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Assessment of immature embryo culture to select for drought tolerance in bread wheat

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Key words: Bread wheat, drought tolerance, immature embryo culture, *in vitro* selection.

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Abstract

In order to evaluate the response of bread wheat genotypes to callus induction and *in vitro* drought stress, an investigation was carried out as a factorial experiment with a completely randomized design and five replications**.** The results of analysis of variance indicated significant differences between the entries and stress levels for callus relative growth (CRG), callus relative growth rate (CRGR), callus growth rate (CGR), percentage of callus chlorosis (PCCH) and percentage of callus water content (PCWC) indicating the presence of genetic variability, different responses of genotypes to different drought intensities and *invitro* selection of drought-tolerant genotypes. Mean comparisons between genotypes revealed that maximum CRG, CGR, CRGR, PCWG, PCCH and INTOL were attributed to genotypes 5, 16, 17, 2, 3 and 20 (drought tolerant), respectively. Graphic observation exhibited that indices of drought tolerance decreased with increase of PEG concentrations. Cluster analysis of genotypes (Ward's method) based on CRG, CGR, CRGR, PCWG, PCCH and INTOL and subsequent discriminant analysis for confirming the number of clusters, grouped the genotypes into four different clusters. The first group included genotypes 1, 3, 6, 7, 12, 13 and 18, the second group included genotypes 2, 4, 8, 9, 10 and 11 and the third group consisted of genotypes 14, 16, 17, 19 and 20, while the genotype 15 formed the fourth group. Superior genotypes 2, 5 and 16 showed drought tolerance at the callus culture level together with their high potential for callus induction leaded us to the conclusion that a hybridization breeding program using these superior plant materials supplemented with *in vitro* selection for drought tolerance might be beneficial for improvement drought tolerance in bread wheat.

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Introduction

Wheat (*Triticum aestivum* L.) is not only a main crop for more than one third of the earth population (Charkaz *et al.,* 2010) but also the most abundant sources of energy and nourishment for mankind(Debasis and Paramjit, 2003), as well as an important staple food crop, dominant grain of world commerce. In addition it is one of the most important cereal and the most critical agricultural crop worldwide (Fahmy *et al.,* 2006; Farshadfar *et al.*, 2013).

Conventional plant breeding has improved yield partly by increasing drought resistance of wheat. Plant biotechnology offer various valuable techniques including cell, anther, pollen, leaves, root, mature and immature culture etc., which improves the breeding methods to improve heritable characters including drought resistance in economical crops. Tissue culture creates a wide range of genetic variation in plant species, which can be combined in plant breeding programs. In addition by *in vitro* selection, mutants with useful agronomic traits, such as disease resistance, salt or water stress tolerance can be obtained in a short duration (Mercado *et al.,* 2000; Jain, 2001; El-Aref, 2002). The adaptation of wheat to a wide range of environmental conditions including drought remains a central objective of breeding and biotechnological programs. Environmental stresses give rise to water deficiency for the plants, thus damaging many biological roles (Sakthivelu *et al.,* 2008). Drought is one of the most common environmental limitations (Boyer, 1982), main factor in reducing growth, development and production of plants and cause significant yield reductions on presently cultivated land, together with major problem the cultivation of crops on arid and semiarid lands (Jain, 2001). Three mechanisms of the plant to water deficit includes drought escape, drought avoidance and drought tolerance (Moayedi *et al.,* 2010). Polyethylene glycol (PEG) of high molecular weights has been used for many years to stimulate water stress in plants (El-Shafey *et al.,* 2009; Kaufman and Eckard, 1971; Corchete and Guerra, 1986). In tissue culture of cereals including wheat, PEG is often used for evaluation of drought tolerance. As a new source of variability, screening somaclonal variation using immature embryo culture offers a great opportunity for drought tolerance in wheat breeding. El-Aref (2002) and Sakthivelu *et al.* (2008) proposed that *in vitro* breeding for water deficit might be conducted at callus culture level efficiently and also can be utilized as an effective tool to find drought tolerant genotypes. Immature embryos are common explants for the initiation of somatic embryogenesis. The first successful immature embryo culture in order to callus induction of wheat was reported by Sears and Deckard (1982). Patel *et al.* (2004), Shariatpanahi *et al.*(2006), Redha and Talaat (2008) and Tamas *et al.* (2004), investigated that the percentage of callus induction and plant regeneration in tissue culture of wheat were usually affected by the explants source, effect of genotype (Fennell *et al.,*1996 Filippov *et al.,* 2006) and effect of medium composition (Przetakiewicz *et al.,* 2003; Tamas*et al.,* 2004). In vitro selected plants with a significant improvement in drought tolerance were obtained in *Triticale* (Birsin and Ozgen, 2004), winter wheat (Ozgen *et al.,* 1998), Maize (El-Aref, 2002), Sorghum (Duncan *et al.,* 1995), wheat (Hsissou and Bouharmont, 1994; Almansouri *et al.,* 2001) and in Rice (Adkins *et al.,* 1995).

