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Full Length Research Paper

Establishment of an efficient callus induction and plant regeneration system in some wheat (*Triticum aestivum* L.*)* cultivars grown in Sudan

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A procedure for callus induction and plant regeneration was developed for wheat (*Triticum aestivum)* cultivars grown in Sudan. Mature embryos of cultivar Khaleefa removed from surface sterilized seeds were used as explants. For callus induction, explants were cultured on Murashige and Skoog medium (MS) supplemented with different levels (1.5 to 4.0 mg/l) of 2,4-dichlorophenoxy acetic acid (2,4-D), indole-3- butyric acid (IBA) and indole-3- acetic acid (IAA). After 4 weeks of culture, the highest callus fresh weight (0.05 ± 0.01 g) was obtained on MS medium supplemented with 2.0 mg/L of 2,4-D. For shoot regeneration, the developed calli were transferred to MS medium without growth regulators or with different levels (0.1 to 3.0 mg/l) of 5-phenylcarbamoylamino-1,2,3-thiadiazole (thidiazuron) (TDZ), benzyladenine (BA) and kinetin (KN) alone or in combination with 2.0 mg/L of 2,4-D. After 6 weeks of culture, the best result for regeneration percentage (40%) was obtained on MS medium without growth regulators. Callus derived shoots were rooted most effectively in half-strength MS medium without or with different levels of IBA. To determine the genotypic effect, the developed procedure was used to evaluate the regeneration ability of other 5 Sudanese wheat cultivars. Significant differences were observed between genotypes for plant regeneration ability, indicating that these criteria are genotype dependent. The acclimatized regenerated plants were morphologically uniform with normal leaf shape and growth pattern under greenhouse conditions.

Key words: *Triticum aestivum*, callus induction, regeneration, 2,4-dichlorophenoxyacetic acid (2,4-D), genotypes.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the major staple food crops grown worldwide (Zhou et al., 2003; Bhalla et al., 2006). In Sudan, wheat is the second most important cereal crop after sorghum (Ishag, 1994). Being a temperate crop, it is not indigenous to Sudan; yet it was traditionally grown since early times in the Northern State (latitude 18 to 22° N) and it enjoys a relatively cooler and longer winter season than in Central Sudan. However, owing to increasing demand for wheat coupled with the limited resources in Northern Sudan, the expansion in wheat cultivation took place in warmer central plains (Mohammed, 2009). Therefore, the average wheat yields in Sudan are very low and uncertain (Saad, 2010). The major reasons for low productivity and instability in addition to heat stress also includes late planting of wheat, non availability of improved inputs like seed, inefficient fertilizer use, weed infestation, shortage of irrigation water and delayed harvesting. Moreover, farmers are not aware of modern technologies because of weak

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Abbreviations: MS, Murashige and Skoog basal medium; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **IBA**, indole-3- butyric acid; **IAA,** indole-3- acetic acid; **TDZ,** 5-phenylcarbamoylamino-1,2,3-thiadiazole (thidiazuron); **BA,** benzyladenine; **KN,** kinetin.

extension services system (Kabesh et al., 2009).

Wheat conventional plant breeding methods in Sudan, although, have been practiced successfully since 1960s for the production of improved wheat varieties and solving of some of these problems, these methods have some limitations such as long time requirement and rather limited gene pool available for wheat breeders.

Modern biotechnology overcomes many of the problems associated with conventional breeding, in comparison to conventional breeding; this technique is more precise and allows transfer of traits across species/genera. These techniques also offer creation of variation through somaclonal and gametoclonal variations and consequently facilitate rapid development of new varieties.

