MOLECULAR AND FIELD ANALYSIS OF SOME BARLEY GENOTYPES FOR WATER STRESS TOLERANCE

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Barley is one of the most important cereal crops grown in many developing countries, where it is often subject to extreme drought stress that significantly affects production (Ceccarelli *et al*., 2007). Barley is grown over a broader environmental range than any other cereals where unfavorable climates prevail.

Drought is the main environmental constraint, which often having devastating effects on crop productivity. Hence, improved tolerance to drought has been an important goal in crop improvement programs (Nazari and Pakniyat, 2010). Drought tolerance is a complex trait and mostly conditioned by many component responses, which may interact and may be different with respect to types, intensity and duration of water deficit. Moreover, most agronomic traits are expressed differently in normal and stress conditions and are known to be affected by environmental factors. Therefore, selection based on the phenotype would be difficult for such traits (Hittalmani *et al*., 2003).

Stress tolerance in plants is a complex trait and direct selection for grain yield under stress conditions has been hampered by low heritability, polygenic control, epistasis, and high genotype-by-environment (G x E) interactions. Determination of the molecular basis of drought tolerance would allow and facilitate the targeted breeding of varieties adapted to stress.

Water deficit and scarcity of rainfall decrease the germination and establishment of barley. In such conditions, the barley encounters drought stress during seed germination and early growth stages. These stages are the most vulnerable to drought stress and presenting a challenge in barley production (Amini, 2013). Drought under high temperature conditions, coinciding with the reproductive stage of the plant, may cause a decrease in the size and number of the grains, and eventually its yield. Identification of the genes responsible for drought tolerance in barley facilitates the genetic improvement of barley through marker-assisted selection (Guo *et al*., 2009). Heat shock proteins (HSPs) expressed in plants during development and in response to stress (DeRocher and Vierling, 1994).

The objectives of this study were to: (1) provide new tools and strategies to better exploit the available genetic variation for drought tolerance evaluation in barley germplasm in early seedling and

advanced growth stages of barley under drought stress conditions and (2) achieve molecular screening for barley genotypes using PCR amplification of late embryogenesis abundant (LEA) gene, simple sequence repeats (SSR) markers which are known to be correlated with drought tolerance.

MATERIALS AND METHODS

Three barley genotypes (2 local varieties i.e., Giza 126 and Giza 129 and California Marriott pure line) were used in this study. The hulled genotypes Giza 126 and California Marriott are known for its adaptability and tolerance to drought stress in the semiarid region, while the hulless variety Giza 129 is grown in the irrigated region. Three genotypes were grown in two experiments; one in lab and one under the field conditions.

Design for simulated water stress conditions

The experiment was conducted at Genetic laboratory, Faculty of Agriculture, Tanta University, Egypt, in 2014. The seeds were kindly provided by Barley Department, FCRI, ARC, Egypt. To assess water stress tolerance during germination, 25 seeds with similar size per genotype in four replications were germinated on two layers of whatman filter paper in Petri dishes containing different potential of osmotic solution created by adding polyethylene glycol-6000 (PEG) at one of four concentrations: 0, 5, 10 and 20%. The factors were priming with polyethylene glycol (PEG 6000) at four osmotic potential levels including 0, -0.5, -1.48 and -4.91 MPa, according to the method presented by (Michel and Kaufmann, 1973). PEG was used because it is an inert, non-toxic and non-penetrating solute in plant research, unlike other osmolytes such as mannitol, sodium chloride and sugar (Kramer and Boyer, 1995). Because this compound has a high molecular weight, it cannot pass through the cell wall and therefore it is used to regulate water potential in germination tests.

The seeds were germinated at 20°C with different drought stress (0, -0.5, -1.48 and -4.91 MPa osmotic potential). The control plants were moistened with distilled water. In each replication, 10 seeds per treatment were analysed. Eight days after incubation, seed germination percentage, root length, and shoot length and seedlings fresh weight were measured. Seeds were considered to have germinated when the radicle emerged.

DNA extraction

Genomic DNA was extracted from barley fresh leaf tissues (10 days old) of the three selected genotypes using EZ-spin column genomic DNA minipreps kit (plant), BIO BASIC INC. Canada.

