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In vitro Evaluation of Bread Wheat (*Triticum aestivum* L.) Genotypes for Drought Tolerance

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Abstract: **Bread wheat (Triticum aestivum L.) is one of the most important food crops cultivated in a wide range agro-ecologies of Ethiopia. Drought remains an ever-expanding problem that has adverse effects on growth and crop production globally, and demands integration of biotechnological and conventional approaches. Somaclonal variations induced by tissue culture can be used for improving drought tolerance of bread wheat. To evaluate drought tolerance, a factorial experiment using 5 levels of polyethylene glycol (PEG) 6000 and 3 bread wheat genotypes was carried out in the tissue culture laboratory of Mekelle Agricultural Research Center. Highly significant effects were observed due to genotypes, PEG levels used and their interactions for traits such as regeneration percentage (PRP), shoot number (SN), shoot length (SL), root number per shoot (RPS) and root length (RL). This indicated the presence of genetic variability and differential responses of genotypes to PEG simulated drought. The results obtained from the mean comparisons of genotype by PEG interaction showed that increasing PEG concentration in the medium significantly reduced the above measured traits. The current study suggests that genotypes G3 (SHARP/3/PRL/SARA//TSI/VEE#5/5/VEE/ LIRA//BOW/3/BCN/4/KAUZ/ 6/HUBARA-5) and G2 (NESMA*2/14-2//2*SAFI-3/4/PASTOR//HXL7573/2*BAU/3/ WBLL1) were relatively drought tolerant genotypes, while genotype G1 (FLAG-6/ICARDA-SRRL-6) appeared to be drought sensitive at the highest level of PEG concentration.**

Keywords: **Callus, Drought stress, Leaf culture, PEG-6000, Regeneration.**

1. INTRODUCTION

Bread wheat is one of the most important agricultural commodities grown all over the world. It is an exotic crop cultivated in a wide range of agro-ecology of Ethiopia. Despite the significant dietary and ecological importance, its productivity in Ethiopia is far below its potential which is 3 ton ha^{-1} (CSA, 2021). Recurrent drought is among the major yield limiting factors of wheat production and productivity in Ethiopia. This calls for a concerted research efforts towards the development of improved cultivars with adaptability to marginal rainfall conditions coupled with appropriate management practices.

Screening for drought tolerance under field conditions involves considerable resources (land, power and suitable environmental conditions) for effective and repeatable phenotypic expression of drought tolerance attributable to the genotype. However, advances in tissue culture techniques, especially callus culture have widened the scope of physiological crop improvement, and is an alternative tool for developing stress-tolerant cultivars with limited time, space and cost (Mahmood *et al*., 2014). In *in vitro* cultures, some regenerants appeared to be no longer precise clonal copies of their parents. The genetic or phenotypic variations among such plants are termed as somaclonal variations, and sometimes provide a very important source of genetic variations for desired traits including drought tolerance, disease resistance, improved quality, quantity and yield parameters. Culture, besides its use as a tool for obtaining drought tolerant plants, may offer potential for quick evaluation of germplasm against drought.

Natural variations for drought tolerance existing among cell lines can be exploited in the presence of suitable concentration of osmoticum and stress duration. In addition, improvements in tissue culture techniques had widened the genetic variability in crops and are clearly a mutagenic procedure (Afrasiab and Iqbal, 2012). Single gene mutation, aneuploidy, transposable elements, cytogenetic changes and DNA methylation are considered as some of the possible causes of somaclonal variations (Obute *et al*., 2007). The success of culture of wheat and other monocot plants depends mainly on genotypes, explant source, the culture medium and the interactions between these factors. Among these factors, the genotype appears to be important factor influencing the efficiency of culture. The choice of a suitable selection agent with appropriate selection pressure and stress duration is a prerequisite to screen drought tolerant callus lines, and subsequently regenerate them into drought tolerant somaclones (Mahmood *et al*., 2012; Verma *et al*., 2013). Polyethylene glycol, (PEG-6000), a non-ionic, non-penetrating and non-toxic water soluble polymer of high molecular weight (mol.wt. 6000) has long been used for this purpose. It lowers the water potential of the nutrient medium similar to soil drying without being taken up by the plants or being phytotoxic, and has been used effectively to explore somaclonal variations for the improvement of crops like wheat (Kacem *et al*., 2017), rice (Verma *et al*., 2013) and sorghum (Yohannes *et al*., 2014) against drought stress. Therefore, the objective of the present study, , were to evaluate bread wheat genotypes for drought tolerance under *in vitro* conditions using different doses of PEG; and to identify the optimum PEG concentaration for *in vitro* drought tolerance screening of bread wheat

