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RESEARCH ARTICLE

GENOTYPIC EFFECTS ON EMBRYOGENESIS AND PLANT REGENERATION IN WHEAT (*TRITICUM AESTIVUM* L.)

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ABSTRACT

Embryogenic callus induction and subsequently plant regeneration using mature embryos as an explant for six Sudanese elite wheat cultivars were investigated in the present study, mature embryos were placed indifferent MS salt strengths supplemented with 2.0 mg/l 2,4-D. Relatively callus induction frequencies were observed over all concentrations and cultivars; however significant differences were detected in callus induction ability, embryogenic callus differentiation and plantlet regeneration among MS salts concentrations and cultivars. Wadi Elnile and Sasaraib cultivars achieved the best value for callus proliferation at the normal concentration (1X); followed by Khaleefacultivarat the same concentration, the least call genesis was obtained in Bohain cultivar. The calluses produced were subjected to somatic embryogenesis and regeneration studies. Maximum embryogenic callus formation was obtained in MS medium supplemented with different concentrations of TDZ singly and TDZ in combination with 0.1 mg/l2,4-D in all treatments, while 0.5 mg/l2,4-D concentration inhibitedembryogenic callus formation. Takana cultivar was the most responsive cultivar among other cultivars; whereas plant regeneration was occurred in almost all TDZ concentrations for this cultivar. The present study cleared that somatic embryogenesis and regeneration abilities are highly genotypic dependent; whereas cultivars responded differently to the different abilities.

INTRODUCTION

In early studies it was observed by various researchers that callus formation was successful in wheat (Shimada *et al*., 1969). Studies on wheat tissue culture revealed that regeneration can be brought about either by somatic embryogenesis or adventitious bud and shoot development with subsequent rooting (Bhaskaran and Smith, 1990). Direct organogenesis has also been studied in wheat (Li *et al*., 1992). The frequencies of callus induction and plant regeneration through *in vitro* systems of wheat (*Triticumae stivum* L.) are commonly influenced by genotype, composition of the culture medium and the explant source (Bi, 2007). Mature embryos were used for callus induction and plant regeneration in several studies (Tang *et al*., 2004). Use of mature embryos has remarkable advantages over immature tissues as explants. The need for growing donor material in greenhouses under controlled environmental conditions, requiring intensive labor, time and space, can be avoided. Moreover, dry seeds are available in large quantities all year round and with no problems due to seasonal influence on tissue culture response.

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Moreover, mature embryos showed better regeneration response than immature embryos (Ozgen *et al*., 1996) and they are being used for transformation studies in the recent years (Patnaik *et al*., 2006). Transformation of wheat to tackle the various biotic and abiotic stresses that cause reduction in their yield is now in practice in many countries of the world (Jones *et al*., 2005). However, these methods are dependent upon successful, reliable and reproducible regeneration of transformed explants through tissue culture (Yu *et al*., 2008). The present study was initiated to assess the effect of different MS inorganic minerals strengths on callus induction and regeneration ability for six wheat cultivars grown in Sudan and to select the suitable cultivars for *in vitro* system, based on their abilities to form callus and regenerate plantlets using mature embryos.

MATERIALS AND METHODS

Plant material

Mature seeds of six Sudanese wheat cultivars namely: Khaleefa, Wadi Elnile, Sasaraib, Takana, Elnileen and Bohain were obtained from the Agricultural Research Corporation (ARC), Wheat Research Program-Wad Madani, Sudan.

Sterilization

Wheat seeds were washed under running tap water for 30 minutes and socked in tap water for five hours at room temperature. Under laminar air flow hood; the embedded seeds were surface disinfested with 70% ethanol for three minutes, and then soaked in commercial sodium hypochlorite 100% supplemented with few drops of tween 20 for twenty minutes, then washed several times with sterile distilled water. Mature embryos were separated from the embedded seeds using sterile forceps and scalpels and placed on the callus induction medium.

Callus induction

The aseptically isolated mature embryos were placed in different MS salts strengths (0.0, 0.25X, 0.5X, 1.0X, 2.0X) supplemented with 2.0 mg/l 2,4-D, the media fortified with 3% sucrose (w/v) and solidified with 0.6 % agar, the pH was adjusted to 5.8 before dispensed in measured amount of 25 ml/bottle and autoclaved at 121º C and 15 psi for 15 minutes. Cultures were kept in the incubation room under dark condition at 25 ± 2 ° C for four weeks.

