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Regeneration of plantlets from unpollinated ovary cultures of Ethiopian wheat (*Triticum turgidum* and *Triticum aestivum*)

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An *in vitro* culture protocol was established for direct regeneration of plantlets from unpollinated ovary cultures of four Ethiopian wheat varieties. Unpollinated ovaries were excised from durum wheat (Yerer and Ude varieties) and bread wheat (Simba and Galama varieties). Analysis of variance (ANOVA) has shown that genotypes, types of media, concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) and kinetin (KIN) and durations of cold pretreatment at 4°C significantly (P≤0.05) affected direct formation of embryonic tissues independently. Stage II of wheat spikes, MS medium containing 1 mg/l of each of 2,4-D and KIN and 15 days of cold pretreatment were found to be the best conditions for direct formation of embryonic tissues. The highest frequency of shoots were regenerated from the cultured embryonic tissues of Yerer (41.6%) and Simba (41.3%) on medium containing 0.1 mg/l 2,4-D. From a total of 14,524 cultured unpollinated ovaries, 1,100 embryonic tissues (7.6 %) and 75 regenerants were obtained. The average percentage of embryonic tissues and regenerants were 9.0 and 1.1% from 3,444; 9.8 and 0.55% from 4,732; 5.6 and 0.17% from 2,988; 4.7 and 0.12% from 3,360 cultured unpollinated ovaries for varieties Yerer, Simba, Ude and Galama, respectively.

Key words: Embryonic tissues, *Unpollinated ovaries*, regenerants, wheat varieties.

INTRODUCTION

Wheat is predominantly a selfing and annual crop plant that originated in the Fertile Crescent of South East of Turkey (Waines and Hegde, 2003). In Ethiopia, wheat is traditionally grown by small scale farmers on heavy black clay soils at altitudes ranging from 1800-2800 m above sea level (masl) (Tesemma and Belay, 1991). Some of the major production constraints of wheat crop that pose a serious threat to global food security are fungal and viral diseases, insect pests and increasing human population (Jauhar, 2006; Getahun et al., 2012). *In vitro* regeneration of plants is one of the pre-requisites for successful genetic transformation, the fastest and the only easy methods for producing homozygous lines (Smale et al., 1996) which are very helpful to mitigate these constraints.

In vitro culture of unpollinated ovaries and ovules have

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxy acetic acid; **KIN,** kinetin; **NAA,** naphthaline acetic acid; **BAP,** benzyl amino purine; **IAA,** indole acetic acid; **DZARC,** Debre Zeit Agricultural Research Center; **HARC,** Holetta Agricultural Research Center; **PGRs,** plant growth regulators; **ET,** embryonic tissues.

been successfully applied to many plant species that are not amenable to androgenesis and to overcome many of the problems associated with androgenesis such as albinism,
inviability and recalcitrant (Shivanna and Mohan, 2005; Jauhar et al., 2009). Successful results of *in vitro* gynogenesis to produce haploids and double haploids have been reported in tef (*Eragrostis tef*) (Gugsa et al., 2006).

Potential use of wheat in tissue culture studies have been reported using various explants such as immature embryos (Sears and Deckard, 1982), anthers (Dogramaci et al., 2001), microspores (Liu et al., 2002) and unpollinated ovaries (Slama and Slim, 2007). However, tissue culture works have not been reported in any of the explant types using Ethiopian wheat genotypes. The objectives of this study were to regenerate plantlets from unpollinated ovary cultures of four Ethiopian wheat varieties and optimize the concentrations of plant growth regulators essential for the formation of embryonic tissues and regenerations of plantlets.

MATERIALS AND METHODS

Plant materials

Two bread wheat varieties (*Triticum aestivum*) varieties namely, Simba, and Galama and two durum wheat (*Triticum turgidum*) varieties (Ude and Yerer) were used in this study. Seeds of these varieties were obtained from Debre Zeit Agricultural Research Center (DZARC) and Holetta Agricultural Research Center (HARC). Seeds were sown in 30 cm diameter pots filled with black soil and grown in a glasshouse. Five seeds were sown per pot and two pots were used for each variety. Seeds were sown every two weeks interval for one year to get continuous source of explants.

