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A Simple and Efficient Protocol for Callus Induction and Regeneration from Wheat (*Triticum aestivum* L.) Mature Embryos

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Abstract: ***The aim of this study was to develop a protocol for callus induction and regeneration of 12 wheat (Triticum aestivum L.) cultivars widely grown in Sudan. Different concentrations/combinations of 2, 4-dichlorophenoxyacetic acid (2, 4-D) and Zeatin were tested for efficient callus, shoot and root inductions using mature embryos. Experiments were laid out in a completely randomized design with ten replicates and Duncans Multiple Range test was used for mean separation. There were significant differences between the means number of calli obtained with different 2, 4-D concentrations tested. The highest frequencies of induced embryogenic calli were recorded in Murashige and Skoog (MS) medium containing 2.0 mg/l of 2,4-D. Different wheat cultivars displayed variable frequencies in their response to each of the six 2,4-D concentrations indicating genetic variability and that wheat regeneration is genotype dependent. Shoots and roots were successfully induced in MS medium supplemented with 2.0 mg/l 2, 4-D and 1.0 mg/l Zeatin. The highest regeneration capacities of more than 40% were recorded for cultivars Sasareeb (49%), Nebta (45%) and Imam (42%), while the lowest (19 %) was recorded for cv. Nasser. This simple and reproducible regeneration protocol can be used in any future biotechnological program for wheat improvement in Sudan.***

Keywords: Wheat, callus induction, embryogenesis, regeneration

1. Introduction

1.1 *Wheat (Triticum aestivum L.), is a member of the family Poaceae, which includes major cereal crops of the world such as maize, millet, barely and rice [10]. Wheat is the main staple food for 35% of the world's population and provides almost 20% of their total food calories [15]. Since the 1960s, wheat consumption has risen almost 5 percent a year in developing countries, faster than any other basic food crop [3]. Wheat is becoming increasingly popular in Africa, with output up by two-thirds in the past 20 years. Africa is also a heavy wheat importer, especially in urban areas as bread becomes more important [12].*

1.2 In Sudan, wheat is mostly grown in irrigated areas in the Gezira and New Halfa schemes where the crop is subjected to severe drought spells, either early in the season or late at the filling stage, and also to pressures of high temperatures. As a result of these stresses yield is very low in Sudan and productivity in irrigated areas is estimated to be around 2471 kg/Ha compared to 622 kg/fed in rainfed areas [1]. Many of the desirable traits, such as drought and tepmrature tolerance, required for improvement of crops cannot be achieved by traditional breeding technologies due to the lack of such traits in the existing particular crop cultivars [31]. In such cases, genetic transformation offers an indispensible alternative for crop improvement [35]. However, any plant genetic transformation program requires a pre-determined reproducible regeneration protocol [21].

1.3 Regeneration capacity which includes callus, shoot and root induction in wheat and other crops are genotype-dependent [20]. They are also influenced by explant type, geographical origin and the concentration/combination of plant growth regulators [13]. In wheat, different explant sources were studied: shoot tips [36]; seeds [17]; inflorescences [30]; young leaves [40]; mature embryos [11]; [37]; immature embryos [24]; and anthers [8]. These tissues vary in their ability to regenerate whole plants [25]. [9] Established highly embryogenic cell suspension cultures from immature embryo-derived calli of winter wheat. [29] Induced callus in excised embryos of spring and winter varieties using different auxins. High frequency of callus induction is also reported through mature embryo culture in wheat [28].

1.4 Successful application of embryo-based transformation systems in wheat requires the use of popular genotypes selected for specific tissue culture response and capacity to regenerate fertile plants. The aim of this study was to develop an in vitro culture protocol for callus induction and proliferation from mature embryos of 12 wheat cultivars that are commonly grown in Sudan.

2. Materials and Methods

2.1 Plant Materials

Seeds of twelve wheat commercial cultivars grown in different regions of Sudan were obtained from Agricultural Research Corporation (ARC), Ministry of Agriculture, and Sudan. These were: Tagana, Nebta, Wadi Elniel, Sasaraib, Argeen, Debaira, Niser, Elnielain, Imam, Condor, Bohain and Khalifa.