Regeneration

Mature embryos were placed in double MS salts strength (2X) medium and supplemented with 2.0 mg/l 2,4-D to form callus. The derived calluses were transferred to (1) MS medium supplemented with 2.0 mg/l 2,4-D,(2) to MS medium free hormones, (3) to MS medium supplemented with different concentrations (0.0, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) of TDZ singly, (4) and to MS medium supplemented with different concentrations (0.0, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) of TDZ in combinations with two concentrations (0.1 and 0.5 mg/l) 2,4-D. Cultures were kept under 16 hours light/8 hours dark photo period at 25 ± 2 °C for eight weeks.

Statistical analysis

Experiments were set up as a completely randomized design and statistically analyzed using ANOVA table and presented as average ± standard error. Means were separated by Duncan's multiple range test (Duncan, 1955) at 0.05 probability level.

RESULTS AND DISCUSSION

Callus induction

In a preliminary experiment callus were maintained in completely dark condition and in 16/8 h (light/dark) photoperiod, results showed that the best callus proliferation was obtained in the totally dark condition compared to photoperiod condition (data not shown). Many external factors affect plant growth and development but among them light is the most important because it regulates the whole process of growth and development (Bi *et al*., 2007); he examine the effect of 16/8 h (light/dark) photoperiod (light intensity, 40 lmol/m2/s) on callus induction and subsequent differentiation were undertaken; he found that the quality of callus was better, the frequencies of callus induction and subsequent differentiation were higher when callus was induced in total darkness rather than in a 16/8 h photoperiod.

(Table 1) showed that all the cultivars had the ability to form callus, the percentage of forming callus ranged between 80-100 %, but the frequencies of callus induction and the quality of callus were significantly different. Wadi Elnile and Sasaraib cultivars achieved the best results of callus proliferation at MS salts normal concentration (1X) with a mean number of (3.0±0.0a); followed by Khaleefa cultivar with a mean number of (2.6±0.2); Bohaincultivar gave the least values for callus proliferation among other cultivars (Table 1). Doubling MS salts strength (2X) enhanced the formation of callus nodules (embroyids); Khaleefa cultivar proved the best effective for inducing embryogenic callus (62%) (Table 1).

Table 1. The effect of MS salts strengths on callus induction of six Sudanese elite wheat cultivars after 4 weeks

Cultivars	MS salts con. (X)	Callus induction (Mean±SE)	Callus/explants %	Nodulated callus %
Khaleefa	0.0	1.0±0.0 ^{p}	90	0.0
	0.25	1.7±0.2 ^{k}	100	6
	0.5	2.0±0.0 ^{h}	100	31
	1.0	2.6±0.2 ^{c}	100	18
	2.0	2.0±0.0 ^{h}	100	62
Wadi-Elnile	0.0	1.6±0.2 ^{l}	95	0.0
	0.25	2.6±0.2 ^{c}	100	6
	0.5	2.8±0.1 ^{b}	100	0.0
	1.0	3.0±0.0 ^{a}	100	0.0
	2.0	2.3±0.2 ^{f}	100	0.0
Sasaraib	0.0	1.5±0.2 ^{m}	90	0.0
	0.25	2.5±0.2 ^{d}	100	12
	0.5	2.2±0.1 ^{g}	100	12
	1.0	3.0±0.0 ^{a}	100	12
	2.0	2.4±0.2 ^{e}	100	12
Takana	0.0	1.0±0.0 ^{p}	90	0.0
	0.25	1.6±0.2 ^{l}	90	6
	0.5	1.5±0.2 ^{m}	95	12
	1.0	2.3±0.2 ^{f}	100	4
	2.0	1.8±0.1 ^{j}	100	0.0
Elnileen	0.0	1.0±0.0 ^{p}	90	0.0
	0.25	1.6±0.2 ^{l}	95	0.0
	0.5	1.9±0.1 ^{i}	95	0.0
	1.0	2.4±0.2 ^{e}	100	0.0
	2.0	1.9±0.1 ^{i}	100	0.0
Bohain	0.0	1.0±0.0 ^{p}	80	0.0
	0.25	1.3±0.2 ^{o}	80	0.0
	0.5	1.4±0.2 ^{n}	80	0.0
	1.0	1.9±0.1 ^{i}	90	0.0
	2.0	1.6±0.2 ^{l}	90	0.0

Means with the same letter (s) in the same column are not significantly different at 0.05 probability level using Duncan Multiple Range Test.

