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Original article

Screening pomegranate (*Punica granatum* L.) genotypes for drought tolerance using physiological and phytochemical characteristics

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Summary

The present study aimed to evaluate physiological, phytochemical, fruit phytonutrients, and fruit yield (FY) of Iranian pomegranate genotypes under drought stress. A split plot experiment was conducted based on a randomized complete block design with three replications, where the three irrigation regimes were allotted to main plots and the ten pomegranate genotypes were allotted to subplots. The three irrigation regimes including control, mild and severe drought stresses were applied from bud burst to fruit harvest, when 25, 50 and 75% of the total available soil moisture was depleted from the root-zone (~40 cm), respectively. The result of present study showed that with increase in drought stress levels fruit yield (FY), relative water content (RWC), total chlorophyll, chlorophyll *b*, total soluble solids and titratable acidity were decreased, while water-soluble carbohydrate (WSC), catalase, superoxide dismutase, chlorophyll *a*, total phenol content, and cyanidin-3-glucoside content were increased. The FY was significantly decreased in mild and severe drought stress conditions by 23.71 and 40.40%, respectively compared to the control. The distribution of genotypes on the biplot of principal component analysis under normal condition revealed a high genetic variation among the Iranian pomegranate genotypes. MTS and PSS genotypes, with high FY, proline content, WSC and catalase activity, were identified as preferable and suitable genotypes for both normal and mild stress conditions. In addition, PSS genotype maintained its FY in mild and severe drought stresses, which suggested as a preferable and superior genotype for cultivation in areas under drought stress.

Keywords
drought stress, drought tolerant, fruit yield, secondary metabolites

Significance of this study

What is already known on this subject?

- There is a high genetic diversity among Iranian pomegranate genotypes, however, drought stress is the main limiting factor in pomegranate fruit production.

What are the new findings?

- 'Post Sefide Shirin' genotype maintained its fruit yield under drought stresses, which suggested as a preferable and superior genotype.

What is the expected impact on horticulture?

- The cultivation of 'Post Sefide Shirin' genotype in areas with water shortage could be helpful to improve the quality and quantity of pomegranate fruit production.

Introduction

Pomegranate (*Punica granatum* L.) is one of the most important fruit crops, due to its high economic value and wide clinical use of its fruit for prevention and treatment of cardiovascular disease as well as some cancers (Afaq *et al.*, 2005; Barman *et al.*, 2011; Johanningsmeier and Harris, 2011). In addition, the higher drought tolerance of this plant makes it appropriate for cultivation particularly in arid and semi-arid regions (da Silva *et al.*, 2013; Dinc *et al.*, 2018). About one third of the world's cultivated lands are in semi-arid and arid regions (Atlin and Frey, 1990). India, China, Turkey, the United States, and Iran are the main pomegranate producing countries, of which Iran is the world's largest producer (da Silva *et al.*, 2013; Mirabolfathy *et al.*, 2012; Olmo-Vega *et al.*, 2017). Also, the country is the most important center of origin with more than 700 pomegranate genotypes; thereby it has one of the richest pomegranate germplasms worldwide (Verma *et al.*, 2010). There are many biotic and abiotic stresses which affect the quality and quantity of crops in Iran (Sheikh-Mohamadi *et al.*, 2017, 2018). Drought stress is one of the major environmental constraints causing deterioration of conditions for survival, growth and final yield of plants (Akbari *et al.*, 2018; Dejahang *et al.*, 2018; Staniak and Kocoń, 2015). In contrast, the plants have developed numerous adaptations at physiological and biochemical levels to cope with drought stress with a variety of escape, avoidance, and tolerance mechanisms (Rokiatou, 2017). Among them, drought tolerance is a very complex mechanism, due to the combined roles of a variety of physiological and biochemical features (Pandey and Shukla, 2015; Wang and Huang, 2004). The production of reactive oxygen species (ROS) is one of the key events occurring during drought stress, and contributes to establishing