2.2 Seeds Sterilization and Embryo Culture

Mature seeds were surface sterilized in 70% (v/v) ethanol for one minute followed by immersion in 10% (v/v) clorox for 15 minutes and were then rinsed several times in sterile distilled water. Seeds were soaked for 1 hour in sterile distilled water so that seeds get fully turgid and embryos swell and increase in size. Turgid seeds were then plotted on moistened filter papers in Petri dishes and embryos were gently separated and 100 of them per cultivar were transferred to sterilized Murashige and Skoog (MS) basal medium [29]. This medium contained: MS, 4.43 g/L; sucrose, 3.0% (w/v); TC agar, 7.0 g/L and supplemented with different concentrations of 2, 4-D (0.5, 1.0, 1.5, 2.0, 3.0 or 4.0 mg/L). In all cases the medium pH was adjusted to 5.7 prior to autoclaving. Callus formation frequency was calculated according to [23] and Callus development was periodically monitored for 6 weeks. Weight of callus (mg) was determined and their colours were indicated for each cultivar in all media. Percentages of shoot induction were calculated and shooted calli were then transferred to rooting media.

2.3 Rooting and elongation of *in vitro* regenerated shoots:

For root induction, 100 shooted embryogenic calli of each wheat cultivar were cultured on MS medium containing different concentrations of 2, 4-D (0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 mg/l) and different concentrations (0.5, 1.0, 1.5, 2.0 mg/L) of Zeatin [ZEA]. The regeneration percentages were calculated as follows:

$$\frac{\text{No. of rooted shoots} \times 100}{\text{Total No. of shoots}}$$

2.4 Statistical Analysis

Two-way Analysis of Variance (ANOVA) was used to analyze the effects of genotype and the concentrations of the PGRs as well as their interaction on the number of regenerated plantlets. The means were compared by Duncan's Multiple Range Test using SPSS v. 16.0 package software.

3. Results and Discussion

3.1 Callus induction

Regeneration capacity of a crop is an essential prerequisite for its genetic improvement through genetic transformation. Regeneration frequency is highly dependent on genotype [16]; [6], [20], explants [18] and regeneration medium used [33]. Frequencies of callus induction from mature embryos of the twelve wheat genotypes were determined weekly for six weeks in six different concentrations of 2,4-D. Results (Table 1) show that there were significant differences ($p<0.05$) between the mean callus % recorded for the 12 cultivars and for the same cultivar at different 2,4-D concentrations. However, no significant difference was observed for the interaction between cultivars and 2, 4-D concentrations. Wheat cultivars display variable frequencies (30 - 86%) in their response to each of the six 2, 4-D concentrations. For all cultivars, the highest callus induction frequencies were obtained when MS medium supplemented with 2.0 mg/L 2, 4-D was used (Plate 1). However, reduction in callus formation frequencies was observed when 2, 4-D concentration was increased beyond this value. Similar results were reported by [19], [39] and [4]. Also, [25] reported that the major problems associated with callus induction and maintenance from wheat explants may be resolved to a great extent by using 2.0 mg/l 2,4-D. Other investigators have reported the highest callus induction frequency from mature embryos of wheat cultivars when 2, 4-D was used at a concentration of 1.5 mg/l [32]; [38].

The highest callus induction frequency (86%) using mature embryos was observed for cultivar Wadi El Niel, followed by Nebta and Elnielain (82% each) and Tagana and Bohain (80% each). Similarly, high frequency of callus induction of 90.6% was also reported by [28] using wheat mature embryos. Although mature embryos are readily available at all times, they are the least to be used for regeneration trials. This may be due to the low callus induction frequency frequently obtained with these explants [2]; [27]. Callus induction was also significantly ($p<0.05$) influenced by wheat genotypes (Table 2). With the exception of its response in 1.0 and 4.0 mg/l 2, 4-D, cultivar Imam showed the lowest frequency of callus induction in all other 2, 4-D concentrations tested. On the other hand, at 1.5 and 2.0 mg/l 2, 4-D, cultivar Wadi El Niel showed the highest callus induction percentage compared to the other tested cultivars. Cultivar Nebta scored the highest overall mean percentage (63.5%) of callus induction while Nasser and Imam scored the lowest (43% each). In accordance with this result, [24] and [14] reported the influence of genotype on callus induction of wheat.

Calli weights were measured after six weeks of incubation. Increments in callus weight were recorded when 2,4-D concentration was increased from 2.0 mg/l to 4.0 mg/l. The heaviest callus weight (0.516 mg) was recorded for cultivar Khalifa when cultured in MS medium supplemented with 4.0 mg/l 2,4-D while the lightest (0.10) was recorded for Elnielain cultivar when cultured in 0.5 mg/l 2,4-D (Table 3).