The role of plant growth regulators in cereal tissue culture is very important. In a previous study (Hala *et al*., 2012) we confirm that the presence of 2.0 mg/l 2,4-D in the culture medium was optimal for callus induction from mature embryos. In general, auxins, usually 2,4-D in the range of 1–3 mg/l is essential for the formation of embryogenic callus from cereal embryos (Bi *et al*., 2007), In agreement with our findings it has been reported by Carman *et al*., 1987 that the double contraptions of macro elements in MS medium enhanced embryogenic callus induction in comparison with other media tested. Also He *et al*., 1989 found that the presence of NH_4NO_3 was essential for the proliferation of embryogenic

callus and a relatively high concentration (10 mM) was found to be optimum for the induction of both scutellum and epiblast callus and the presence of $CaCl_2$, $MgSO_4$ and KH_2PO_4 was not essential for the initiation of epiblast callus but strongly affected the further development of this callus and its differentiation potential.

mature embryo culture was pointedly influenced by the genotypes and growth regulators type and concentration, whereas callus became visible as compact greenish callus in MS medium free hormones and at all treatments of Khaleefacultivar; which achieved the highest percentage of nodulated callus/explants (Table 2), while the 2.0 mg/l 2,4-D

Table 2. The effects of 2,4-D, MS basal medium and different concentrations of TDZ on embryogenesis and plantlet regeneration after 8 weeks

Cultivars	2,4-D con. mg\l	TDZ con. mg\l	Nodulated callus\explants (%)	Regeneration (%)	Callus morphologic response
Khaleefa	2.0	0.0	0.0	0.0	Compact yellow callus
	0.0	0.0	90	0.0	Compact greenish callus
	0.0	0.5	90	0.0	Compact greenish callus
	0.0	1.0	90	0.0	Compact greenish callus
	0.0	2.0	90	0.0	Compact greenish callus
	0.0	3.0	90	0.0	Compact greenish callus
	0.0	4.0	90	0.0	Compact greenish callus
Wadi-Elnile	2.0	0.0	0.0	0.0	Compact yellow callus
	0.0	0.0	70	0.0	Compact yellow callus
	0.0	0.5	70	0.0	Compact yellow callus
	0.0	1.0	70	0.0	Compact yellow callus
	0.0	2.0	70	0.0	Compact yellow callus
	0.0	3.0	70	0.0	Compact yellow callus
	0.0	4.0	80	0.0	Compact yellow callus
Sasaraib	2.0	0.0	0.0	0.0	Compact yellow callus
	0.0	0.0	80	0.0	Compact greenish callus
	0.0	0.5	80	0.0	Compact greenish callus
	0.0	1.0	70	0.0	Compact yellow callus
	0.0	2.0	70	0.0	Compact yellow callus
	0.0	3.0	70	0.0	Compact yellow callus
	0.0	4.0	70	0.0	Compact yellow callus
Takana	2.0	0.0	10	0.0	Compact yellow callus
	0.0	0.0	30	0.0	Compact yellow callus
	0.0	0.5	30	10	Regenerated shoots
	0.0	1.0	50	20	Regenerated shoots
	0.0	2.0	30	0.0	Compact yellow callus
	0.0	3.0	20	10	Regenerated shoots
	0.0	4.0	10	0.0	Compact yellow callus
	2.0	0.0	0.0	0.0	Compact yellow callus
Elnileen	0.0	0.0	80	0.0	Compact greenish callus
	0.0	0.5	0.0	0.0	Compact yellow callus
	0.0	1.0	0.0	0.0	Compact yellow callus
	0.0	2.0	0.0	0.0	Compact yellow callus
	0.0	3.0	0.0	0.0	Compact yellow callus
	0.0	4.0	0.0	0.0	Compact yellow callus
Bohain	2.0	0.0	0.0	0.0	Compact yellow callus
	0.0	0.0	80	0.0	Compact greenish callus
	0.0	0.5	60	0.0	Compact greenish callus
	0.0	1.0	50	0.0	Compact yellow callus
	0.0	2.0	50	0.0	Compact yellow callus
	0.0	3.0	30	0.0	Compact yellow callus
	0.0	4.0	10	0.0	Compact yellow callus