SSR assay and LEA gene amplification

From nine tested primers only two primers were selected as molecular marker for drought tolerance in the studied barley genotypes, i.e., primer for late embryogenesis abundant (LEA) gene and the SSR primer HVB23D:

1. LEA specific primer, F5`-ATggCTCgCTgCTCTTACTC-3` and R 5`-TCAgTgAgAggATCgATTgAAC-3`. (Wang *et al*., 2006).
2. SSR primer HVB23D, F5`-ggTAgCAgACCgATggATgT-3`and R 5`-ACTCTgACACgCACgAACAC-3`. (Varshney *et al*., 2007).

Primers were constructed by Metabion International AG, D-82152 Martinsried, Germany**.**

PCR analysis

DNA amplification was performed in 25 µl volumes containing 12 µl of PCR Master mix 2x (CinnaGen/ Iran), 2 µl of each primer (10 pmol/ µl), 1 µl genomic DNA (50 ng/ µl) and 10 µl sterile deionized water were added. PCR reactions were achieved in Thermal Cycler (LongGene-MG96G/China). The initial denaturation temperature of the first cycle was 94°C for 4 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, with a final extension of 4 min at 72°C, then the reaction was held at 4°C.

PCR products (15 µl of each reaction) and 100 bp DNA ladder (Larova GmbH-Germany) as DNA size marker were resolved by electrophoresis on 2 % agarose gel containing ethidium bromide for 90 min. at 70 volt, visualized via UV transilluminator and then photographed. Molecular sizes of the amplified fragments separated on gels were measured by gel images with Gel Analyzer software package version 2010a.

Protein analysis by SDS-PAGE

1-Total soluble Protein

The total soluble proteins were extracted from 9 days old plants (whole plant) using the extraction solution (50 mM Tris-base pH 8.0 and 10% sucrose), then samples of the four PEG treatments of each studied barley genotype were separated on SDS-PAGE according to Laemmli (1970).

2-Heat-stable proteins

To fractionate the heat-stable proteins, aliquot of the extracted total soluble proteins of each treatment was incubated (in water bath) at 100°C for 3 min., then centrifugated for 2 min. at 8000 rpm. The supernatant containing the heat stable proteins (non-denaturated proteins) was transferred into new tube and aliquots were separated on SDS-PAGE (DeRocher and Vierling, 1994).

Field experiment

Field experiment was conducted at the Experimental Farm of Sakha Agricultural Experiment Station, (ARC), Egypt, during the two successive seasons 2011/12 and 2012/13, which has a semiarid Mediterranean climate (Table 1). The soil was classified as clay loam, with a pH of 8.2, electrical conductivity of 2.1 dS cm^{-1}. Seeds were hand drilled at the recommended sowing rate of barley in Egypt (50 kg/fed.). Each genotype was sown in six rows of 3.5 m, with 20 cm between rows. This experiment was laid out in randomized complete block design with four replications. The 1^{st} treatment (control) was

under full irrigation (three irrigations), the 2nd was under severe stress (only one irrigation at sowing time). Sowing was done in 15th of December in both seasons. The preceding crop was cotton in the two seasons.

The phenotypic traits i.e., days to maturity, plant height, spike length, and spikes number m^2 and seed index, 1000-grain weight, number of grains per spike, grain yield and biological yield were estimated.

RESULTS AND DISCUSSION

Field experiment

Data obtained clearly indicated that the differences between the three barley genotypes under investigation were significant in all studied parameters viz., number of days to maturity, plant height, spike length, no. of spikes/m^2, No. of grains/spike, 1000-grain weight and biological yield as well as grain yield.

The days required for maturity were not similar in the two years of this study Table (2), due to the difference in (rainfall and irrigation water) water applied. Moreover, the maximum temperature was high and the relative humidity and rainfall were low in the second season, compared with the first season Table (1). The results showed that the genotypes under stress condition were matured earlier than irrigated condition. These results were in agreement with those obtained by Vaezi *el al.* (2010) and El-Seidy *et al.* (2012).

Grain yield and yield components

Analysis of variance showed that water deficit significantly reduced the 1000-grain weight, number of spikes/m^2, number of grains/spike and grain yield (Table 2). The 1000-grain weight and its size reduction are possibly due to a decrease in the assimilation rate and lower photoasimilate translocation to physiological sinks. Water deficit is known to reduce the 1000-grain weight by shortening the grain-filling period (Mamnouie *et al.*, 2006). These findings are in agreement with Maktoobian *et al.* (2013) who found that water stress at the flowering stage reduced 1000 grain weight significantly. There were significant differences in the number of grains/spike between barley genotypes (Table 2).