Surface sterilization and culture initiation

Immature spikes were sterilized with 70% ethanol for 1 min, followed by 20% sodium hypochlorite of the original stock 5.25% and four drops of Tween 20 for 10 min, and then rinsed with sterile double distilled water. Unpollinated ovaries were excised aseptically using sterilized scissors, forceps and scalpel blades under stereomicroscope. Twenty ovaries were placed in each sterilized Petri-dish (15 x 100 mm) containing 20 ml of MS medium containing 0.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 30 g/l sucrose. Before autoclaving the medium was adjusted to pH of 5.8 and 0.8% agar was added. The Petri-dishes were kept in growthroom incubated under 16 h photoperiod and a light intensity of 1032-1557 lux flourescent intensity and 8 h dark at 24 ± 4°C.

Identifying the appropriate stages of unpollinated ovary explant

Spikes taken from variety Simba were categorized into three stages and unpollinated ovaries were excised from each stage. These were before the emergence of own from the boot (stage I), spike length of 5-9 cm which might be equivalent to uninucleate stage of wheat anther. When the owns emerged out from the boot, stage II (spike length of 10-14 cm) which might be equivalent to late uninucleate to binucleate stage of wheat anther. When the owns completely emerged out from the boot, stage III (spike length of 15- 18 cm) which might be equivalent to the trinucleate stage of wheat anther.

Effect of cold pretreatment durations on the formation of embryonic tissues

Spikes at stage II of the four varieties were cold pretreated at 4°C for 5, 10, 15, 20 and 25 days. Unpollinated ovaries were plated in a disposable Petri-dish containing 20 ml MS medium supplemented with 1 mg/l of 2,4-D. Unpollinated ovaries from spikes of all varieties without cold pretreatment were cultured in the same way as a control.

Effect of culture media on induction of embryonic tissues

Three types of media MS (Murashige and Skoog, 1962), N_6 (Chu, 1978) and B_5 (Gamborg et al., 1968) were used as embryo induction media. Each medium was supplemented with 1.0 mg/l 2,4-D and 30 g/l maltose.

Effect of 2,4-D and kinetin (KIN) on the formation of embryonic tissues

Unpollinated ovaries from stage II of the four varieties were cold pretreated at 4°C and cultured in Petri-dishes containing MS medium supplemented with 12 different concentrations and combinations of 2,4-D and KIN and 30 g/l maltose.

Effect of plant growth regulators (PGRs) on regeneration of shoots

Embryonic tissues of all varieties were transferred into MS regeneration medium supplemented with different concentrations and combinations of PGRs. All treatments were supplemented with 60 g/l sucrose.

Acclimatization of plantlets

Plantlets of varieties Yerer and Simba were removed from Majenta jar using forceps. The roots were washed with distilled water to remove the gelrite and placed into pots filled with 3:2:1 ratios of black soil, compost and sand, respectively.

Data collections and analyes

Each independent experiment had 3 replications and repeated 2-3 times. In induction media, 20 ovaries and in MS regeneration medium, 5 embryonic tissues were used as a unit of replication. Subculturing was carried out every 2-3 weeks. The frequency of responsive unpollinated ovaries was assessed in terms of percentage of developed ovaries and formation of embryonic tissues. All the experiments were carried out in complete random design (CRD). The analysis of variance (ANOVA) was conducted using SAS computer software. The possible pairs of treatment means were compared using LSD/Duncan test.