Regeneration

Auxins and cytokinins are the major growth regulators that affect various aspects of plant cell division, differentiation and organogenesis (Fehe'r *et al*., 2003; Nikolic *et al*., 2006). The present study investigated the effects of various types of growth regulators on embryogenesis and plant regeneration abilities for six Sudanese wheat cultivars, for this purpose; four weeks old healthy callus initiated from mature embryos and placed in (2X) MS medium and then transferred to: MS medium free hormones or to MS medium supplemented with 2,4-D and TDZ singly or in combinations. After eight weeks; the differences in callus morphological response, embryogenesis and plant regeneration abilities were clearly observed and recorded. Results showed that the efficiency of

concentration inhibited nodules formation and visible as compact yellow callus at all concentrations and cultivars (Table 2).

Takana cultivar gave the highest proportion of plants regeneration across other cultivars; regeneration occurred at the concentrations 0.5, 1.0 and 3.0 mg/l TDZ with regeneration percentage of 10%, 20% and 10% respectively (Table 2, Figure 1). (Table 3) showed wide variations in the potential of forming nodules among cultivars. Plant regeneration was occurred in Takana cultivar at the concentration 0.1 mg/l 2,4-D in combination with 0.5 mg/l TDZ; 0.1 mg/l 2,4-D in combination with 1.0 mg/l TDZ and 0.1 mg/l 2,4-D in combination with 4.0 mg/l TDZ with regeneration percentage of 27%, 7% and 20% respectively (Table 3, Figure 1),

Table 3. Effect of 0.1 mg/l 2,4-D in combination with different concentrations of TDZ on embryogenesis and plantlet regeneration in some Sudanese wheat cultivars after 8 weeks

Cultivars	2,4-D con. mg\l	TDZ con. mg\l	Nodulated callus\ Explants (%)	Regeneration (%)	Callus morphologic response
Khaleefa	0.1	0.0	100	0.0	Compact greenish callus
	0.1	0.5	90	0.0	Compact greenish callus
	0.1	1.0	85	0.0	Compact greenish callus
	0.1	2.0	80	0.0	Compact greenish callus
	0.1	3.0	70	0.0	Compact yellow callus
	0.1	4.0	70	0.0	Compact yellow callus
Wadi-Elnile	0.1	0.0	50	0.0	Compact yellow callus
	0.1	0.5	50	0.0	Compact yellow callus
	0.1	1.0	50	0.0	Compact yellow callus
	0.1	2.0	55	0.0	Compact yellow callus
	0.1	3.0	60	0.0	Compact yellow callus
	0.1	4.0	60	0.0	Compact yellow callus
Sasaraib	0.1	0.0	70	0.0	Compact greenish callus
	0.1	0.5	70	0.0	Compact greenish callus
	0.1	1.0	70	0.0	Compact yellow callus
	0.1	2.0	55	0.0	Compact yellow callus
	0.1	3.0	50	0.0	Compact yellow callus
	0.1	4.0	50	0.0	Compact yellow callus
Takana	0.1	0.0	0.0	0.0	Compact yellow callus
	0.1	0.5	30	27	Regenerated shoots
	0.1	1.0	30	7	Regenerated shoots
	0.1	2.0	30	0.0	Compact yellow callus
	0.1	3.0	20	0.0	Compact yellow callus
	0.1	4.0	20	20	Regenerated shoots
Elnileen	0.1	0.0	0.0	0.0	compact Yellow callus
	0.1	0.5	10	10	Compact yellow callus
	0.1	1.0	0.0	0.0	Compact yellow callus
	0.1	2.0	0.0	0.0	Compact yellow callus
	0.1	3.0	0.0	0.0	Compact yellow callus
	0.1	4.0	0.0	0.0	Compact yellow callus
Bohain	0.1	0.0	30	0.0	Compact yellow callus
	0.1	0.5	40	0.0	Compact yellow callus
	0.1	1.0	40	0.0	Compact yellow callus
	0.1	2.0	30	0.0	Compact yellow callus
	0.1	3.0	10	0.0	Compact yellow callus
	0.1	4.0	10	0.0	Compact yellow callus

Table 4. Effect of 0.5 mg/l 2,4-D in combination with different concentration of TDZ on embryogenesis and plantlet regeneration in some Sudanese wheat cultivars after 8 weeks