2. MATERIALS AND METHODS

Plant Materials

The experiment was carried out on three bread wheat (*Triticum aestivum* L.) genotypes selected from previous field drought screening experiments. The genotypes were classified as drought sensitive G1 (FLAG-6/ICARDA-SRRL-6), moderately drought tolerant G2 (NESMA*2/14-2//2*SAFI-3/4/PASTOR//HXL7573/2*BAU/3/WBLL1) and drought tolerant G3 (SHARP/3/PRL/SARA//TSI/VEE#5/5/VEE/LIRA//BOW/3/BCN/4/KAUZ/6/HUBARA-5).

Experimental Treatments and Design

The experiment was conducted at the Tissue Culture laboratory of Mekelle Agricultural Research Center in 2018..The treatments comprised entire factorial combinations of the three bread wheat genotypes described above and five levels of polyethylene glycol (PEG 6000) drought stress intensities of (0, 5, 10, 15 and 20% (w/v)). The treatments were laid out using completely randomized design (CRD) with six replications

Experimental Procedure

Mature seeds were surface-sterilized for 5 min in 70% ethanol and rinsed with sterile distilled water for five times and kept in 5% sodium hypochlorite supplemented with 1 to 2 drops of tween for 20 for 20 minutes, followed by five washes in sterile distilled water. The sterilized seeds were transferred to glass jars of 250 ml capacity containing 40 ml of solidified basal MS medium (Murashige and Skoog,1962) supplemented with 30 g/l sucrose and 8 g agar. After 5 days, young seedling basal leaves were used for callus induction.

Six explants of basal leaf about 3-4 mm long were aseptically excised and then incubated on MS callus induction medium supplemented with 2 mg l^{-1} 2.4-dichlorophenoxyacetic acid (2.4-D), 8 g agar and 30 g sucrose for 5 weeks. Afterward, induced calli were sub-cultured to callus multiplication media the same as callus induction medium for another period of 5 weeks until enough callus material was obtained to initiate the drought stress treatment stage. The calli obtained were then transferred to the same medium as callus induction containing different concentrations of PEG (0, 5, 10, 15 and 20% (w/v) for four weeks. For shoot regeneration, the calli were transferred into glass jar containing MS basal salt supplemented with 1.0 mg l^{-1} Kinetin for about 6 weeks. Rooting was initiated on half strength MS media supplemented with 1.0 mg l^{-1} indole-3- butyric acid (IBA) for about 5 weeks. The culture media were refreshed every 16 to 21 days.

All the operations and inoculation were performed under aseptic conditions in a laminar airflow cabinet. The pH value of the medium was adjusted to 5.8 prior to autoclaving at 121 ˚C for 20 min. The cultures were maintained in a growth chamber under 16:8 h (light: dark) photoperiod regime with light intensity of 2000 - 2500 lux provided by Mahtab, Iran, 40 W white bulbs at 25 ˚C ± 2 ˚C and 80% relative humidity (RH). Rooted plantlets were taken out from the culture bottles and washed gently with sterile water to remove the adhering medium completely. Thereafter, they were transferred to pots containing a mixture of garden soil and sand (2:1) for acclimatization in a greenhouse. The plantlets were covered with transparent polyethylene bags to prevent desiccation.

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Data Collected

The following characteristics were recorded for all the treatments.

1) Callus induction efficiency (CIF) was recorded as the number of calli induced divided by the total number of cultured explant and expressed as per cent

2) Plant regeneration (%) (PRP) was recorded as number of plantlets obtained divided by the total number of calli and expressed as per cent

3) Total number of shoot per culture (SN) was recorded as the average number of shoots counted per culture

4) Shoot length (cm) (SL) was measured using an autoclaved square paper and a well sterilized measuring tape

5) Root length (cm) (RL)was measured using an autoclaved square paper and a well sterilized measuring tape.