Variations in calli colours were also observed and indicated. Different rates of shoot proliferation were observed from white (80.63%), green (79.29%), creamy (73.58%), brown (77.56%) and yellow (73.26%) calli. [38] Reported that the frequency and duration of plant regeneration is markedly increased if a visual distinction is made between embryogenic callus which is smooth, compact, milky white to yellow in color, and non-embryogenic callus which is rough, yellow to brown in colour. Similar results were also reported by [32] and [34].

3.2 Shoot initiation and Regeneration Capacity:

Significant differences were observed in shoot initiation rates recorded at different 2, 4-D concentrations (Table 1). Variation in the overall mean percentages between the cultivars ranged between 33% in 0.5 mg/l to 61% in 2.0 mg/l

2, 4-D. The highest shooting percentages, ranging between 53 – 74% were recorded in MS medium supplemented with 2.0 mg/l 2, 4-D (Table 4). Significant differences ($p<0.05$) in shoot induction frequency were detected at different 2, 4-D concentrations and among cultivars tested. The highest overall mean (48.5%) of shoot induction frequency was recorded for cultivar Argeen while the lowest (31.5%) was recorded for cultivar Bohain.

Embryogenic calli that initiated shoots after six weeks in 2.0 mg/L 2,4-D were transferred, separately, to MS media each containing a combination of 2.0 mg/L 2,4-D and one of four (0.5, 1.0, 1.5 and 2.0 mg/l) Zeatin concentrations. Root initiation and elongation was monitored and rooting percentages were recorded weekly for six weeks, for each shooted callus, in each of the MS-2, 4-D/Zeatin medium combination, results are shown in Plate 2 and Fig. 1. The highest regeneration percentage for each cultivar was recorded when 1.0 mg/l Zeatin was used. At this concentration, cultivar Sasaraib showed significantly high regeneration percentage (49%) compared to the other cultivars tested. The highest regeneration percentages were recorded for cultivar Imam in Zeatin concentrations of 1.5 and 2.0 mg/l, while the lowest frequencies of 5, 6, 7 and 19% were recorded for cultivar Nasser in the four Zeatin concentrations used. It has been reported that cytokinins, such as Zeatin, 6-benzylaminopurine (BAP) and Kinetin when incorporated in the regeneration medium could improve shoot regeneration [22]; [5]. In this study, the addition of zeatin in the regeneration medium significantly improved regeneration capacity of callus which is consistent with the findings above.

The transfer of foreign genes into wheat by genetic engineering techniques requires the development of an efficient in vitro regeneration system. Results presented in this study are interesting because it may increase efficiency of gene transfer technology to genotypes that have low tissue culture response from immature embryos. An efficient regeneration system such as mature embryo culture may provide enough material for direct gene transfer studies. Therefore, mature embryos, which are readily available throughout the year, can be used as an effective explant source in wheat tissue culture.

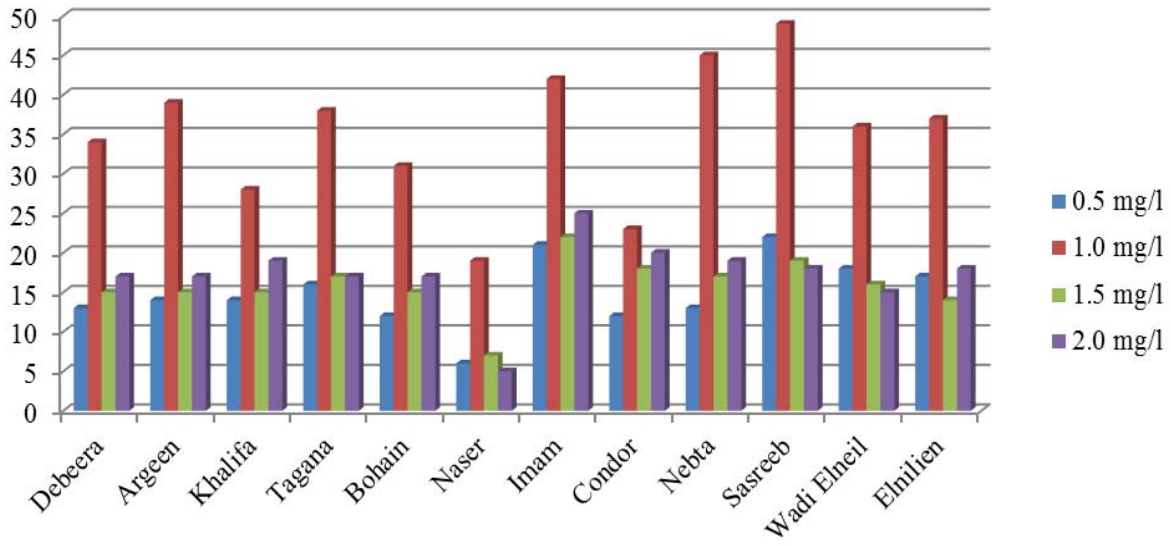

Figure 1: Regeneration of different wheat cultivars in MS medium supplemented with 2.0 mg/l 2, 4-D and 1.0 mg/l Zeatin