Efficient plant regeneration from cultured cells and tissues is required for the successful application of modern biotechnology in crop improvement. Therefore, the success of cell and tissue culture research depends upon reliable callus culture and plant regeneration procedures (Murat and Arpacioğlum, 2003). Callus induction and *in vitro* regeneration of wheat is possible from different explants such as mature and immature embryos, seeds, endosperm, leaves, shoot bases and root tips (Sarker and Biswas, 2002). Among these, the immature embryo was reported as the best for callus induction and shoot regeneration (Sarker and Biswas, 2002; Arzani and Mirodjagh, 1999; Hou et al*.,* 1997). But availability of immature embryo is limited by wheat growing season or requires expensive and sophisticated growth chambers. On the other hand, mature seeds of wheat are readily available throughout the year, hence can be used for plant regeneration in any convenient time (Rahman et al., 2008). With this in view, the aim of this study, therefore, was to determine the callus induction and plant regeneration potential of various Sudanese wheat cultivars by using mature embryos as the explant source.

MATERIALS AND METHODS

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

Seeds surface sterilization and explant preparation

Wheat mature dry seeds were washed under running tap water for 30 min, and then socked in tap water for 4 to 5 h at room temperature before embryos dissection so that the embryos can swell and become distinctly visible and easy to remove. Under laminar air flow hood, the soaked seeds were surface disinfested with ethanol (70%) for three minutes, then soaked in commercial sodium hypochlorite (clorox) 100% supplemented with 1 to 2 drops of Tween 20 for twenty minutes with continuous shaking. After washing several times with sterile distilled water, embryos were separated from the embedded seeds using a sterile scalpel and forceps and placed in glass bottle containing nutrient medium (Figure 1a).

Callus induction, shoot and root initiation

For callus formation, mature embryo explants were transferred to culture bottles containing MS (Murashige and Skoog, 1962) medium supplemented with various concentrations of auxins (2,4-D, IBA and IAA). The concentrations of plant growth regulators are shown in Table 1. The MS medium was fortified with 3% sucrose (w/v), solidified with 0.6% agar and pH was adjusted to 5.8 before been dispensed in measured amount of 25 ml/bottle and autoclaved at 121°C and 15 psi for 15 min. Explants were placed in bottles and incubated under total darkness conditions and temperature was adjusted to 25 ± 2°C. After 4 weeks, callus were picked out from bottles using forceps and placed on sensitive balance to measure the fresh weight.

For shoot regeneration, calli obtained with 2.0 mg/L 2,4-D were either subcultured in the same medium or transferred to MS medium without or with different concentrations (0.1 to 3.0 mg/l) of cytokinines (BA, Kin and TDZ) alone or in combinations with 2,4-D (2.0 mg/L) and incubated under photoperiod of 16 h light under cool-white fluorescent lamps at 25 ± 2°C.

For root initiation, the regenerated shoots were excised from callus and placed on half strength MS media supplemented with different concentrations of IBA.

Experimental design and statistical analysis

Each experiment was set up as a completely randomized design. Data were subjected to one-way ANOVA and statistically analyzed using ANOVA table on excel program and presented as average ± standard error. Means were separated according to Duncan's multiple range test (Duncan, 1955) at 0.5 probability level. For the determination of callus fresh weight, 10 replicates were used. Callus were transferred on sterile pre-weighted aluminum foil and weighed again. The whole experiment was repeated twice.

RESULTS AND DISCUSSION

After four weeks of culture, the mature embryo explants of the cultivar Khaleefa cultured on MS medium without growth regulators or supplemented with IBA and IAA failed to induce callus, instead vegetative growth into seedling was observed (Figure 1b, c and d). However, explants cultured on MS media supplemented with different concentrations of 2,4-D formed a yellow compact callus after 4 weeks of culture (Figure 1e). In general, wheat callus induction and plant regeneration from tissue culture are influenced by culture medium, explant and genotype (Maddock et al., 1983; He et al., 1989; Bommineni and Jauhar, 1996; Özgen et al., 1998). Here, in this study, our result shows that only 2,4-D induced yellow compact callus on wheat mature embryo. The superiority of 2,4-D for wheat callus induction was reported in a previous study (Abdrabou and Moustafa, 1993). Moreover, in others studies (Barro et al., 1998; Arzani and Mirodjagh, 1999), 2,4-D was reported as the most widely used growth regulator for callus induction and callus maintenance in wheat.