The objectives of the present investigations were to (i) screen bread wheat genotypes for drought tolerance under *in vitro* condition (ii) evaluate the ability of genotypes to induce callus using immature embryo culture and (iii) screening *in vitro* indicators of drought tolerance.

Materials and methods

Plant genetic materials

In order to evaluate the response of bread wheat (*Triticum aestivum* L.) genotypes (Table 1) to callus induction and *in vitro* drought stress, an investigation was carried out as a factorial experiment with a completely randomized design and five replications in the Campus of Agriculture and Natural Resource, Razi University, Kermanshah, Iran. Plants from these genotypes were grown in the field and the immature

kernels (14-15 days after pollination) were collected and brought to the laboratory for tissue culture procedure.

In vitro culture conditions

Seeds were disinfected for 1 min in a 70% ethanol, and then washed three times by sterile distilled water. Afterwards for 20 minutes by submerging in a 2% sodium hypochlorite solution, and then rinsed three times by sterile distilled water. Immature embryos were aseptically isolated and placed on the solid culture medium (callus induction medium) with the rounded scutellar side exposed and the flat plumule-radical axis side in contact with the medium. Callus was initiated and maintained on MS basal medium (Murashige and Skoog, 1962) supplemented with Fe-EDTA 1.00ml L^{-1}, 2.00mg L^{-1} glycine, 1.00mg L^{-1} L-asparagine, 1.00ml L^{-1} nicotinic acid, 1.00ml L^{-1} thiamine-HCl, 1.00ml L^{-1} pyridoxine-HCl, 5.2 mg L^{-1} 2,4-D, 30.00g L^{-1} sucrose, and 8.00 g L^{-1} Agar, pH 8.5. The cultures were incubated at 25°C with 16/8 photoperiods and sub-cultured after four weeks to the same medium. For identification of drought tolerant genotypes, the entries were exposed to different concentrations of polyethylene glycol (PEG 6000) (0, 5, 10, 15 and 20%) (Merck, Germany).

Data on immature embryo culture were collected and the following callus characteristics were measured under stress conditions:

Callus relative growth (CRG)

CRG = [LnW2-LnW1]/GP (Birsin and Ozgen, 2004)

Where W1 and W2 are the initial and final weight of callus and GP is the growth period, respectively.

Callus relative growth rate (CRGR)

CRGR = [(W2-W1)]/W1(Chen *et al.,* 2006)

Where W1 and W2 are the initial weight of callus before and after four weeks, respectively.

Callus growth rate (CGR)

CGR (mm/day) of cultured embryos on MS medium were measured at 7, 14, 21 and 28 days after transferring calli to medium.

Percentage of callus chlorosis (PCCH)

PCCH was determined visually as percentage of necrotic callus, 16 days after moving callus to the PEG containing medium.

Percentage of callus water content (PCWC) callus samples of known fresh weight were dried in an oven set at 70°C for 24 h and RWC was calculated by following formula (Errabii *et al.,* 2006):

PCWC = [(W2-W1)/W2] × 100

Where W2 and W1 are the callus dry weight and fresh weight, respectively.