6) Number of roots per shoot (RPS) was taken as the average number of roots counted per shoot

7) Rooting percentage was recorded as the number of rooted shoots obtained divided by total number of shoots transferred to rooting medium after five weeks.

8) Survival Percentage: The survival % was recorded as the percent of survival plants after four weeks of transfer to pot experiment

Data Analyses

Analysis of variance of data and mean comparisons with LSD test were done using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS) software 9.2 . Principla component analysis was done using XLSTAT (2014).

3. RESULTS AND DISCUSSION

Callus Induction

Callus induction was observed following five weeks of culture. The analysis of variance (Table 1) revealed the presence of highly significant differences ($P \leq 0.01$) among the genotypes for mean callus induction percentage. This indiactes the presence of genetic variation or different responses of genotypes in callus induction and possibility of selection for callus induction in bread wheat using young seedling basal leaves. Although young seedling basal leaves are readily available at all times, they are the least to be used for regeneration trials due to the low callus induction frequency obtained with these explants.

Mean comparison for the genotype (Table 1) indicated that the range of callus induction frequency was between 66.97 and 51.53%. G3 possessed the highest callus induction frequency (66.97%). In contrast, G2 (54.9%) and G1 (51.53%) possessed lower values. These results confirmed that callus induction is genotype dependent. Culture response was greatly influenced by the wheat genotypes and also emphasized a marked effect of genotypes on callus induction capacity, which is in agreement with reports of callus induction in wheat (Yadav *et al.*, 2000; Farshadfar *et al.*, 2012)

Table 1. Analysis of variance and mean comparisons for callus induction percentage of three bread wheat genotypes

Source of variation	DF	Mean squares	Mean comparisons	
			Genotypes	Mean
Genotype	2	394.3950617**	G1	51.53[b]
Error	15	11.5675926	G2	54.97[b]
CV (5.88%)			G3	66.972[a]
Mean (57.82)				

**: significant at 1% level of probability

Drought Tolerance

Highly significant differences were observed among the genotypes, PEG concentrations (0, 5,10 ,15 and 20% w/v) and their interactions for plant regeneration percentage (PRP), number of shoots per culture (SN), shoot length (SL), number of roots

per shoot (RPS) and root length (RL). This indicated diffrences among the genotypes and also differential responses of the bread whet genotypes to PEG-simulated drought stress. Highly significant difference was observed over the drought stress levels for rooting percentage (R%), while survival percentage (S %) was stable and independent of different genotypes and drought levels (Table 2). Ehsaneh *et al*. (2014) found significant difference of genotype by PEG interactions for number of roots, root length, shoot length and regeneration percentage in wheat genotypes Similar findings were reported between sorghum genotypes for the same traits (Yohannes *et al.*, 2014).

Table 2. Mean squares from the nalyis of variance of in vitro culture responses of bred wheat genotypes and PEG levels

Source of variation	Mean Square							
	DF	PRP	SN	SL	RPS	RL	R%	S%
Genotype	2	1436.83**	6.10**	8.23**	20.71**	7.21**	124.74^{ns}	19.12^{ns}
PEG	4	4280.64**	12.14**	22.34**	1.41**	4.35**	1212.20**	141.82^{ns}
Genotype *PEG	8	115.36**	0.40**	0.25**	0.21**	0.10**	55.71**	120.65^{ns}
Error	17	29.319	0.02	0.06	0.04	0.02	220.01	270.71
CV		11.12	5.81	3.90	4.56	3.63	19.65	17.77
Mean		48.7	2.53	6.44	4.14	4.73	75.48	92.56

ns: Non-significant, **: highly significant, PRP: regeneration percentage, SN: number of shoot per culture, SL: shoot length, RPS: number of roots per shoot, RL: root length, R%: rooting percentage and S%: survival percentage.

The results obtained from the mean comparisons of genotype by PEG interaction (Table 3) showed that increasing PEG in the medium significantly reduced the plant regeneration frequency. The highest plant regeneration frequency (83.33%) was recorded for calli grown on the control medium (MS without PEG) of G3 and decreased gradually to 11.11% for G1 and 19.45% for G2 in medium with 20% PEG. No significant difference was observed between the control and 5% PEG concentrations for the tested genotypes indicating that 5% PEG concentrations was not effective to select resistant cell lines. However, MS medium supplemented with 10%, 15% and 20% PEG showed the highest effect for all the genotypes except G3 at 10% PEG. A parallel decrease in plantlet regeneration with increasing *in vitro* osmotic stress were reported in wheat (Imran *et al*., 2012) and rice (Wani *et al*., 2010; Anindita *et al*., 2018).