Among the different concentrations of 2,4-D used in this study, the result showed that 2,4-D at 2.0 mg/L induced the best result (0.05 ± 0.01 g) for callus fresh weight (Table 1). These results are consistent with the

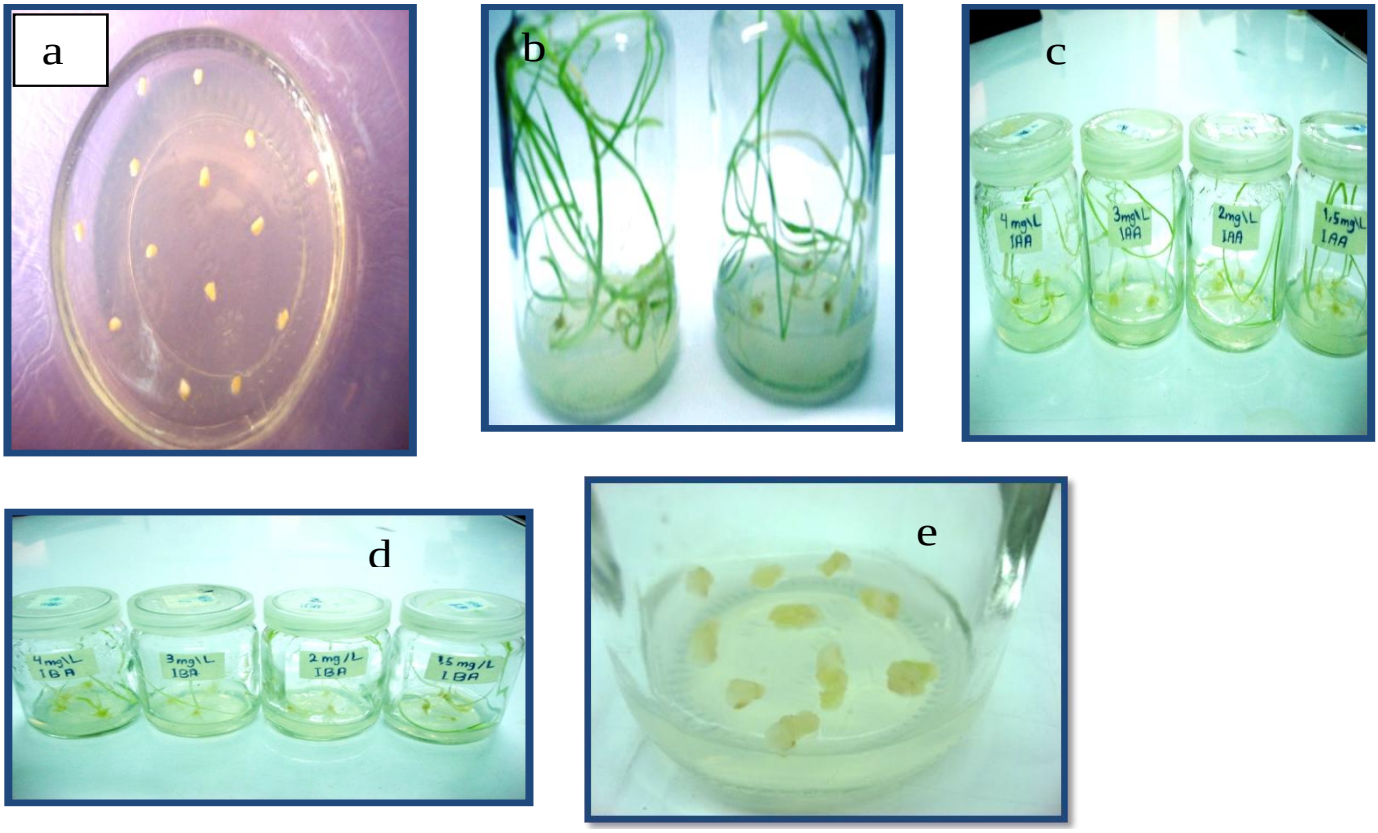

Figure 1. Callus induction of wheat cultivars Khaleefa. (a) Mature embryo explant of wheat used for callus formation; (b) explant cultured on MS medium without growth regulators; (c) explant cultured on MS medium supplemented with IAA after 4 weeks of culture; (d) explant culured on MS medium supplemented with IBA after 4 weeks of culture; (e) callus induction on MS medium supplemented with 2.0 mg/L 2,4-D after 4 weeks of culture.

Table 1. Effect of different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D), indole-3- butyric acid (IBA) and indole-3-actic acid (IAA) on callus formation from mature embryos of wheat (Khaleefa c.v.) after 4 weeks of culture.