The number of grains/spike of Giza 129 variety was the lowest due to its naked type grain feature. Water deficit reduced the number of spikes m^2 in barley genotypes. Under stress condition the average number of spikes/m^2 was relatively high in Giza 126 which was related to its higher drought tolerance. However, the lowest spikes/m^2, was observed in Giza 126 variety. Grain yield in Giza 126 variety was the highest under stress condition as well as in well-irrigated condition. These results are in a partial agreement with those of Noaman *et al.* (1995), Ali *et al.* (2009) and El-Seidy *et al.* (2012) who concluded that Giza 126 was high stable and had a great drought tolerance. Therefore, the Egyptian cultivar Giza 126 and California Marriott as drought tolerant

genotypes could be used in the future breeding programs to increase yield ability under drought stress.

Drought stress during different stages of growth in rainfed and at terminal stages in irrigated cereals is the first limiting factor to reduce performance of these crops. Under drought stress conditions final crop grain yield is affected by the effects of water stress on net photosynthesis, respiration, soluble protein and metabolism of nutrient (Bahavar *et al*., 2009; Abdul Jaleel *et al*., 2009). Water stress in the grain filling period accelerates leaf senesces and decreases grain filling period, mean grain weight and yield (Santvari *et al*., 2002).

SSR assay and LEA gene amplification

HVB23D SSR primer and the specific LEA primer generated specific bands in the studied drought tolerant barley genotypes (Giza 126 and California Marriott), which were absent in the sensitive genotype Giza129 (Fig. 1 A and B).

The generated banding pattern of HVB23D SSR primer in Fig. (1A) showed the presence of 153 bp band in lanes 1 and 2 (California Marriott and Giza 126), while it was absent in lane 3 (Giza 129). Furthermore, Fig. (1B) showed the presence of 720 bp band in lanes 1 and 2 (California Marriott and Giza 126), while it was absent in lane 3 (Giza 129). These results indicated that we can use these specific bands as markers for drought tolerance especially in the studied barley genotypes.

Total soluble proteins and hot shock protein analysis

The results in Fig. (2A) showed the banding pattern of the total soluble proteins under the four different treatments, 0, 5, 10 and 20% PEG 6000. This data indicated the high response of California Marriott and Giza 126 genotypes as increasing of number of protein bands and protein concentration (band density) associated with PEG concentration compared with the control, while the genotype Giza 129 showed lower response, e.i., lower protein concentration and lower number of protein bands. Moreover, Fig. (2B) showed the heat-stable proteins banding patterns fractioned from the total extracted protein. Figure (2B) show clearly the lack of protein band at 107 KDa in Giza 129 (drought sensitive) comparing with California Marriott and Giza 126 which are drought tolerant. These heat-stable proteins may contain heat shock proteins that expressed in response to water stress.

The germination percentage and shoot length decreased with increasing PEG concentration, with the sharpest reductions occurred under the highest water stresses comparing with the control (Fig. 3 A - B, $P \leq 0.05$).

The remarkable reduction of the germination percentage indicated that the response of the tested genotypes varied among stress treatments (Fig. 3A). The maximum decrease in germination percentage occured in Giza 129 (1.6%) at -4.91 MPa.

Shoot length was substantiality affected by water stress levels, especially at levels higher than 10% (Fig. 3 B). Increasing stress levels caused remarkable reduction in shoot length of Giza 129 (the decrease in the shoot length ranged between 8% to 44% in the severe stress).The observed data of shoot length indicated that Giza 129 is highly sensitive to water stress, while Giza 126 and California Marriott were tolerant, with slight decrease in the shoot length ranged between 6% to 14% (Fig. 3 B). High PEG treatment was more discriminating between drought tolerant and drought sensitive genotypes than the low drought treatment (Fig. 3). These results corroborated with those of Khan *et al*. (1997), Alam *et al*. (2005) and Ghazi *et al*. (2007) who reported that germination rate decreased as stress increased as differences between tolerant and sensitive genotypes became more evident.