The highest values of plant regeneration percent were observed for G3 with means of 83.33%, 72.22%.and 69.44% under the control, 5% and 10% PEG, respectively and for G2 with means of 77.78% and 69.45% under the control and 5% PEG, respectively (Table 3). At 15% PEG, G3 gave the highest value (38.89%) followed by G2 (36.11%), whereas relatively the lowest value was recorded for G1 genotype (30.55%). At 20% the highest plant regeneration was recorded for G3 (27.78%) followed by G2 (19.45%), indicating that these genotypes were more tolerant to high concentrations of PEG . whereas the lowest was recorded for G1 (11.11%) which indicated the relative susceptibility of this genotype to PEG. A decrease in PRP is a typical response of explants of crop genotypes when subjected to PEG stress, and a parallel decrease in plantlet regeneration with increasing osmotic stress was reported in wheat (Hemaid and Mohamed, 2013; Mandour *et al.*, 2015), rice (Wani *et al.*, 2010) and sorghum (Yohannes *et al.*, 2014).

The interaction effects of genotypes and PEG concentrations on number of shoots varied significantly (Table 3). All the genotypes regenerated the highest number of shoots in the control compared to all the PEG levels. The highest number of shoots per callus was noted for G3 (4.77) followed by G2 (4.75) and G1 (2.9) under the control treatment. The differences in the performances of the genotypes under the control might be due to genetic variability of the genotypes. Increasing level of PEG from 0 to 15% had lowest effect on the mean number of shoots per callus for G3 indicating the relative tolerance of this genotype for drought stress. In contrast, the highest effect of PEG was observed on G1 which indicated the relative susceptibility of this genotype to PEG. The number of shoots decreased significantly with increasing levels of PEG supplemented into the medium. Begum *et al*. (2011) in sugarcane and Berhan *et al*. (2016) in cactus found similar reduction in number of shoots with increasing concentration of PEG.

Regarding the shoot length, the mean comparisons for the genotype by PEG interaction showed significant differences among the genotypes. The shortest shoot length was observed for G2 and G1 from medium containing 25% PEG, whereas G3 had the longest shoot length. At 15% PEG stress, the longest shoot was produced in G3 followed by G2. With increasing PEG levels, the length of the shoots decreased significantly. Likewise, Githinji *et al*. (2016) in wheat, Yohannes *et al*. (2014)

in sorghum and Berhan *et al*. (2016) in cactus reported similar findings in relation to the reduction in shoot elongation. Reduction in shoot length in cereal crops is mostly linked to drought tolerance (Bibi *et al.*, 2012).

The trend in the number of roots were different for all the genotypes. Genotype G2 possessed higher value for number of roots per shoot under all PEG concentrations (Table 3). In contrast, G1 possessed lower values for the same trait under all PEG concentrations. The number of roots per shoot was highest in G2 (5.29) at the control medium followed by G2 at 5% PEG, G2 (5.03) at 15% and G3 (5.02) at the control. The lowest was recorded in G3 with means of 3.12, 3.07, 2.87, 2.83 and 2.23 from control to 20% PEG levels, respectively, while the reduction in this genotype due to PEG stress level was not significant. At 20% PEG, the number of roots per shoot for G2 and G3 were 4.83 and 3.37, respectively. The number of roots per shoot decreased with increasing levels of PEG. Berhan *et al*. (2016) found similar results in cactus.

Genotypes and PEG interacted significantly on root lenth reponses. Root length ranged between 6.13 cm and 4.64 cm for genotypes G3 and G1 at PEG free (control), respectively (Table 3). At 5% PEG concentration, the genotypes did not exgibit varying root length as compared to control, but at 10% PEG the root length decreased. Moreover, at 15% PEG level the root length also declined for G3 and G2 genotypes with the longest root recorded for G3 (4.87 cm) followed by G2 (4.83 cm), while no significant difference was observed among them. The 20% PEG level affected more to G1 as compared to G2 and G3. The extent of root development is closely related to the ability of the plant to absorb water and the tolerant genotypes have higher capacity of these character. The findings of the present study are in line with earlier studies where severe water stress reduced root length in cereals (Kamran *et al.*, 2009; Ali *et al.*, 2007).