Auxin type	Concentration (mg/L)	Callus fresh weight (g) (Mean ± SE)	Morphology
Control	0.0	0.00 ± 0.0^{d}	Vegetative growth
2,4-D	1.5	0.03 ± 0.0^{c}	Yellow compact callus
	2.0	0.05 ± 0.01^{a}	Yellow compact callus
	3.0	0.04 ± 0.0^{b}	Yellow compact callus
	4.0	0.03 ± 0.01^{c}	Yellow compact callus
IBA	1.5	0.00 ± 0.0^{d}	Vegetative growth
	2.0	0.00 ± 0.0^{d}	Vegetative growth
	3.0	0.00 ± 0.0^{d}	Vegetative growth
	4.0	0.00 ± 0.0^{d}	Vegetative growth
IAA	1.5	0.00 ± 0.0^{d}	Vegetative growth
	2.0	0.00 ± 0.0^{d}	Vegetative growth
	3.0	0.00 ± 0.0^{d}	Vegetative growth
	4.0	0.00 ± 0.0^{d}	Vegetative growth

Means with the same letter(s) in the same column are not significantly different at 5% using Duncan multiple range test.

Table 2. Effect of benzyl adenine (BA), kinetin (KN) and thidiazuron (TDZ) alone or in combination with 2,4-dichloro phenoxy acetic acid (2,4-D) on shoot regeneration of wheat (Khaleefa c.v.).

