the type of auxin on callus induction and somatic embryogenesis using immmature embryos which proved to be the most reliable explant used for efficient regeneration *via* somatic embryogenesis in wheat was documented in several studies on wheat plants [12,17,19,22, 24].

3.2. Plant regeneration

The embryogenic calli of each treatment were transferred to the regeneration medium supplemented with 1 mg/l BA and 0.1 mg/l TDZ. The germination of somatic embryos was appeared after two weeks and developed the shoots and roots during the next four weeks as shown in (Fig 1. G-I). Then the regenerated plantlets were transferred to the root development medium to promote the lateral roots on the main root of the plant (Fig. 1 J-K). The results of the percentage of regeneration exhibited the same trend of the percentage of somatic embryogenesis, thus, the combination of 2,4-D with 0.1 mg/l BA + 0.1 mg/l TDZ recorded the highest percentage of regeneration 47.33 % and 46.00 % for Misr 3 and Sids 12, respectively. Whereas, the combination of Dicamba with 0.1 mg/l BA + 0.1 mg/l TDZ recorded 45.66 % and 43.66 % for Misr 3 and Sids 12, respectively.

Table 1: Effect of different callus induction media on percentage of somatic embryogenesis, the Average No. of regenerated plantlets/callus and percentage of regeneration for bread wheat cv. Misr 3

Treatments Concentrations mg/l		No of explants (immature embryos)	Percentage of callus induction	Percentage of somatic embryogenesis (No. of calli with s. embryos / total No. of explants)	Avg. No. of regenerated plantlets / callus	Regeneration % (No. of calli producing shoots / total No. of explants)
Auxin	Cytokinin					
Dic. 2 mg	-	150	100	37.25	7.21	39.66
Dic. 2.5 mg	-	150	100	39.11	8.26	41.66
Dic. 3 mg	-	150	100	38.36	8.15	41.00
Dic. 2 mg	0.1 BA + 0.1 TDZ	150	100	39.29	9.22	43.66
Dic. 2.5 mg	0.1 BA + 0.1 TDZ	150	100	41.28	10.93	45.66
Dic. 3 mg	0.1 BA + 0.1 TDZ	150	100	40.87	10.24	44.33
2,4-D 2 mg	-	150	100	35.22	8.11	40.33
2,4-D 2.5 mg	-	150	100	38.19	8.92	41.66
2,4-D 3 mg	-	150	100	37.58	8.67	41.33
2,4-D 2 mg	0.1 BA + 0.1 TDZ	150	100	39.74	10.25	42.66
2,4-D 2.5 mg	0.1 BA + 0.1 TDZ	150	100	42.85	11.41	47.33
2,4-D 3 mg	0.1 BA + 0.1 TDZ	150	100	40.23	10.56	43.66
L.S.D. at 0.05				1.15	0.74	1.54

Table 2: Effect of different callus induction media on percentage of somatic embryogenesis, the average No. of regenerated plantlets/callus and percentage of regeneration for bread wheat cv. Sids 12

Treatments Concentrations mg/l		No of explants (immature embryos)	Percentage of callus induction	Percentage of somatic embryogenesis (No. of calli with s. embryos / total No. of explants)	Avg. No. of regenerated plantlets / callus	Regeneration % (No. of calli producing shoots / total No. of explants)
Auxin	Cytokinin					
Dic. 2 mg	-	150	100	33.77	6.22	35.33
Dic. 2.5 mg	-	150	100	35.95	7.59	38.11
Dic. 3 mg	-	150	100	35.21	7.16	37.84
Dic. 2 mg	0.1 BA + 0.1 TDZ	150	100	35.42	7.82	40.57
Dic. 2.5 mg	0.1 BA + 0.1 TDZ	150	100	37.29	8.75	43.66
Dic. 3 mg	0.1 BA + 0.1 TDZ	150	100	37.16	7.96	41.65
2,4-D 2 mg	-	150	100	32.54	6.41	35.86
2,4-D 2.5 mg	-	150	100	34.26	7.35	39.24
2,4-D 3 mg	-	150	100	33.12	7.13	38.87
2,4-D 2 mg	0.1 BA + 0.1 TDZ	150	100	36.62	8.11	41.36
2,4-D 2.5 mg	0.1 BA + 0.1 TDZ	150	100	39.96	9.03	46.00
2,4-D 3 mg	0.1 BA + 0.1 TDZ	150	100	38.23	8.51	42.18
L.S.D. at 0.05				1.32	0.62	2.13