The regenerated plantlets were successfully rooted. Highly significant differences among the PEG levels for rooting percentage were observed. However, no significant effect was observed due to the interaction between the genotypes and PEG levels and among the genotypes. An overall mean of 75.48% rooting percentage was observed (Table 3). For acclimatization, derived plantlets were removed from rooting medium after 6 weeks of incubation and transferred to plastic pots containing autoclaved soil and covered with transparent polyethylene to maintain humidity. The acclimatization procedures applied was successful, and 92.56% of the plants survived.

Table 3. Effect of genotype x PEG interaction on plant regeneration percentage (PRP), number of shoot per culture (SN), shoot length (SL), number of roots per shoot (RPS) and root length (RL)

Genotype	PEG level	PRP	SN	SL	RPS	RL (cm)
G1	Control	55.55^{c}	2.9^{de}	7.23^{c}	3.12^{ef}	4.64ef
	5%	50^{c}	2.1^{f}	7.33^{bc}	3.07^{ef}	4.57^{f}
	10%	38.89^{d}	1.9^{f}	5.53^{f}	2.87^{f}	4.03^{g}
	15%	30.55^{de}	1.13^{g}	4.63^{g}	2.83f	3.53^{h}
	20%	11.11^{g}	1^{g}	3.87^{h}	2.23^{g}	2.97^{i}
G2	Control	77.78^{ab}	4.57^{a}	7.67^{b}	5.29^{a}	5.45^{b}
	5%	69.45^{b}	3.5^{bc}	7.6^{bc}	5.27^{ab}	5.43^{b}
	10%	52.78^{c}	2.73^{e}	6.73^{d}	4.97^{bc}	5.07^{cd}
	15%	36.11^{de}	1.83^{f}	5.83^{ef}	5.03^{abc}	4.83^{def}
	20%	19.45^{fg}	1.13^{g}	4.13^{h}	4.83^{cd}	4.2^{g}
G3	Control	83.33^{a}	4.77^{a}	8.9^{a}	5.02^{abc}	6.13^{a}
	5%	72.22^{b}	3.7^{b}	8.8^{a}	4.9^{c}	6.03^{a}
	10%	69.44^{b}	3.23^{cd}	7.33^{bc}	4.8^{cd}	5.22^{bc}
	15%	38.89^{d}	2.6^{e}	6.17^{e}	4.53^{d}	4.87^{de}
	20%	27.78^{ef}	1.2^{g}	4.8^{g}	3.37^{e}	4.03^{g}

Principal Component Analysis

The results of the principal components (PCs) analysis of the drought- tolerance indices and five traits of the three bread wheat genotypes are presented on Table 4. Principal component analysis (PCA) was performed to assess the relationships between all the attributes so as to identify superior genotypes for stress conditions. The two PCs explained 87.97 % of the total variation of the standardized data. PC1 alone explained 75.01% of the total variation with high contribution due to drought sensitivity index (DSI) for shoot length (7.04%), shoot length reduction due to stress (7.08%), root length reduction due to stress (7.61%), plant regeneration percentage (7.51%), shoot number (7.01%), shoot length (7.62%) and root length (7.76%)). PC2 explained 12.96%% of the gross variation with high contribution due to drought sensitivity index for shoot number (15.30%), shoot number reduction due to stress (13.75%) drought sensitivity index for number of roots per shoot (11.16%), drought sensitivity index for root length and numper of roots per shoot (12.34%).