In vitro tolerance (INTOL)

INTOL was calculated according to the following formula(Al-Khayri and Al-Bahrani, 2000)

INTOL=RGRtreatment / RGRcontrol

Where RGR = relative growth rate and was measured by the formula of Birsin and Ozgen (2004).

Statistical analysis

Analysis of variance, mean comparison using Duncan's multiple range test (DMRT), correlation analysis and cluster analysis were performed by MSTAT-C and SPSS ver. 16.

Results and discussion

Analysis of variance

In vitro research was evaluated to induce variability in the bread wheat genotypes using PEG-6000 stress. The culture responses were extremely influenced by the genotype in the immature embryo cultures. Therefore, in the tissue culture programs of wheat, genotypes should be chosen according to a high callus regeneration capacity. The results of analysis of variance for different characters (Table 2) in the stress medium indicated significant differences between the entries and stress levels for callus relative growth (CRG) (based on diameter mm/day), callus relative growth rate (CRGR) (based on fresh weight), callus growth rate (CGR), percentage of callus chlorosis (PCCH) and percentage of callus water content (PCWC) indicating the presence of genetic variability, different responses of genotypes to different drought intensities and *in vitro* selection of

drought-tolerant genotypes. As the effect of drought is quantitative different regression relations were calculated and separated into different regression degrees. The response of all characters to different drought levels was linear, but non-linear for CGR, PCCH and INTOL indicating that the response of these criteria to the increase of drought level will be constant or even decline in a specific level.

Table 1. Names of wheat genotypes used in the experiment.

Genotype No.	Name/pedigree
1	Pishtase
2	Croosalborz
3	Aazar-2
4	Sardari
5	Shi#4414.Crow"S".Fow-1
6	Ww33G.Vee"S".Mrn.3.Atilla.Tjn
7	Shi#4414.Crow"S".Vee"s:.Nac
8	CHAM-4DOVIN-2ICW93-0001-AP-OL-OBR-1AP-2AP-OAP
9	Ww33G.Vee"S".Mrn.4.HD2172.Bloudan.Azd.3.san.Ald"s".Avd
10	Zagross
11	Azd.HD2172.Kayson.Glenson.3.170-28.Ning8201
12	TEVEE S. KARAWAN S
13	Ww33G.Vee"S".Mrn.3.Atilla.Tjn
14	CHAM-8.MAYON"S'.CW93-0031-1AP-OL-OBR-2AP-1AP-OAP
15	Ns732.HER.Darab
16	T.AEST.SPRW"S".CA8055.3.BACANORA88.CW92-0477-...
17	TEVEE S. KARAWAN "S"ICW93-0073-1AP-OL-8AP-OL-...
18	URES.3.FURY.SLN.ALDAN"S".4.NS732.HER ICW93-0531-...
19	T.AEST.SPRW"S".CA8055.3.BACANORA88ICW92-0477-...
20	AZD.HD2172.Pltoma.Cucurp88

The stress × genotype (G × S) interaction was significant for CRG, CGR, PCCH and PCWC except for CRGR displaying different responses of characters to different levels of drought(PEG), while CRGR was stable and independent of different drought levels. El-Aref (2002) reported a significant difference between maize genotypes for the same characteristics.

Table 2. Analysis of variance of evaluated traits on immature embryo calli under drought stress conditions.

S.O.V	DF	Mean squares					
		CRG	CRGR	CGR	PCCH	PCWC	INTOL
Genotype(G)	19	23.045**	0.004**	0.020**	73.288**	176.582**	0.027*
Stress(S)	4	375.726**	0.439*	0.097**	2303.073**	1019.025**	1.677**
Linear(degree 1)	1	1316.874**	0.654**	0.174**	4929.021**	3593.062**	4.311**
degree of 2	1	26.615ns	0.0003ns	0.134**	2547.527**	0.620ns0.277**	
degree of 3	1	84.654**	0.129**	0.050**	1379.214**	5.893ns0.060ns	
degree of 4	1	6.759ns	0.002ns	0.030**	356.530**	476.526*	-
G × S	76	11.076*	0.002ns	0.005**	28.206**	160.132**	0.013ns
Error	200	7.519	0.002	0.002	4.197	76.513	0.015
Total	300	-	-	-	-	-	-
CV%		11.82	6.38	19.71	4.172	11.178.03	

*; ** : Significant at the *0.05 and **0.01 level of probability; MS= Mean square; CRG= Callus relative growth; CRGR= Callus relative growthrate; CGR= Callus growth rate; PCCh = Percentage of callus chlorosis; PCWC= Percentage of callus water content.