RESULTS

Determination of appropriate harvesting stages of spikes

Ovaries (Figures 1a and b) taken from stage II induced maximum percentage of embryonic tissues (25%) (Figure 1c) followed by stage I (11.6%), however, overies taken from stage III could not sprouted out to give amenable

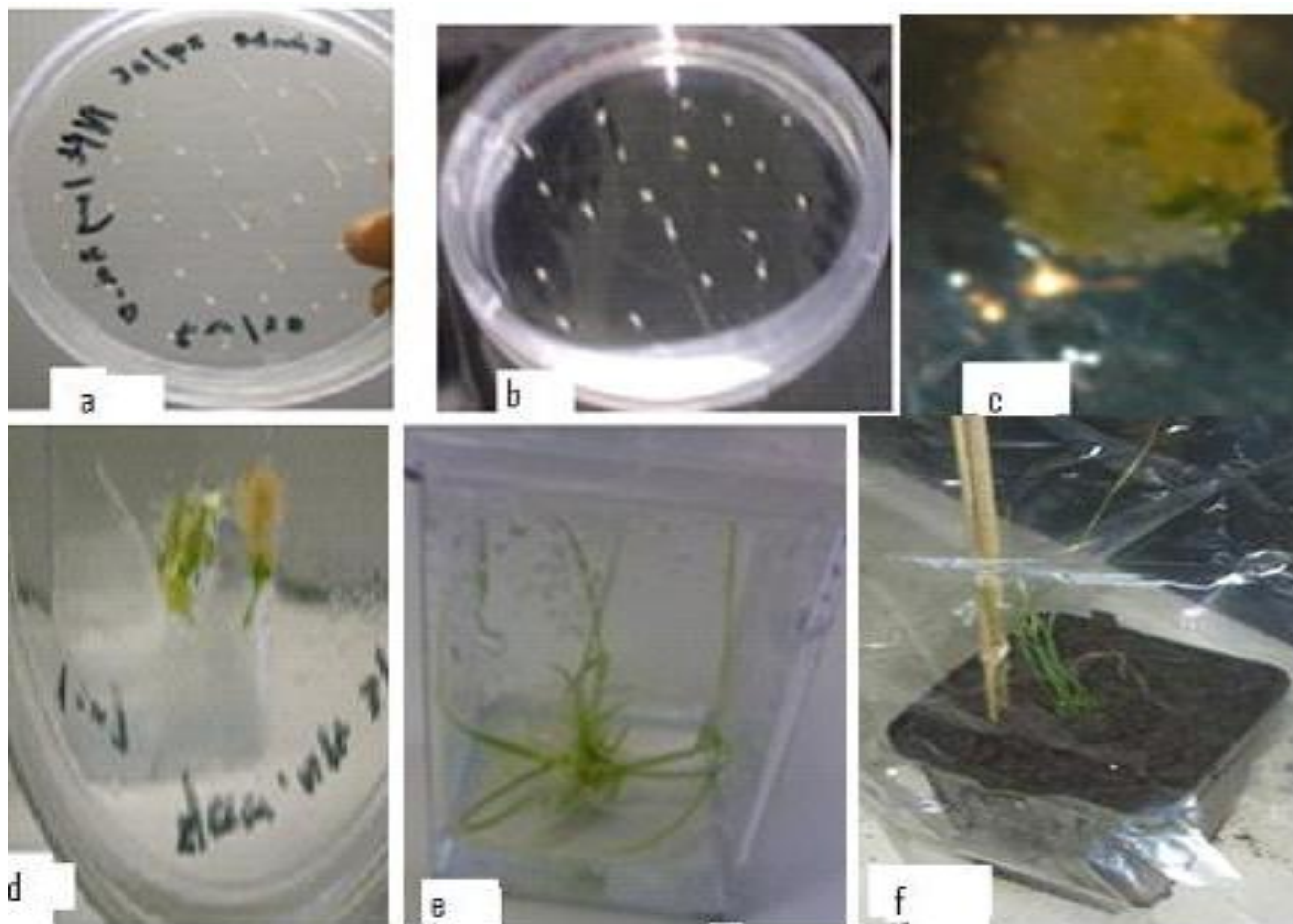

Figure 1. Embryonic tissues and plantlets of varieties Simba and Yerer.**a,** Ovary culture of variety Simba; **b,** ovary culture of variety Yerer; **c,** embryonic tissues of variety Simba; **d,** embryonic tissues of variety Yerer; **e,** plantlets of variety Yerer; **f,** acclimatization of plantlet of variety Yerer.