BA (mg\l)	KN (mg\l)	TDZ (mg\l)	2,4-D (mg\l)	Regeneration (%)	Number regenerated shoot (Mean ± SE)
0.0	0.0	0.0	0.0	40	0.8 ± 0.4[a]
0.0	0.0	0.0	2.0	0.0	0.0 ± 0.0[d]
0.1	0.0	0.0	2.0	0.0	0.0 ± 0.0[d]
0.25	0.0	0.0	2.0	0.0	0.0 ± 0.0[d]
0.5	0.0	0.0	2.0	0.0	0.0 ± 0.0[d]
1.0	0.0	0.0	2.0	0.0	0.0 ± 0.0[d]
2.0	0.0	0.0	2.0	0.0	0.0 ± 0.0[d]
3.0	0.0	0.0	2.0	0.0	0.0 ± 0.0[d]
0.1	0.0	0.0	0.0	0.0	0.0 ± 0.0[d]
0.25	0.0	0.0	0.0	10	0.31 ± 0.3[b]
0.5	0.0	0.0	0.0	20	0.31 ± 0.2[b]
1.0	0.0	0.0	0.0	0.0	0.0 ± 0.0[d]
2.0	0.0	0.0	0.0	0.0	0.0 ± 0.0[d]
3.0	0.0	0.0	0.0	0.0	0.0 ± 0.0[d]
0.0	0.1	0.0	2.0	0.0	0.0 ± 0.0[d]
0.0	0.25	0.0	2.0	0.0	0.0 ± 0.0[d]
0.0	0.5	0.0	2.0	0.0	0.0 ±0.0[d]
0.0	1.0	0.0	2.0	0.0	0.0 ± 0.0[d]
0.0	2.0	0.0	2.0	0.0	0.0 ± 0.0[d]
0.0	3.0	0.0	2.0	0.0	0.0 ± 0.0[d]
0.0	0.1	0.0	0.0	0.0	0.0 ± 0.0[d]
0.0	0.25	0.0	0.0	0.0	0.0 ± 0.0[d]
0.0	0.5	0.0	0.0	0.0	0.0 ± 0.0[d]
0.0	1.0	0.0	0.0	10	0.21 ± 0.2[c]
0.0	2.0	0.0	0.0	0.0	0.0 ±0.0[d]
0.0	3.0	0.0	0.0	0.0	0.0 ± 0.0[d]
0.0	0.0	0.1	2	0.0	0.0 ±0.0[d]
0.0	0.0	0.25	2	0.0	0.0 ± 0.0[d]
0.0	0.0	0.5	2	0.0	0.0 ± 0.0[d]
0.0	0.0	1.0	2	0.0	0.0 ±0.0[d]
0.0	0.0	2.0	2	0.0	0.0 ± 0.0[d]
0.0	0.0	3.0	2	0.0	0.0 ± 0.0[d]
0.0	0.0	0.1	0.0	0.0	0.0 ± 0.0[d]
0.0	0.0	0.25	0.0	0.0	0.0 ± 0.0[d]
0.0	0.0	0.5	0.0	0.0	0.0 ± 0.0[d]
0.0	0.0	1.0	0.0	0.0	0.0 ± 0.0[d]
0.0	0.0	2.0	0.0	0.0	0.0 ± 0.0[d]
0.0	0.0	3.0	0.0	0.0	0.0 ± 0.0[d]

Means with the same letter(s) in the same column are not significantly different at 5% using Duncan multiple range test.

findings of Yu et al. (2008), who reported that wheat embryogenic callus induction frequency peaked with 2.0 mg/L 2,4-D in the induction medium.

Shoot regeneration is crucial and important in the realization of potential of cell and tissue culture techniques for plant improvement (Nasircilar et al., 2006). After 4 weeks of culturing, mature embryo on MS basal medium containing 2.0 mg/L 2,4-D, the embryogenic calli were transferred to regeneration medium for shoot induction. The regeneration media were MS basal media without growth regulators or supplemented with different concentrations of BA, KN or TDZ alone or in combinations with 2,4-D at 2.0 mg/L. Our result shows that after 4 weeks of culture on the regeneration media, the best regeneration percentage (40%) and the highest mean number of the regenerated shoots (0.8 ± 0.4) was obtained from the callus transferred to MS basal medium without growth regulators followed by 20, 10 and 10% regeneration percentage obtained on calli transferred to MS medium supplemented with 0.5, 0.25 mg/L BA or 1.0 mg/L KN, respectively (Table 2). In agreement with our findings, Purnhauser et al. (1987) stated that auxins