Means comparison

Mean comparisons between genotypes (Table 4) indicated that maximum CRG, CGR, CRGR,PCWG, PCCH and INTOL were attributed to genotypes 5, 16, 17, 2, 3 and 20 (drought tolerant),respectively. While the lowest amount of CRG, CGR, CRGR, PCWC, PCCH and INTOL were belonged to genotypes 13, 10, 15, 4, 15 and 15 (drought sensitive), respectively. Therefore, because of the genetic variability between drought tolerant and drought sensitive genotypes they can be used as parents for the genetic analysis of *in vitro* indicators of drought tolerance using diallel mating design and mapping quantitative trait loci (QTLs) using molecular markers.

Table 3. Means comparison of wheat genotypes on immature embryo calli under drought stress.

Genotypes	CGR	CGR	CRGR	PCWC	PCCP	INTOL
1	0.214 c-e	4.927 c	0.057 ab	81.698 ab	50.667 bc	0.190 c
2	0.309 a	10.545 a-c	0.092 ab	83.258 a	45.172 b-e	0.298 bc
3	0.199 c-f	4.355 c	0.064 ab	81.524 ab	62.093 a	0.286 bc
4	0.286 ab	11.007 a-c	0.099 a	67.753 ab	45.662 b-e	0.371 bc
5	0.289 ab	14.851 ab	0.103 a	77.839 ab	45.207 b-e	0.342 bc
6	0.236 c-e	5.794 bc	0.078 a-b	80.582 ab	39.333 e-g	0.333 bc
7	0.198 d-f	4.748 c	0.064 ab	76.350 ab	41.422 d-e	0.235 c
8	0.233 c-e	6.003 bc	0.074 ab	74.372 ab	41.400 d-e	0.273 bc
9	0.204 c-f	3.787 c	0.060 ab	72.184 b	51.789 b	0.322 bc
10	0.240 cd	3.047 c	0.047 b	73.768 ab	50.333 b-c	0.335 bc
11	0.233 c-e	3.509 c	0.072 ab	83.103 a	40.111 d-f	0.624 ab
12	0.227 c-e	7.210 bc	0.081 ab	78.921 ab	47.133 b-d	0.300 bc
13	0.186 e-f	7.735 a-c	0.069 ab	79.320 ab	39.067 e-g	0.224 b-c
14	0.249 bc	6.436 bc	0.069 ab	79.904 ab	47.067 b-d	0.235 c
15	0.266 ef	3.727 c	0.044 b	72.474 b	33.333 g-h	0.094 c
16	0.230 c-e	16.901 a	0.106 a	82.129 ab	44.665 c-e	0.313 bc
17	0.165 f	4.898 c	0.281 ab	76.333 ab	34.200 f-h	0.281 bc
18	0.221 c-e	9.154 a-c	0.087 ab	81.185 ab	45.933 b-e	0.419 bc
19	0.204 c-f	5.898 bc	0.089 ab	78.373 ab	44.200 c-e	0.430 bc
20	0.269 d-f	12.144 a-c	0.086 ab	76.148 ab	32.067 h	0.842 a

*Means followed by similar letters in each column are not significantly different.

Various amount of CRG, CGR, CRGR, PCWG, PCCH and INTOL in different genotypes exhibited that the characters measured were genotype dependent. Similar results were found by Zouzou *et al.* (2008) who reported that, response in tissue culture such as callus initiation is highly genotype dependent. Sakthivelu *et al.* (2008) reported that a meaningful decrease in the relative growth rate of callus cultures was observed for soybean cultivars, with increasing PEG concentrations to the MS medium.