Cultivars	2,4-D con. mg\l	TDZ con. mg\l	Nodulated callus\ explants (%)	Regeneration (%)	Callus morphologic response
Khaleefa	0.5	0.0	0.0	0.0	Compact yellow callus
	0.5	0.5	0.0	0.0	Compact yellow callus
	0.5	1.0	0.0	0.0	Compact yellow callus
	0.5	2.0	0.0	0.0	Compact yellow callus
	0.5	3.0	0.0	0.0	Compact yellow callus
	0.5	4.0	0.0	0.0	Compact yellow callus
Wadi-Elnile	0.5	0.0	50	0.0	Compact yellow callus
	0.5	0.5	50	0.0	Compact yellow callus
	0.5	1.0	50	0.0	Compact yellow callus
	0.5	2.0	50	0.0	Compact yellow callus
	0.5	3.0	50	0.0	Compact yellow callus
	0.5	4.0	50	0.0	Compact yellow callus
Sasaraib	0.5	0.0	0.0	0.0	Compact yellow callus
	0.5	0.5	0.0	0.0	Compact yellow callus
	0.5	1.0	0.0	0.0	Compact yellow callus
	0.5	2.0	0.0	0.0	Compact yellow callus
	0.5	3.0	0.0	0.0	Compact yellow callus
	0.5	4.0	0.0	0.0	Compact yellow callus
Takana	0.5	0.0	0.0	0.0	Compact yellow callus
	0.5	0.5	30	0.0	Compact yellow callus
	0.5	1.0	30	10	Regenerated shoot
	0.5	2.0	20	0.0	Compact yellow callus
	0.5	3.0	20	0.0	Compact yellow callus
	0.5	4.0	20	0.0	Compact yellow callus
Elnileen	0.5	0.0	0.0	0.0	Compact yellow callus
	0.5	0.5	0.0	0.0	Compact yellow callus
	0.5	1.0	0.0	0.0	Compact yellow callus
	0.5	2.0	0.0	0.0	Compact yellow callus
	0.5	3.0	0.0	0.0	Compact yellow callus
	0.5	4.0	0.0	0.0	Compact yellow callus
Bohain	0.5	0.0	0.0	0.0	Compact yellow callus
	0.5	0.5	0.0	0.0	Compact yellow callus
	0.5	1.0	0.0	0.0	Compact yellow callus
	0.5	2.0	0.0	0.0	Compact yellow callus
	0.5	3.0	0.0	0.0	Compact yellow callus
	0.5	4.0	0.0	0.0	Compact yellow callus

shoot regeneration also observed in Elnileen cultivar at the concentration 0.1 mg/l 2,4-Din combination with 0.5 mg/l TDZ with regeneration percentage of 10% (Table 3).