Table 1: Analysis of variance for callus and shoot induction frequencies of the 12 wheat cultivars in MS medium supplemented with different 2, 4-D concentrations.

Source of Variation	*Callus induction frequency*				*Shoot induction frequency*		
	df	*SS*	*MS*	*F*	*SS*	*MS*	*F*
Cultivars	11	296.949	26.995	10.659**	175.349	15.941	6.795**
PGRs Concentrations	5	811.074	162.215	64.052**	680.340	136.068	58.004**
Cultivars x PGRs Concentrations	55	79.376	1.443	0.570^{NS}	121.976	2.218	0.945^{NS}

**means are highly significant ($p<0.05$), ^{NS} means are not significant

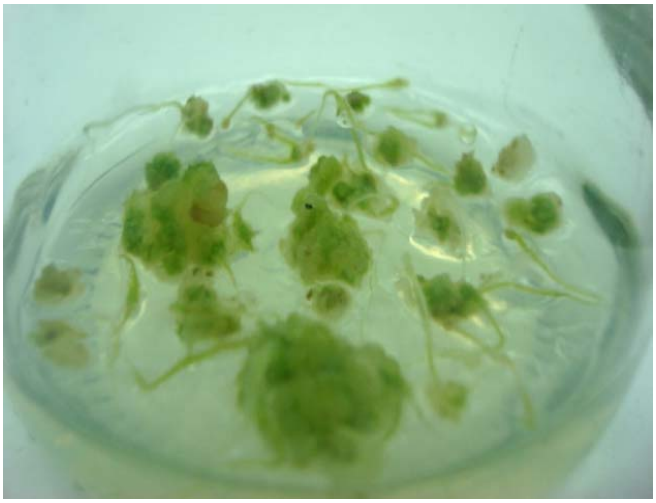

Plate 1: Callus induction and shoot initiation in MS medium supplemented with 2.0 mg/l 2, 4-D

Table 2: % callus induction of wheat cultivars in six 2,4-D concentrations

Cultivars	*2,4-D concentration (mg/L)*						*Mean* ± SD*
	0.5	**1**	**1.5**	**2**	**3**	**4**	
Debeera	5.6	4.7	5.4	7.7	5.2	4.8	$5.57^{b} \pm 0.21$
Argeen	5.4	5.8	6.1	7.6	4.9	5.0	$5.80^{bc} \pm 0.21$
Khalifa	4.4	5.7	6.7	7.9	5.7	4.9	$5.88^{bc} \pm 0.21$
Taganna	4.5	5.3	6.1	8.0	5.0	4.8	$5.62^{b} \pm 0.21$
Bohain	4.9	5.5	6.3	8.0	5.4	5.7	$5.97^{bc} \pm 0.21$
Nasser	3.3	4.0	5.3	6.5	3.7	3.0	$4.30^{a} \pm 0.21$
Imam	3.1	4.4	4.9	6.3	3.7	3.4	$4.30^{a} \pm 0.21$
Gondor	4.4	5.0	5.9	7.9	6.0	5.1	$5.72^{bc} \pm 0.21$
Nabta	5.3	6.1	6.7	8.2	5.8	6.0	$6.35^{c} \pm 0.21$
Sasreeb	4.4	5.1	5.9	7.8	5.8	4.8	$5.63^{b} \pm 0.21$
Wadi EL Niel	5.3	6.0	7.2	8.6	5.0	5.2	$6.22^{bc} \pm 0.21$
Elnielien	4.9	5.8	6.7	8.2	5.0	4.9	$5.9^{bc} \pm 0.21$
Mean* ± SD	$4.7^{a} \pm 0.15$	$5.3^{c} \pm 0.15$	$6.1^{d} \pm 0.15$	$7.8^{e} \pm 0.15$	$5.1^{bc} \pm 0.15$	$4.8^{ab} \pm 0.15$	5.63 ± 0.21

* means with different letters are significantly different at $p = 0.05$.