Table 4. Drought levels comparison of data mean.

Drought level	CRG	CGR	CRGR	PCWC	PCCP	INTOL
0%	0.272 a	16.51 a	0.166 a	82.83 a	9.183 c	-
5%	0.258 a	14.34 a	0.159 a	82.28 a	53.76 a	1.001 a
10%	0.184 c	5.447 b	0.070 b	76.24 b	49.53 b	0.489 b
15%	0.188 c	0.218 c	-0.004 c	76.99 b	52.71 a	-0.029 c
20%	0.223 b	0.151 c	0.013 c	73.24 b	55.03 a	-0.108 c

*Means followed by similar letters in each column are not significantly different.

Mean comparison for the effect of different stress (PEG) levels (Table 3) indicated that the effect of stress level on CRG, CGR, PCWC and INTOL were decreased with increment of drought percentage. Likewise, El-Aref (2002) reported that these characters reduced while studying somaclones

achieved from calli immature embryo culture of maize under drought stress with PEG. CRGR and INTOL were minimum at 15% level and became stable (no effect) at 20%. The reason for this may be due to the reduction of osmotic potential of the environment. Maximum PCWC was observed at 0% and minimum at 20% PEG level. Biswas *et al.* (2002)and Al-Khayri and Al-Bahrany (2000) reported that increasing the levels of PEG(0-30%) reduced CRGR, PCWC and INTOL in date palm (*Phoenix dactylifera* L.). Turhan and Baser (2004) and Lutts *et al.* (2004) also reported the same results in bread and durum wheat and Sakthivelu *et al.* (2008) in soybean, respectively, which is in consistent with the results of this experiment.

Table 5. Simple correlation coefficient matrix of wheat genotypes in water stress conditions to indices and some of characteristics related to drought tolerance.

Characteristics	Correlation coefficients between characteristics				
	1	2	3	4	5
1. CRG	1	870.00**	-123.00^{ns}	496.00*	259.00^{ns}
2. CRGR		1	-077.00^{ns}	358.00^{ns}	414.00^{ns}
3.PCCH			1	-129.00^{ns}	-242.00^{ns}
4.CGR				1	173.00^{ns}
5.INTOL					1

Significant at the *0.05, **0.01 level of probability and ns is non-significant.

Graphic interpretation of in vitro drought tolerance indicators

Graphic observation (Fig. 1) revealed that indices of drought tolerance decreased with increase of PEG concentrations (Fig. 1A). Osmotic stress due to PEG application significantly decreased the fresh weight of calli in response to 5 and 20% PEG, as compared with the control (Fig. 1A). The decrease in the growth of wheat calli, as a result of treatment with PEG, is consistent with those found in sunflower and maize (Navari-Izzo *et al.*, 1990). RuiqinBai *et al.*, (2011) reported that the salt tolerance indices decreased with increasing salt concentrations.

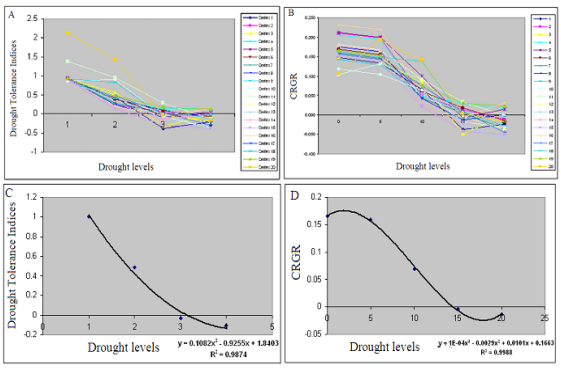

Fig. 1. Drought tolerance indices (A) and Callus relative growth rate or CRGR (B) of bread wheat genotypes in drought different levels.Regression graph of Drought tolerance indices (C) and Callus relative growth rate (D) in drought different levels.