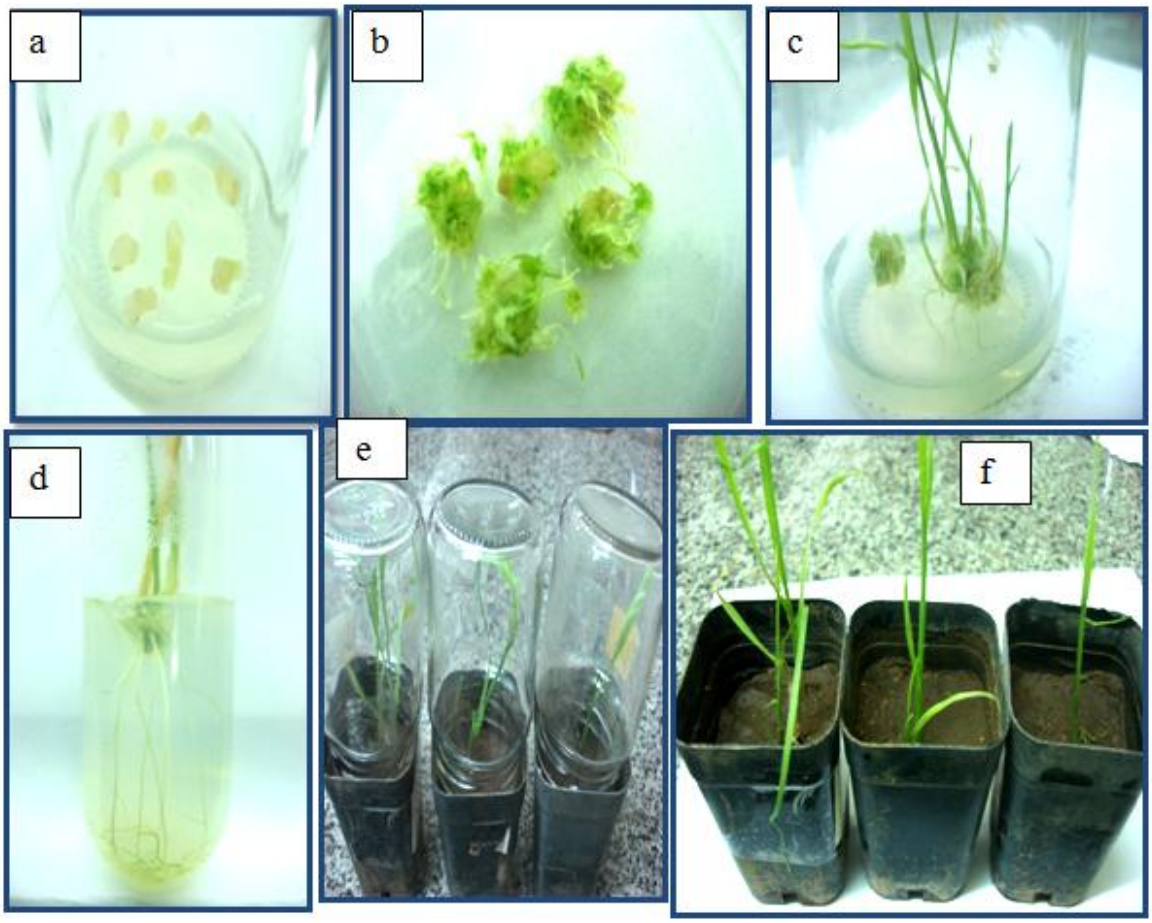

Figure 2. Plant regeneration from callus culture of wheat cultivars Khaleefa. (a) One month old wheat callus induced on MS medium supplemented with 2.0 mg/L 2,4-D; (b) two month old wheat callus with leaf like green spots; (c) plantlet regeneration on MS basal medium without growth regulators; (d) *in vitro* rooting of regenerated shoots on half strength MS medium supplemented with 1.0 mg/L IBA; (e) acclimatization of plantlets under room conditions; (f) wheat plant established successfully in soil.

which are essential for callus induction play a negative role in plant regeneration and are generally reduced or excluded from culture media used for shoot regeneration. Furthermore, previous studies (Bregitzer, 1992; Daaloul et al., 1992) reported that wheat regeneration was obtained when calli were transferred to MS basal salts without hormones.

The regenerated plantlets were successfully rooted in half strength MS medium supplemented with different concentrations of IBA; 100% rooting were observed in all concentrations. The best result for mean number of roots per shoot (4.0 ± 0.0) was obtained on half strength MS supplemented with 1.0 mg/L IBA (Figure 2), the longest roots were observed on half strength MS basal medium without hormones and on half strength MS medium supplemented with 0.5 mg/L IBA with mean number of 17. 3 ± 0.9 for both of them (Table 3). For rooting of *in vitro* derived shoot, auxin IBA has been reported to be useful for inducing rooting in a variety of plants (Hammatt and Ridout, 1992; Perez-Parron et al., 1994; Ramanayake et al., 2006; Faisal et al., 2007; Mishra et al., 2008).