Table 3: Weight and colour of induced calli from mature embryos of different wheat cultivars in MS medium supplemented with different 2,4-D concentrations

Cultivars	*2,4-D concentrations (mg/L)*												Mean
	0.5		*1*		*1.5*		*2*		*3*		*4*		
	Weight (mg)	*Colour*	*Weight (mg)*	*Colour*	*Weight (mg)*	*Colour*	*Weight (mg)*	*Colour*	*Weight (mg)*	*Colour*	*Weight (mg)*	*Colour*	
Debeera	0.030	yellow	0.034	green	0.047	yellow	0.051	green	0.402	creamy	0.430	yellow	0.166
Argeen	0.245	white	0.255	creamy	0.026	white	0.027	white	0.350	yellow	0.400	green	0.217
Khalifa	0.135	brown	0.140	white	0.155	brown	0.167	yellow	0.492	white	0.516	brown	0.268
Tagana	0.023	yellow	0.033	yellow	0.035	white	0.044	creamy	0.363	brown	0.430	cream	0.155
Bohain	0.015	creamy	0.016	green	0.017	creamy	0.018	brown	0.340	white	0.350	brown	0.126
Nasser	0.011	green	0.012	creamy	0.013	green	0.013	green	0.330	yellow	0.340	creamy	0.120
Imam	0.019	brown	0.020	yellow	0.022	yellow	0.022	yellow	0.340	creamy	0.395	green	0.136
Condor	0.018	green	0.019	creamy	0.019	creamy	0.020	brown	0.375	yellow	0.380	white	0.138
Nebta	0.017	yellow	0.018	yellow	0.019	green	0.020	white	0.360	white	0.375	yellow	0.135
Sasreeb	0.045	creamy	0.056	creamy	0.057	yellow	0.058	creamy	0.435	creamy	0.510	creamy	0.193
Wadi El Niel	0.035	white	0.036	green	0.037	white	0.037	creamy	0.360	brown	0.410	yellow	0.152
Elnilien	0.010	brown	0.011	yellow	0.012	brown	0.013	brown	0.310	green	0.320	white	0.113
Mean	0.050		0.054		0.038		0.041		0.371		0.405		

Table 4: Shoot initiation frequency of different wheat cultivars at different 2,4-D concentrations

Cultivars	*2,4-D Concentration (mg/L)*						*Mean* ± SD*
	0.5	**1.0**	**1.5**	**2.0**	**3.0**	**4.0**	
Debeera	3.9	3.4	4.0	5.6	3.8	3.5	$4.03^{abc} \pm 0.20$
Argeen	4.0	4.8	5.2	6.9	4.2	4.0	$4.85^{a} \pm 0.20$
Khalifa	3.1	4.8	5.3	5.3	5.1	3.4	$4.50^{a} \pm 0.20$
Taganna	2.8	4.0	4.6	6.2	3.8	2.6	$4.0^{a} \pm 18.0$
Bohain	4.0	4.5	5.6	6.8	4.8	3.3	$31.5^{a} \pm 12.4$
Nisr	2.7	3.2	4.1	5.5	2.8	2.3	$25.3^{a} \pm 19.2$
Imam	2.6	3.6	3.8	5.6	2.8	3.1	$44.7^{a} \pm 14.2$
Gondor	3.5	4.1	4.1	5.9	3.9	3.5	$34.2^{a} \pm 8.0$
Nabta	2.8	3.9	5.4	6.5	5.0	4.8	$36.3^{a} \pm 18.7$
Sasreeb	2.3	2.9	4.5	5.7	4.1	3.5	$36.7^{a} \pm 22.8$
Wadi EL Niel	3.7	4.7	5.9	7.4	3.9	4.1	$32.8^{a} \pm 14.9$
Elnielien	3.6	4.3	5.5	6.3	3.8	3.3	$32.8^{a} \pm 15.9$
Mean* ± SD	$3.3^{a} \pm 0.14$	$4.0^{b} \pm 0.14$	$4.8^{c} \pm 0.14$	$6.1^{a} \pm 0.14$	$4.0^{b} \pm 0.14$	$3.5^{a} \pm 0.14$	33.9 ± 15.7

* means with different letters are significantly different at p = 0.05.

Plate 2: Regeneration in MS medium supplemented with 2.0 mg/l 2, 4-D plus 1.0 mg/l Zeatin.

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