These results indicated that the sensitivity/tolerance of the tested genotypes varied among the measured traits. The root length indicated that the differences between genotypes were higher in the absence of stress and declined under PEG treatments. Giza 126 and California Marriott exhibited high root length in all stress levels; Giza 126 was high stable under different stress levels. Root length was increased under mild and severe water stress treatments, where California Marriott showed increasing in root length under stress levels ranged between 10% to 27%, while the sensitive variety Giza 129 showed high increasing in root length under stress levels ranged between 44% to 58% (Fig. 3 C). These results are in partial agreement with Quisenberry (1982), Sullivan (1983), Turner (1986), ShuYing *et al.* (2009) and Nejad *et al.* (2010), they found positive correlations between seed yield and root development in cereals, especially in barley.

Water stress showed increase of dry mater percentage as compared to control in all studied varieties ($p < 0.05$): Giza 126 (14.62 and 19.64 %, respectively), California Marriott (14.91 and 17.33%, respectively) and Giza 129 (13.82 and 14.77%, respectively) (Fig. 3 D).

Water deficit and unsuitable distribution of rainfall decrease the germination and establishment of barley. In such conditions the barley encounters with drought stress during seed germination and early growth stages. These stages are the most vulnerable to drought stress and presenting a challenge in barley production.

SUMMARY

Water stress effects on three barley (*Hordeum vulgare* L.) genotypes in field and lab were studied. The genotypic responses varied among growth stages according to water stress. Lab experiments showed that germination percentage, shoot length, root length and total dry mater were the most effective traits between sensitive and tolerant genotypes. Giza 126 and California Marriott were tolerant and stable under different stress levels, while the sensitive variety Giza 129 showed sharp decrease in germination percentage, shoot length and total dry mater. Giza 126

recorded the highest yield and yield component values under stress condition as well as in well-irrigated condition, followed by California Marriott under the stress condition. Giza 129 ranked last under stress condition. Our results showed that the HVB23D SSR primer and the specific primer LEA generated specific bands occurred in the studied drought tolerant barley genotypes (Giza 126 and California Marriott), while they were absent in the sensitive genotype Giza 129.

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Table (1): Maximum, minimum temperature and rainfall during the growing seasons of barley at Sakha Agricultural Experiment Station, (ARC), Egypt

Month	Temperature (°C)				Relative humidity (%)		Rainfall (mm)	
	2011/12		2012/13					
	Max.	Min.	Max.	Min.	2011/12	2012/13	2011/12	2012/13
Dec.	20.2	6.40	22.0	8.3	71.3	73.6	34.95	15.5
Jan.	20.3	5.80	21.5	7.8	69.3	69.1	0.00	18.3
Feb.	21.7	6.90	24.5	9.4	65.6	70.0	27.20	22.4
Mar.	22.5	6.70	24.3	10.0	60.2	70.2	0.00	12.6
Apr.	26.4	9.91	28.2	11.0	68.4	66.0	0.00	12.0

Table (2): The effect of water stress on days to maturity, plant height, spike length, no. of spike m^2, no. of grains/spike, 1000 grain weight, biological yield and grain yield of barley genotypes (Giza 126, California Marriott and Giza 129).

Genotypes	Season 1			Season 2		
	Normal	Stress	Reduction%	Normal	Stress	Reduction%
Days to maturity (day)						
Giza 126	120.00	118.04	1.63	124.38	122.65	1.41
California Mariout	117.50	115.43	1.76	121.75	120.25	2.42
Giza 129	118.33	114.58	2.32	123.40	121.25	2.78
LSD 0.05	0.33	0.88		0.47	1.05	
Plant height (cm)						
Giza 126	106.13	99.67	6.09	119.75	108.25	9.60
California Mariout	105.70	102.38	3.14	116.25	104.75	9.89
Giza 129	97.80	81.96	16.2	107.00	82.25	23.13
LSD 0.05	2.86	4.73		2.76	4.04	
Spike length						
Giza 126	8.75	7.75	11.43	9.25	8.5	8.11
California Mariout	8	7.25	9.38	8.73	7.4	15.23
Giza 129	8.5	6.75	20.59	8.71	6.5	25.37
LSD 0.05	0.33	0.66		0.37	0.52	
No. of Spikes/m^2						
Giza 126	437.68	362.6	17.15	495.00	413.75	16.41
California Mariout	408.18	347.31	14.91	444.09	388.03	12.62
Giza 129	423.63	307.2	27.48	463.33	341.25	26.35
LSD 0.05	31.54	21.54		22.31	28.18	
No. of grains/spike						
Giza 126	57.1	54.18	5.11	62.50	61.27	1.97
California Mariout	52.6	50.95	3.14	55.25	54.00	2.26
Giza 129	53.48	41.53	22.34	61.47	53.45	13.05
LSD 0.05	2.48	4.67		2.64	3.50	
1000-grain weight (g)						
Giza 126	52.5	48.95	6.76	53.84	51.43	4.48
California Mariout	49.1	46.77	4.75	51.09	48.74	4.60
Giza 129	46.64	41.33	11.39	47.41	41.96	11.50
LSD 0.05	1.37	0.85		2.12	0.69	
Biological yield (kg $fed.^{-1}$)						
Giza 126	9339	6775	27.45	10669	7263	31.92
California Mariout	9114	6281	31.08	10215	6875	32.70
Giza 129	9188	5883	35.97	10630	6109	42.53
LSD 0.05	174	279		211	118	
Grain yield (kg $fed.^{-1}$)						
Giza 126	3390	2623	22.63	4278	3187	25.50
California Mariout	3182	2295	27.88	4153	3047	26.63
Giza 129	3198	1915	40.12	4025	2163	46.26
LSD 0.05	152	77		169	85	