Moreover, the highest significant number of regenerated plantlets/callus was recorded using the combination of 2,4-D with 0.1 mg/l BA + 0.1 mg/l TDZ 11.41 plantlets/callus and 9.03 plantlets/callus for Misr 3 and Sids 12, respectively. In addition, the combination of dicamba with 0.1 mg/l BA + 0.1 mg/l TDZ recorded 10.93 plantlets/callus 8.75 plantlets/callus for Misr 3 and Sids 12, respectively.

The composition of the culture medium used in this study during callogenesis and plant regeneration contains an elevated level of $CuSO_4 .5H_2O$ (5 µM) which about fifty times as the concentration in MS medium. This treatment proved for a long time of intensive studies on different cereals to be essential to enhance callogenesis and regeneration *via* somatic embryogenesis [22,42,45]. In addition, regeneration *via* somatic embryogenesis resulted in a high number of green plants with developed roots derived from germinated somatic embryos as shown in (Fig. 1 H-I) which confirm evidently that this protocol is efficient for plant regeneration through somatic embryogenesis.

Furthermore, the regeneration medium supplemented with 1 mg/l BA and 0.1 mg/l TDZ may enhanced the germination of somatic embryos due to the presence of TDZ which known as an inhibitor to the cytokinin oxidase/dehydrogenase; CKX, EC 1.5.99.12, [46,48] through preventing the catabolism of purine compounds such as zeatin, dihydrozeatin and N6-isopentyladenosine [46, 49]. In addition, the obtained results are in accordance with those obtained by [50-51] whereas, they indicated that the use of TDZ at 0.2 mg/l is the optimal concentration for wheat regeneration. In addition, the auxin-cytokinin ratio used in callogenesis stage in particular 2.5 mg/l 2,4-D + 0.1 mg/l BA + 0.1 mg/l TDZ proved to be most suitable treatment for both cultivars and resulted in the highest percentage of embryogenesis and the highest percentage of regeneration which accompanied with the highest average number of regenerated plants. It is well known that the optimization of plant growth regulators in culture medium is the most important factor to reduce the degree of recalcitrance in wheat genotypes [12]. Therefore, the optimized medium used in this study with an optimal auxin-cytokinin ratio resulted in a pronounced reduction in the recalcitrance of both cultivars Misr 3 and Sids 12 to tissue culture and generated high number of plants/explant *via* enhancing somatic embryogenesis.