Table 1. Determination of appropriate harvesting stages of spikes for formation of embryonic tissues.

Stages of spike	Stage I	Stage II	Stage III
Cultured ovaries	120	120	120
ET	14	30	6
%ET±SD	11.6±4.7	25.0±9.6	5.0±2.9

ET, Embryonic tissues; SD, standard deviation.

amount of embryonic tissues and were found to be the least responsive (5.0%) (Table 1).

Effect of cold pretreatment durations on the formation of embryonic tissues

The highest percentage of embryonic tissues (8.3-20.0%) was obtained from unpollinated ovaries of all varieties that were pretreated at 4°C for 15 days followed by 10 days (5.8-10.8%). There was significant difference among all pretreatment durations for all varieties (Table 2).

Effect of culture media on the formation of embryonic tissues

The formation of embryonic tissues of variety Simba indicated that there was significant difference (P≤0.05) among MS, N6 and B5 media on the percentage of induced

Table 2. Effect of cold pretreatment durations on the percentage of embryonic tissues.

Variety	Percentage of embryonic tissues						
	0	5[p]	10	15	20	25	Mean of the means
Simba	6.0±3.5	8.3±2.4	10.0±4.0	20.0±4.0	9.2±1.9	5.8±1.9	9.2[a]
Yerer	8.3±4.4	9.2±4.2	10.8±2.5	16.6±4.5	3.3±2.4	0.8±2.5	8.6[a]
Ude	5.0±2.9	5.8±2.4	5.8±2.4	8.3±2.4	4.2±2.5	1.6±2.4	5.6[b]
Galama	3.3±2.4	6.0±2.4	6.0±2.4	13.3±2.4	1.7±3.2	0.0±0.0	4.7[b]
Mean of the means	4.2[c]	7.9[b]	8.8[b]	14.6[a]	4.6[c]	2.9[c]	

ANOVA for the effect of cold pretreatment on % of ET formation

Source of variation	DF	MS	F-value	P
Variety	3	87.4	6.6	0.0006**
Pretreatment	5	192.0	14.5	<0.0001**
Rep	2	1.4	0.1	0.9006
Error	61	13.2		
Total	71	294.0		

[p] Pretreatments (days).
Means with the same letter along the row and column are not significantly different at p≥0.05 using Duncan multiple range test. It is shown as mean of % ET±SD. **, Significant difference at p ≤0.1.

Table 3. Analysis of variance for the effect of different media on the formation of embryonic tissues from ovary cultures of variety Simba.

Source of variation	DF	MS	F-value	P
Media	2	304.2	24.3	0.04*
Rep	2	18.8	1.5	0.4
Error	2	12.5		
Total	6	335.5		

*, Significant at p≤0.05.

induced embryonic tissues (Table 3). The highest percentage of induced embryonic tissues were better in MS medium (20.0 %) followed by N_6 medium (5.0%) (data not shown).

Effect of PGRs on the formation of embryonic tissues

Maximum percentage of embryonic tissues were induced in MS medium supplemented with 1.0 mg/l 2,4-D combined with 1.0 mg/l of KIN for all varieties except variety Galama (Table 4). In this treatment, the highest percentage (35%) and the second highest (26.6%) percentage of embryonic tissues were obtained from varieties Yerer and Simba, respectively. Moreover, these varieties produced high quality embryonic tissues that could be easily differentiated into shoots (Figures 1c and d). Analysis of variance has shown that there was significant difference among varieties and treatments on the formation of embryonic tissues (Table 4).