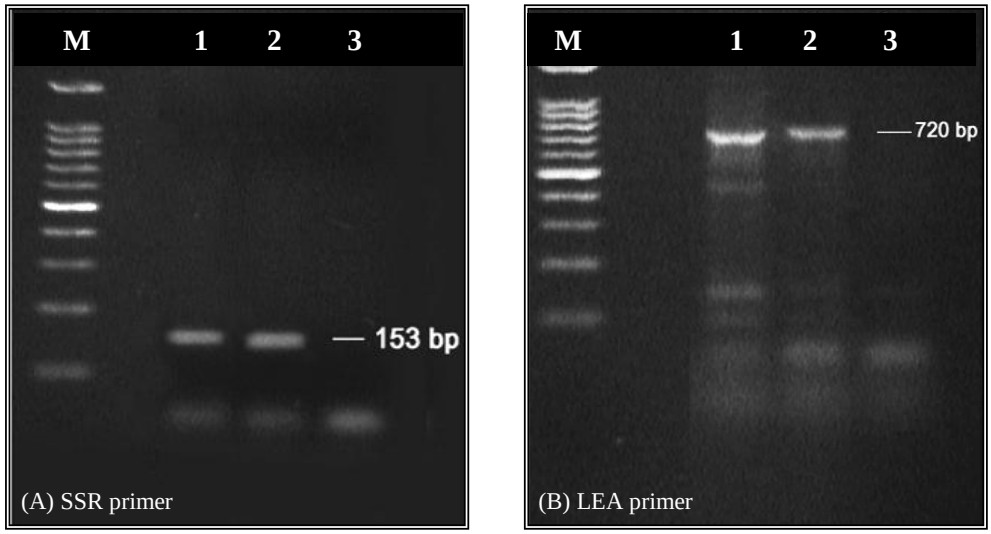

Fig. (1): (A) is the banding pattern of HVB23D SSR primer and (B) LEA primer. M, is 100 bp DNA ladder and lane 1, 2 and 3 are for California Marriott, Giza 126 and Giza 129 barley genotypes, respectively.