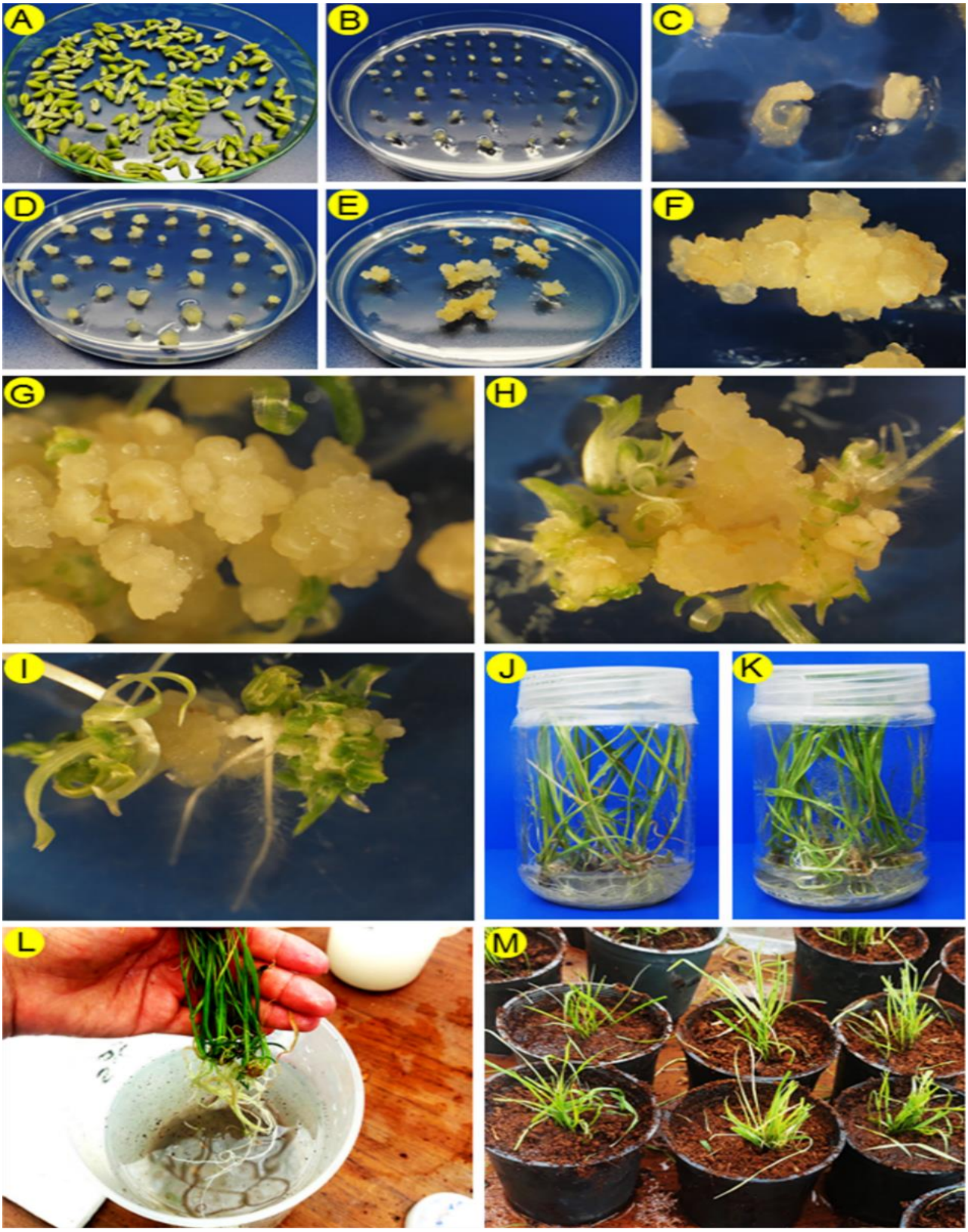

Fig. 1: Regeneration *via* somatic embryogenesis using immature embryos of bread wheat cv. Sids 12 and Misr 3. Sterilized immature grains of Sids 12 (A). Scutellum of immature embryos after removing the embryo axis (B). In focus immature scutellum after 4 days on callus induction medium (CIM) (C). The embryogenic callus of the scutellum of Misr 3 after 2 weeks on CIM (D). The embryogenic callus of Sids12 after 4 weeks on CIM (E). In focus embryogenic callus of Misr 3 after 4 weeks on CIM (F). Somatic embryo like structures on embryogenic callus of Misr 3 after 2 weeks on plant regeneration medium (PRM) (G). Germination of somatic embryos and development of green plantlets of Misr 3 after 3 weeks on PRM (H). Regenerated plantlets of Sids 12 with developed roots after 4 weeks on PRM (I). The developed plants of Misr 3 after 4 weeks on rooting medium (J). The developed plants of Sids 12 after 4 weeks on rooting medium (K). Preparation of Mirs 3 regenerated plants for adaptation stage (L). Regenerated plants of Sids 12 in small pots during preparation for adaptation stage (M).

4. Conclusions

In this study the inclusion of TDZ at 0.1 mg/l in both callus induction and regeneration media proved has positive impact on formation and germination of wheat somatic embryos for both cultivars Misr 3 and Sids 12. Accordingly, an efficient regeneration protocol *via* somatic embryogenesis for these two elite cultivars of bread wheat was established and this efficient protocol will facilitate further improvement of these cultivars through genetic engineering.

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