Effect of PGRs on shoot and plantlet regeneration

A total of 462, 158, 168 and 312 embryonic tissues for varieties Simba, Galema, Ude and Yerer were induced on induction media under different experimental conditions, respectively (Table 6). Eleven combinations of auxins and cytokinins were selected and continuously supplemented in the regeneration medium for the regeneration of shoots (Table 5 and Figure 1e). Maximum responsive embryonic tissues, 41.6 and 41.3% were obtained in MS medium supplemented with 0.1 mg/l 2,4-D without cytokinin (Table 5) from varieties Yerer and Simba, respectively.

Variety Yerer gave better percentage of regenerants (1.1%) from a total of 3,444 cultured ovaries. Variety Simba produced the second highest percentage of regenerants (0.55%) and the maximum percentage of embryonic tissues (9.8%) from a total of 4,732 cultured unpollinated ovaries followed by variety Yerer (9.0%) (Table 6). Using all varieties from a total of 14,524 cultured unpollinated ovaries, 1100 embryonic tissues (Table 6) were produced and 75 regenerants were obtained (data not shown).

Acclimatization

Two plantlets of variety Simba and 16 plantlets of variety Yerer were successfully transferred into pots containing

Table 4. Effect of 2,4-D and KIN on the formation of embryonic tissues using all varieties.

2,4-D (mg/l)	KIN (mg/l)	Percentage % of embryonic tissues				
		Simba	Galama	Ude	Yerer	Mean
0.5	0.0	6.7±2.3	1.7±2.3	2.5±2.5	10.0±0.0	5.2[d]
0.5	1.0	6.7±2.3	2.5±2.5	3.3±1.3	11.6±2.0	6.0[d]
0.5	2.0	9.2±3.4	5.8±1.9	4.2±0.8	11.6±4.7	7.7[cd]
1.0	0.0	20.0±4.0	14.2±1.9	8.3±2.3	16.6±2.4	14.8[ab]
1.0	1.0	26.6±4.6	8.3±2.9	13.3±3.3	35.0±9.4	20.8[a]
1.0	2.0	14.2±5.2	5.8±1.8	9.2±0.8	15.8±3.5	11.3[bc]
1.5	0.0	11.7±4.7	5.0±2.8	8.3±2.4	14.2±4.6	9.8[cd]
1.5	1.0	8.3±3.2	5.0±2.8	8.3±2.4	10.8±1.9	8.1[cd]
1.5	1.0	7.5±2.5	7.5±3.8	6.7±2.4	6.7±2.4	7.1[cd]
2.0	0.0	5.8±1.8	9.2±1.9	7.5±2.7	5.8±2.5	7.1[cd]
2.0	1.0	5.0±1.3	7.5±2.5	5.8±2.5	5.0±2.9	5.8[d]
2.0	2.0	7.5±2.5	5.0±2.8	5.8±2.5	3.3±1.7	5.4[d]
Mean		10.8[ab]	6.5b	6.9b	11.2[a]	

ANOVA for the effect of 2,4-D and KIN on the formation of ETs

Source of variation	DF	MS	F-value	P
Variety	3	202.5	3.11	0.0134 *
Pretreatment	11	278.5	5.11	<0.0001**
Rep	2	25.5	0.47	0.6274
Error	127	54.5		
Total	143	561.0		

Means with the same letter along the row and column are not significantly different at p≥0.05 using Duncan multiple range test. ET, Embryonic tissues; *, significant at p≤0.05; **, very significant at p≤0.01. Mean values are shown as ± SD.

Table 5. Effect of PGRs on the percentage of responsive embryonic tissues. Their effect is shown as mean value of % RET±SD.