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Table 4. Eigenvectors, eigenvalues and proportion of the total varience explained by two principal component for 15 traits of 3 bread wheat genotypes tested under PEG 6000 (5%, 10%, 15% and 20% w/v) stress

Traits	PC1	PC2
PRP DSI	-0.859 (6.55%)	-0.383 (7.53%)
PRP RID	-0.847 (6.37%)	-0.143 (1.05%)
SN DSI	-0.803 (5.74%)	0.545 (15.30%)
SN RID	-0.825 (6.05%)	-0.517(13.75%)
SL DSI	-0.890 (7.04%)	0.413 (8.78%)
SL RID	-0.893 (7.08%)	-0.375 (7.22%)
RPS DSI	-0.838 (6.24%)	0.466 (11.16%)
RPS RID	-0.847 (6.37%)	-0.143 (1.05%)
RL DSI	-0.860 (6.58%)	0.478 (11.77%)
RL RLD	-0.925 (7.61%)	-0.215 (2.38%)
PRP	0.919 (7.51%)	0.210 (2.28%)
SN	0.888 (7.01%)	0.183 (1.72%)
SL	0.926 (7.62%)	0.224 (2.57%)
RPS	0.711(4.49%)	-0.490 (12.34%)
RL	0.935 (7.76%)	-0.147 (1.11%)
Eigenvalue	11.25	1.94
Variability (%)	75.01	12.96
Cumulative %	75.01	87.97

Biplot based on principal component analysis was was employed to identify superior genotypes for stress environments (Figure 1). It showed highly significant positive association between RL and RPS and among SL, SN and PRP, as indicated by the acute angles.. These traits could be used to select and identify genotypes with high tolerance to PEG 6000 simulated stress under *in vitro* culture. Based on RL and RPS, G2 at 10% and15% and G3 at 15% PEG 6000 levels were drought tolerant genotypes. Considering SL,SN and PRP G3 at 5% and 10%, G2 at 5% and G1 at 5% PEG concentrations were drought tolerant genotypes. In contrast G1 at 10%, 15% and 20% PEG levels was drought sensitive genotype, whereas G2 and G3 were drought tolerant at the highest (20%) PEG concentration.

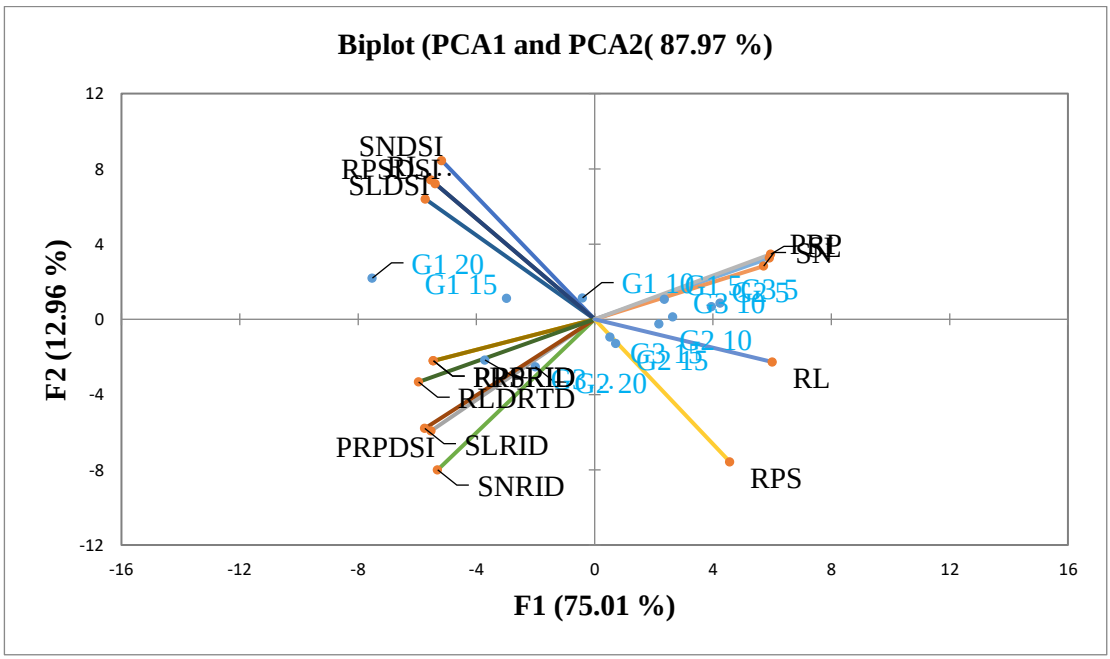

Figure 1. Biplot showing the relationship of in vitro culture response traits of 3 bread wheat genotypes at 5%, 10%, 15% and 20% (w/v) PEG 6000 concentrations in the medium,

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