For acclimatization, *in vitro* derived plantlets were removed from rooting medium after 4 weeks of incubation and transferred to plastic pots containing autoclaved soil and covered with glass bottles to maintain humidity and were kept under culture room conditions for one week (Figure 2e). After three weeks, glass bottles were removed and transferred to green house and placed under shade until growth was observed. One hundred percent of the plants survived and all were morphologically normal (Figure 2f).

Genotype has been well documented to affect callus induction and plant regeneration in all major cereal species, including wheat (Ozgen et al., 1998). Here, in this study, in order to determine the effect of genotype on plantlet regeneration, the optimized procedure obtained for Khaleefa cultivar was then utilized in different

Table 3. Effects of different concentrations of indole 3-butyric acid (IBA) on *in vitro* rooting of regenerated shoots of wheat (Khaleefa c.v) after 4 weeks of culture on half strength MS medium.

IBA (mg/L)	Root length (mean ± SE)	Number root (mean ± SE)	Rooting (%)
0.0	17.3 ± 0.9[a]	3.5 ± 0.3[a]	100
0.5	17.3 ± 0.9[a]	3.5 ± 0.3[a]	100
1.0	17.0± 0.7[ab]	4.0 ± 0.0[a]	100
1.5	16.8 ± 0.6[abc]	3.8 ± 0.8[a]	100
2.0	16.0 ± 0.6[bc]	2.0 ± 0.4[b]	100

Means with the same letter(s) in the same column are not significantly different at 5% using Duncan multiple range test.

Table 4. Effect of elite Sudanese wheat cultivars on callus induction and shoot regeneration from mature embryo explant culture on MS medium supplemented with 2.0 mg/L 2,4-D.

Genotype	Number of explant cultured	Callus induction (%)	Regeneration capacity of callus (%)	Number of regenerated shoot
Khaleefa	274	100	9.12	29
Takana	259	100	-	-
Sasareeb	191	100	2.61	11
Wadi Elnile	168	100	4.17	17
Bohain	211	100	0.94	5
Elnileen	167	100	3.59	10

regeneration experiments for Khaleefa and other five Sudanese elite wheat cultivars. Mature embryo calli obtained on MS medium supplemented with 2.0 mg/L 2,4-D was cultured on MS basal medium. Not all genotypes responded similarly to this medium, however, different responses were observed among them, Khaleefa showed more proliferate callus than other genotypes with regeneration percent of 9.12, Wadi Elnile came second with regeneration percent of 4.17, followed by Elnileen and Sasareeb with regeneration percent of 3.59 and 2.61, respectively; Bohain showed the least regeneration percent (0.94), while no regeneration was observed in Takana (Table 4), these different responses may be attributed to genotypic effect, this results are in agreement with those of Machii et al. (1998) who reported that wheat regeneration via tissue culture varies with the genotype. Generally, in wheat both somatic embryogenesis and regeneration are complex morphogenic processes and have been shown to be genotype-dependent (Mathias and Simpson, 1986; Fennell et al., 1996; Maes et al., 1996; Afsharsterle et al., 1996) and influenced by the components of the culture media used (He et al., 1989; Fellers et al., 1995).

In conclusion, an efficient protocol for wheat regeneration from mature embryos was developed. Six elite wheat cultivars widely grown in Sudan were investigated for their regeneration capacity from mature embryos for the first time. The choice of responsive wheat cultivars is important for plant improvement programs by shortening the time needed to breed new varieties, by using modern biotechnology techniques. The results presented here demonstrated the different capacity of callus induction and plant regeneration of the evaluated genotypes, which makes it possible to choose the best cultivars to achieve such characteristics, by optimizing the growing process of mature embryo culture and the plant regeneration process. The effective use of mature embryos could compensate for the scarcity of immature embryos used in wheat transformation and, to a certain extent, facilitate future research of wheat genetic improvement.

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