Treatment		Variety				Mean of the means
		Simba	Yerer	Ude	Galama	
0.0 2,4-D	0.0 KIN	5.1±1.2	0.0±0.0	5.8±2.4	0.0±0.0	2.7[b]
0.05 mg/l 2,4-D	2.0mg/l KIN	3.3±1.9	23.8±5.5	0.0±0.0	0.0±0.0	6.8[b]
0.05 mg/l 2,4-D	3.0 mg/l KIN	0.0±0.0	29.2±4.1	0.0±0.0	0.0±0.0	7.3[b]
0.1 mg/l 2,4-D	0.0 mg/l KIN	41.3±13.3	41.6±14.4	0.0±0.0	0.0±0.0	20.7[a]
0.1 mg/l 2,4-D	1.0 mg/l KIN	20.6±10.6	0.0±0.0	0.0±0.0	0.0±0.0	5.2[b]
0.1 mg/l 2,4-D	2.0 mg/l KIN	0.04±0.0	0.06±0.0	0.0±0.0	0.0±0.0	0.0[b]
0.1 mg/l 2,4-D	2.0 mg/l BAP	0.042±0.0	9.0±5.0	5.6±2.4	0.0±0.0	3.7[b]
0.1 mg/l NAA	0.0 mg/l KIN	0.0±0.0	4.3±1.1	0.0±0.0	0.0±0.0	1.1[b]
0.1 mg/l NAA	1.0 mg/l KIN	28.0±11.0	25.9±9.8	17.6±6.7	26.6±13.3	24.5[a]
0.1 mg/l NAA	2.0 mg/l KIN	4.2±2.1	0.06±0.0	0.0±0.0	0.0±0.0	2.1[b]
0.5 mg/l IAA	1 mg/l BAP	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0[b]
Mean of the means		9.4[ab]	12.2[a]	2.6[b]	2.4[b]	

3:2:1 proportions of soil, compost and sand (Figure 1f). Each pot was covered with transparent plastic bags and kept in the growthroom for acclimatization. After 2 weeks, 11 plantlets of variety Yerer were transferred to glasshouse.

DISCUSSION

Determination of appropriate harvesting stage of spike

Sibi et al. (2004) have reported that better gynogenic res-

Table 6. The effect of genotypes on the percentage of responded embryonic tissues and regenerants from the total cultured ovaries of each genotype.

Variety	Total cultured unpollinated ovary	Number of embryonic tissue	Embryonic tissue (%)	Regenerant (%)
Simba	4732	462	9.8	0.55
Yerer	3360	158	4.7	0.12
Ude	2988	168	5.6	0.17
Galama	3444	312	9.0	1.10
Total	14, 524	1100	7.6	0.50

ponse was found when the spikes were harvested at bi- or trinucleate stage of microspores of wheat. Slama and Slim (2007) reported the highest regeneration frequency of gynogenic response from unpollinated ovary cultures of durum wheat. This was obtained when the microspore population was in late uninucleate to binucleate stages. These reports may be in consistent with stage II of the present study.

Effect of cold pretreatment durations on the formation of embryonic tissues

Seven days of cold pretreatment were taken as the optimum for the formation of embryonic tissues (Sibi et al., 2004). Slama and Slim (2007) have also reported that in durum wheat, 14 days pretreatment at 4°C gave the highest response of ovary development and callus induction. The above reports were in agreement with the present study. For all varieties, better number of gynogenic embryos were responded at 15 days of cold pretreatment (4°C) followed by 10 days.

Effect of culture media on the formation of embryonic tissues

In the present study, three types of induction media, MS, N_6, and B_5, were compared. From a total of 60, 40 and 40 ovaries cultured in MS, N6 and B5 media, the percentage of induced embryonic tissues were highest in MS medium (20.0 %) followed by N6 medium (5.0 %). In B5 medium, none of the enlarged ovaries induced embryonic tissues (0.0 %). MS medium supplemented with different combinations and concentrations of growth regulator has been commonly used as induction and regeneration medium in wheat crops. Immature embryo culture of durum wheat (Sears and Deckard, 1982; Satyavathi et al., 2004), spike and immature embryo cultures of bread wheat (Lin et al., 2006), and somatic embryo clulture of F1 hybrids of teff (Getahun et al., 2012) were some of them which are in agreement with the present study.