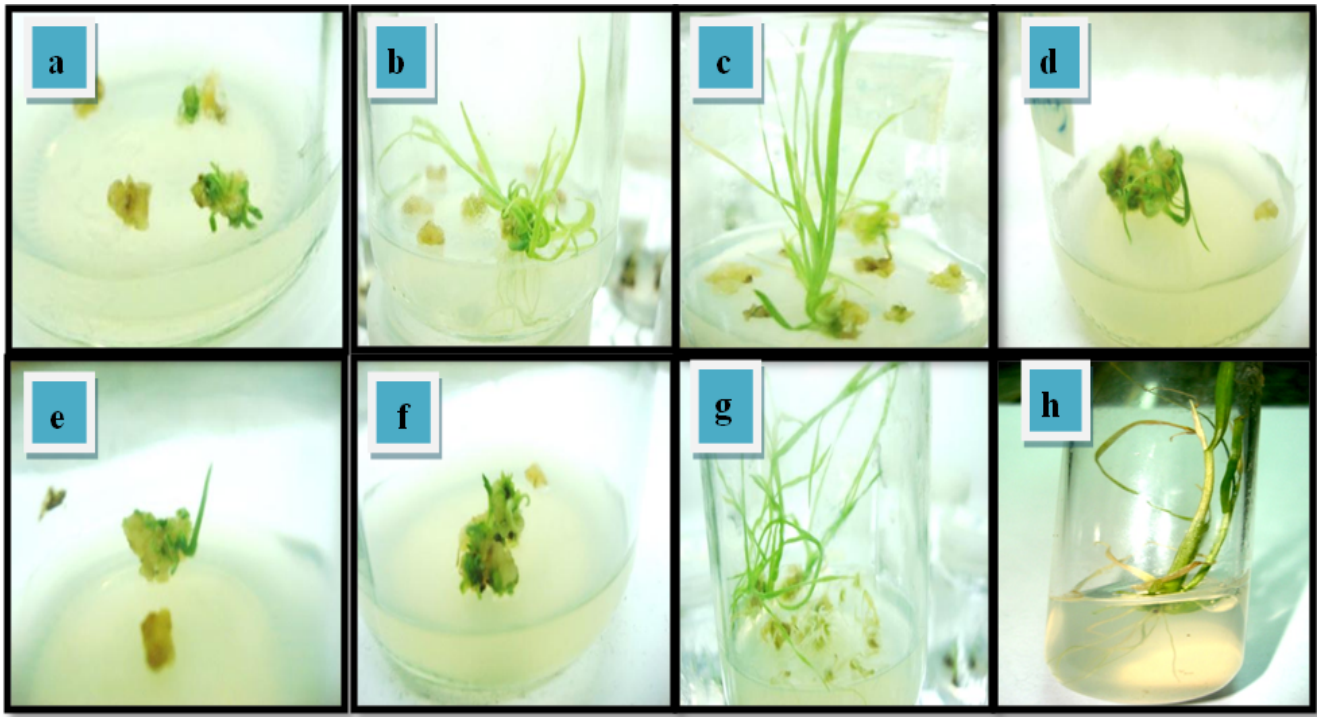

Figure 1. Plant regeneration and rooting in Takana cultivar

a) Takana shoot regeneration in 0.5 mg/l TDZ, (b) Takana regeneration in 1.0 mg/l TDZ, (c) Takana regeneration in 3.0 mg/l TDZ (d) Takana regeneration in 0.1 mg 2,4-D in combination with 0.5 mg/l TDZ, (e) Takana regeneration in 0.1mg/l 2,4,D in combination with 1.0 mg/l TDZ, (f) Takana regeneration in 0.1mg/l 2,4,D in combination with 4.0 mg/l TDZ, (g) Takana regeneration in 0.5 mg/l 2,4-D in combination with 1.0 mg/l TDZ, (h) Rooting of the regenerated shoot of Takanacultivarin 0.5 mg/l IBA

The maintenance of the initiated callus in 0.5 mg/l 2,4-D in combination with different TDZ concentrations were deleterious to nodules formation and plant regeneration, it promoted callus browning and decreased culture efficiency (Table 4).However shoot regeneration was obtained at the concentration 0.5 mg/l 2,4-D in combination with 1.0 mg/l TDZ in Takana cultivar with regeneration percentage of 10% (Table 4, Figure 1).

Plant regeneration from wheat mature embryo culture through organogenesis has been investigated for more than 40 years, but no significant progress has been achieved so far (Yin *et al*., 2011).The recalcitrant nature of wheat mature embryos, such as low regeneration via somatic embryogenesis, has slowed down the application of this explant type for biotechnological programs, although many methods have been developed for regeneration of plants in wheat mature embryos callus cultures, the frequency of the regenerated plants are still relatively low (Ren *et al*., 2010). Even though a lot of researches on wheat mature embryo culture have been conducted (Li *et al*. 2006; Wang and Fan 2006; Yu *et al*. 2007), the regeneration efficiency is still low and less reproducible, and efficient regeneration system with high repeatability has not been established yet. More over the previous studies had showed that the frequencies of callus induction and plant regeneration in tissue culture of wheat were commonly influenced by the explant source (Shariatpanahi *et al*., 2006; Redha and Talaat, 2008; Liu *et al*., 2008), genotype (Filippov *et al*., 2006) and medium composition (Tamas *et al*., 2004).

Conclusion

Establishment of reliable tissue culture protocols for callus induction, somatic embryogenesis and plant regeneration is desired in order to improve wheat yield. The present study revealed that the frequencies of callus induction, embryogenesis and plant regeneration abilities are highly genotypic dependent; hence each cultivar needs a separate protocol to achieve these abilities, this study helps in understanding the response of different Sudanese wheat cultivars under *in vitro* condition, also the success in using mature embryos as an explant in wheat *in vitro* studies needs more efforts and research to provide solid base for molecular breeding of wheat.

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