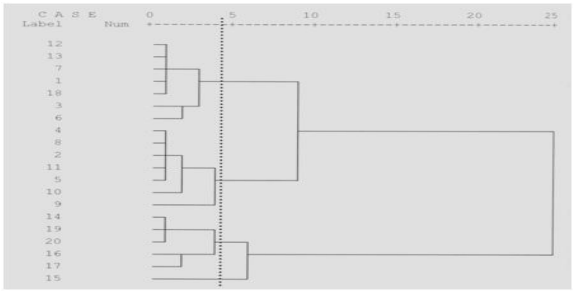

Fig. 2. Dendrogram resulting from cluster analysis of bread wheat genotypes based on *in vivo* characteristics of callus induction under stress condition.

Correlation analysis

Correlation coefficient analysis (Table 5) showed significant positive correlation between CRG with CRGR and CGR. Similarly Zouzou *et al.* (2008) reported in cotton that callus percentage is positively correlated with dry weight of callus. Chlorosis percentage had negative correlation with callus chlorosis percentage. No significant correlation coefficients was detected between the other criteria.

Similar results were reported by Arzani *et al.* (1999). In contrast, Birsin *et al.* (2004) reported negative correlation among percentage of callus induction with callus weight and culture efficacy, also between regeneration percentage and number of regenerated plants.

Cluster analysis of in vitro characteristics

Cluster analysis of genotypes (Ward's method) based on CRG, CGR, CRGR, PCWG, PCCH and INTOL and subsequent discriminant analysis for confirming the number of clusters grouped the 20 genotypes into four different clusters. The first group included genotypes 1, 3, 6, 7, 12, 13 and 18, the second group included genotypes 2, 4, 8, 9, 10 and 11 and the third group consisted of genotypes 14, 16, 17, 19 and 20, while the genotype 15 formed the fourth group (Fig. 2).Within-group genotypes show similar minimum variance and genetic distance, while between-group genotypes are dissimilar with maximum genetic distance. Genotype 4 (Sardary) is a drought-tolerant landrace of Iran and is located in group 2, hence genotypes of group 2 are considered to be drought-tolerant at the *in vitro* level. Superior genotypes2, 4, 5, 8, 9, 10 and 11 showed drought tolerance at the callus culture level together with their high potential for callus induction lead us to the conclusion that a hybridization breeding procedure using these superior plant materials supplemented with *in vitro* selection for drought tolerance might be beneficial for improving this trait in bread wheat. Embryo culture can be useful to speed up wheat improvement, especially the material to be advanced more aggressively, such as, for transferring genes, determining inheritance, producing F_1 hybrid plants and following the single seed descent method (Konieczny *et al.*,2003; Bajji *et al.,* 2004; Yadav *et al.,* 2004; Wu *et al.,* 2005; Gawande *et al.,* 2006; Yao *et al.,* 2007).

Conclusion

In conclusion, genotype was one of the main significant factors for successful callus induction percentage from immature embryos of wheat under drought stress.Callus induction varied from 10 to 100% in different genotypes, therefore the different genotypes had different response to the PEG concentration, hence callus induction is genotype dependent and can be considered as an index for *in vitro* screening drought tolerant plants. On the other hand, the genotypes with the lowest callus relative growth under medium drought stress could be categorized as low-tolerant to drought at the cellular levels. In addition, means comparisons of drought different stress levels indicated that increase of PEG concentrations up to 10% reduced the *in vitro* parameters but higher levels tend towards stability and growth was almost stopped. The reason for this may be reduction of osmotic potential of the environment. Superior genotypes 2, 5 and 16 showed drought tolerance at the callus culture level together with their high potential for callus induction lead us to the conclusion that a hybridization breeding procedure using these superior plant materials supplemented with *in vitro* selection for drought tolerance might be beneficial for improving this trait in bread wheat. Thus, it is obvious that *in vitro* selection can be used as an effective tool to screen a large number of genotypes to water deficit. More investigations such as field and hydroponic conditions studies are needed to corroborate this thought. However, we suggest that breeders do not generally select for specific traits to improve yield under drought principally because drought is unpredictable from year to year and this also means that the physiological responses to drought are also complex and unpredictable

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