Effect of PGRs on the formation of embryonic tissues

The combination of kinetin and 2,4-D was evaluated for callus induction of immature embryo cultures of wheat (Jones et al., 2005). In immature embryo cultures of bread wheat, callus was initiated by 1 mg/l 2,4-D (Sears and Deckard, 1982). In seedlings, roots and stem explants of common wheat, callus growth was also vigorous when 0.5-2.0 mg/1 2,4-D was added (Shimada et al., 1969). In the present study, MS medium containing 1 mg/l 2,4-D in combination with KIN was the most effective PGR combination for all genotypes except for variety Galema.

The response of genotypes on the formation of embryonic tissues and regeneration

Unpollinated ovary cultures of durum wheat (Sibi et al., 2004), immature embryo cultures of durum wheat (Satyavathi et al., 2004), spike and immature embryo cultures of bread wheat (Lin et al., 2006) and unpollinated ovary cultures of durum wheat (Slama and Slim, 2007) and somatic embryo cultures of F1 hybrids of teff with its wild relatives (Getahun et al., 2012) revealed that plantlet regeneration was influenced by genotypes. In the present study, the embryonic tissue formation and regeneration of plants were affected by genotypes.

In anther cultures of durum wheat, from a total of 86,400 cultured anthers, 324 (0.38%) plants were obtained (Doramaci et al., 2001). Out of 324 plantlets, 248 were green plants (0.29 %) and 76 were albino plants (0.09%). In microspore cultures of bread wheat, 27.2%, were green and 72.8% were albino plants (Kim et al., 2003). In the present study, ovary cultures of three genotypes from the four wheat genotypes (75%) induced green plants which were better than the anther cultures of durum wheat (Doramaci et al., 2001). They induced a minimum and a maximum regenerants of 0.12 and 1.1% from ovary cultures of varieties Galema and Yerer, respectively. From a total of 14,524 cultured ovaries using four varieties, 75 regenerants (0.50 %) were obtained which were better than the work of Doramaci et al. (2001). All the plantlets were green and no albino plants were obtained. This is considered as an advantage over androgenic cultures reported by Doramaci et al. (2001) and Kim et al. (2003).

The effect of plant growth regulators on regeneration

In immature embryo cultures of wheat, shoots were initiated by reducing the 2,4-D from 1 to 0.1 mg/l. Complete plants were regenerated by transferring to 2,4-D free medium (Sears and Deckard, 1982) and somatic embryos of F1 hybrids of teff regenerated into plantlets in MS medium without plant growth regulators (Getahun et al., 2012). In the present study, the best response of plant regeneration was obtained from Simba and Yerer varietis on MS medium containing 0.1 mg/l 2,4-D. To some extent, these results are in agreement with the works of Sears and Deckard (1982) and Getahun et al. (2012).

Growth conditions of regenerants

Out of 18 green plantlets that were transferred into pots, 68.8% of the regenerants survived and 32.2% of the regenerants died in the growthroom. Out of 11 plantlets of variety Yerer, four plantlets (36.4%) survived in the glasshouse.The death of plantlets in the glasshouse was mainly due to high temperature of the glasshouse (38-40°C) as the glasshouse did not have temperature regulation system. As reported by Leone et al. (2006) the temperature of the glasshouse for wheat should have been between 21-25°C in Ethiopia.

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REFERENCES

Chu C (1978). The N_6 medium and its applications to anther culture of cereal crops. Pro. Symp. Plant tissue culture, Beijing 1978, Botson, pp. 43-50.

Dogramaci AM, Peterson S, Jauhar P (2001). Anther culture-derived regenerants of durum wheat and their cytological characterization. J. Hered. 92:56-64.