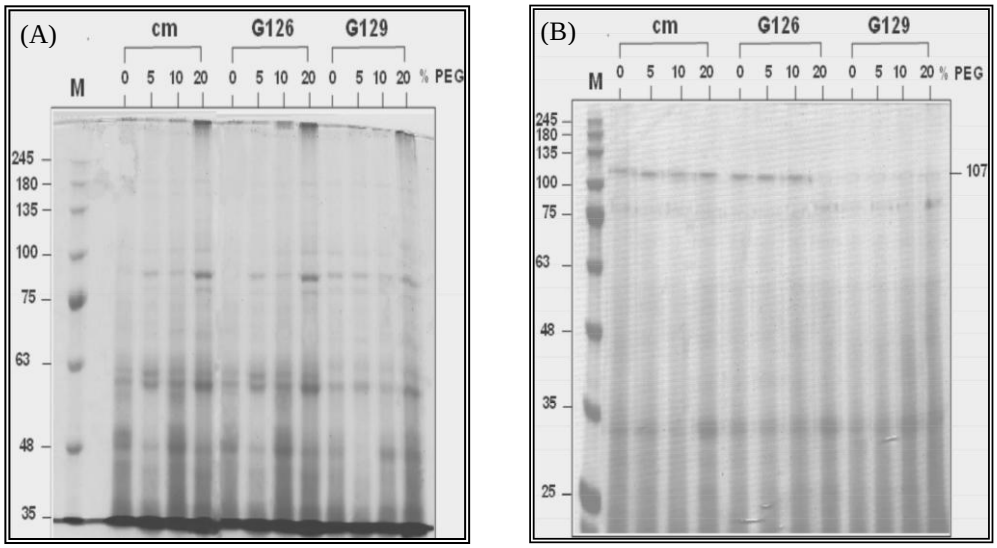

Fig. (2): (A) is a PAGE banding patterns showing the total soluble proteins from the tested three barley genotypes, California Marriott (Cm), Giza 126 (G 126) and Giza 129 (G 129) under PEG 6000 concentrations 0, 5, 10 and 20%. (B) show the heat-stable proteins from the same genotypes under the same PEG 6000 concentrations.

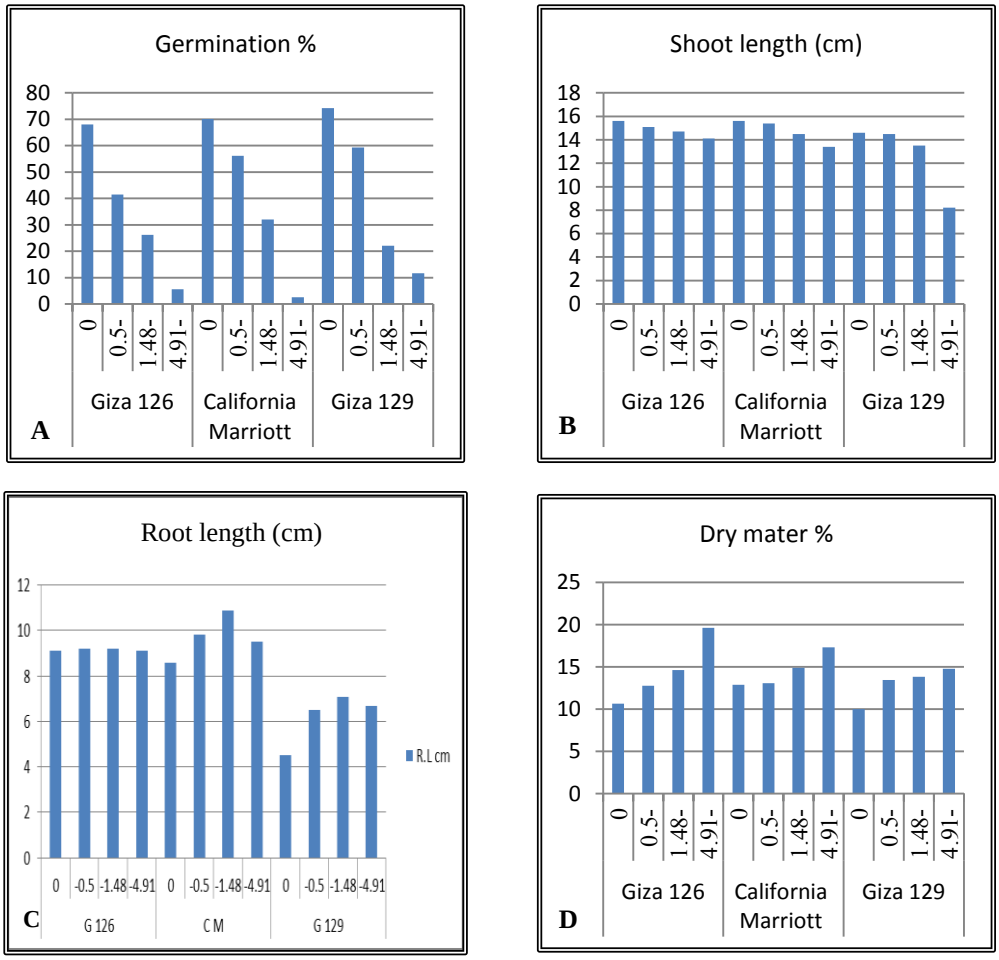

Fig. (3): The effect of water stress (0, -0.5, -1.48 and -4.91 MPa osmotic potential) on (A): germination percentage, (B): shoot length, (C): root length and (D): dry mater% of barley (*Hordeum vulgare* L.) seeds, $P \leq 0.05$.