Gamborg O, Miller R, Ojim K (1968). Nutrient requirement of Suspension culture of soybean root cells. Explan. Cell Res. 50:151-158.

Getahun T, Feyissa T, Gugsa L (2012). Somatic embryogenesis and plant regeneration from embryo rescue cultures of F1 hybrids of teff with its wild relatives. Afri. Crop Sci. J. 20(3):189-196.

Gugsa L, Sarial K, Lörz H, Kumlehn J (2006). Gynogenic plant regeneration from unpollinated flower explants of teff [*Eragrostis tef* (Zuccagni) Trotter]. J. Plan. Cell Rep. 25:1287-1293.

Jauhar P (2006). Modern biotechnology as an integral supplement to conventional plant breeding. Am. Soc. Crop Sci. 46:1841-1859.

Jauhar P, Xu S, Baenziger S (2009). Haploidy in cultivated wheats: Induction and utility in basic and applied research. Crop Sci. 49:737-755.

Kim M, Baenziger S, Rybczynski J, Arumuganathan K (2003). Characterization of ploidy levels of wheat microspore-derived plants using laser flow cytometry. *In vitro* Cell. Dev. Biol. 39:663-668.

Leone E, Marin S, Barro F (2006). Establishment of a highly efficient regeneration system for *in vitro* culture of young wheat spikes. CSIC 25:580-583.

Lin SZ, Lu XL, Jing W, Qi CZ, Hui JG, Zhao SR, Wen HC (2006). Establishment of a highly efficient regeneration system for *in vitro* culture of young wheat spikes. Chin. J. Agri. Biotech. 3:147-154.

Liu W, Zheng Y, Polle A, Konzak F (2002). Highly efficient doubled-Haploid production in wheat (*Triticum aestivum* L.) via Induced Microspore Embryogenesis. Crop Sci. 42:686-692.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Plan. Physiol. 15:475-497.

Satyavathi V, Jauhar P, Elias M, Rao B (2004). Effects of Growth Regulators on *in Vitro* Plant Regeneration in Durum Wheat. Crop Sci. 44:1839-1846.

Sears G, Deckard L (1982). Tissue culture variability in wheat: Callus induction and plant regeneration. Crop Sci. 22:546-550.

Sibi M, Kobaissi A, Shekafandeh A (2004). Green haploid plants from unpollinated ovary culture in tetraploid wheat (*Triticum durum* Defs.). Euphyt. 22:351-359.

Shivanna R, Mohan RY (2005). Contributions of Panchanan Maheshwari's school to angiosperm embryology through an integrative approach. Curr. Sci. p.89.

Shimada T, Sasakuma T, Tsunewaki K (1969). *In vitro* culture of wheat tissues. Callus formation, organ redifferentiation and single cell culture. Genom. 11:294-304.

Slama AO, Slim AH (2007). Production of doubled haploids in durum wheat (*Triticum durum* Desf.) through culture of unpollinated ovaries. Plan. Cell Tiss. Org. Cult. 91:125-133.

Smale M, Aquino P, Crossa J, Toro E, Dubin J, Fischer T, Fox P, Khairallah M, Mujeeb KA, Nightingale K, Ortiz-Monasterio I, Rajaram S, Singh R, Skovmand B, van Ginkel M,Varughese G, Ward R (1996). Understanding Global Trends in the Use of Wheat Diversity and International Flows of Wheat Genetic Resources. Economics Working Paper 96-02. Mexico, D.F.: CIMMYT, p.61.

Tesemma T, Belay G (1991). Aspects of Ethiopian Tetraploid wheat with emphasis on Durum wheat Genetics and Breeding Research. In: wheat research in Ethiopia: A historical perspective, (Hailu GM, Tanner D, Mengistu H eds), IAR/CIMMYT, Addis Ababa. pp.47-71.

Waines G, Hegde G (2003). Intraspecific gene flow in bread wheat as affected by reproductive biology and pollination ecology of wheat flowers. Crop Sci. 43:451-463.