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adaptive signaling pathways (Ba *et al.*, 2013). In addition, external or endogenous overproduction of these compounds induces a disruption of redox signaling which can seriously disrupt normal metabolism through oxidative damage to lipids, proteins, and nucleic acids (Zuluaga *et al.*, 2017). The role of many physiological factors such as antioxidative enzymes, chlorophyll content, and relative water content (RWC), and different types of organic and inorganic solutes such as water-soluble carbohydrates (WSCs) and proline has been supported in oxidative damage (Rout and Shaw, 2001). These features could be applied as reliable indicators in screening genotypes for drought tolerance. Superoxide dismutase (SOD) as the first line of defence system against the oxidative stress, is a major antioxidant enzyme which exists in the intracellular and extracellular environment to detoxify ROS and convert superoxide radicals to H_2O_2 and molecular oxygen which are then scavenged by catalase (CAT) enzyme (Koike *et al.*, 2018). CAT is a potent scavenger of H_2O_2 and a constituent of peroxisomes that degrades H_2O_2 into water and molecular oxygen (Kumar *et al.*, 2011). In addition, when ROS are produced in plants as a response to drought stress, the photosynthetic apparatus are damaged and the chlorophyll content is diminished (Fu and Huang, 2001). The ability of plants to synthesize more chlorophyll could be applied as a reliable indicator in screening genotypes for drought tolerance (Li *et al.*, 2006). RWC is another important character considered in determining the drought tolerance in the crops. Selection based on higher RWC led to higher yielding and drought tolerant genotypes (Ebrahimiyan *et al.*, 2013). The role of sugar metabolism enzymes and concentrations of WSCs have also been supported in drought tolerance. Accordingly, the ability to store and remobilize large amounts of WSC was used as a physiological index for dehydration tolerance in plants (Ruuska *et al.*, 2006). Accumulation of proline as an organic compatible solute can also to be related to osmotic adjustment during environmental stresses (Bajji *et al.*, 2001). Although its actual role in plant osmo-tolerance remains controversial, it is also thought that this compound has significant effects on enzyme and membrane integrity along with adaptive roles in mediating osmotic adjustment (Khalifa *et al.*, 2016). Valuable compounds such as polyphenolics, as well as total soluble solids (TSS) and titratable acidity (TA) in different parts of the pomegranate fruit have nutritional value and medicinal effects such as antioxidant, anticancer and anti-atherosclerotic effects (Akbari *et al.*, 2018; Mertens-Talcott *et al.*, 2006). Some studies showed that the drought stress can have detrimental or beneficial effects on fruit quality via changes in the concentrations of chemical compounds (Laribi *et al.*, 2013). Galindo *et al.* (2017) indicated that a very short period of irrigation restriction at the end of ripening period induces early harvest time and enhances the bioactive compounds content such as anthocyanins and phenolic compounds. Although there are some studies concerning irrigation of pomegranate trees in the world, there is little information documented on the effects of deficit and full irrigation on pomegranate trees in Iran, despite the wide geographical distribution of *P. granatum* and also existence of rich and unique pomegranate germplasm. The present study aimed to evaluate the physiological responses, antioxidant defence system, phytochemical antioxidants, phytonutrients and fruit yield of ten pomegranate genotypes under drought stress conditions. The genotypes were selected based on the percentage of cultivated area, and the share in commercial fruit production for export (Table 1).

Materials and methods

Field experiment

The experiment was conducted at the research orchard of the Pomegranate Research Station, Saveh, Iran (35°1'N, 50°21'E, 960 m a.s.l.) on 9-year-old pomegranate trees during the growing season 2016. This region has a sandy loam soil (pH 7.7) with an average bulk density of 1.48 g cm^{-3} in the top 40-cm soil surface. The range of annual temperature and precipitation are 12.4 to 24.1 °C 76.6 to 271.2 mm and with the averages of 20.5 °C and 151 mm, respectively. Also, the electrical conductivity (EC) of irrigation water was 1.3 dS m^{-1}. In this study, ten commercial local pomegranate genotypes were selected (Table 1). A split plot experiment was conducted based on a randomized complete block design with three replications, where the three irrigation regimes were allotted to main plots and the ten genotypes were allotted to subplots. The three irrigation regimes including control (non-stress), mild and severe stresses were applied from bud burst to fruit harvest, when 25, 45 and 65% of the total available soil moisture was depleted from the root-zone (~40 cm), respectively. Soil moisture was measured based on standard gravimetric methods at three depths: 0–20, 20–40, and 40–60 cm (Gregorich and Carter, 2007). Irrigation was applied with a basin irrigation system, in which water was delivered to the field via a pump station and polyethylene pipes. The water quantity was determined according to the following equation (Singh, 2008):

Total quantity of water to be applied per tree (liters) = A×d
where, A: Basin area to be irrigated (m^2); d: depth of irrigation (mm).

Table 1. Information of 10 pomegranate genotypes investigated in this study.

Genotype	Origin	Flavor	Peel color	Aril color	Seed hardness
Post Siyah (PS)	Yazd, Iran	Sweet-sour	Black	Yellow	Hard
Agha Mohamad Ali (AMA)	Markazi, Iran	Sweet-sour	Dark pink	Pink	Hard
Alake Shirin (AS)	Markazi, Iran	Sweet	Red	Red	Hard
Alake Torsh (AT)	Markazi, Iran	Sour	Red	Red	Hard
Malas Shirin Saveh (MSS)	Markazi, Iran	Sweet	Red	Red	Hard
Malase Torshe Saveh (MTS)	Markazi, Iran	Sweet-sour	Red	Red	Hard
Post Sefide Torsh (PST)	Markazi, Iran	Sour	White	Pink	Hard
Post Sefide Shirin (PSS)	Markazi, Iran	Sweet	White	Pink	Hard
Post Sefide Bihaste Shomal (PSBS)	Markazi, Iran	Sour	White	Pink	Soft
Tabestaniye Torsh (TT)	Markazi, Iran	Sour-sweet	Dark pink	Pink	Hard

The depth of irrigation water for each application was calculated by the following formula:

$$d = \frac{Pw \times Bd \times D}{100},$$

where, Pw: moisture percentage to be raised; Bd: bulk density of the soil (1.48 g cm^{-3}); and D: depth of root-zone to be moistened (40 cm).

The characters measured

The characters were recorded on three randomly selected trees per genotype in each plot (each replication) with three replications at the maturity stage of fruits based on commercial harvesting times for each genotype, which were September 15 for 'Tabestani Torsh' (TT) and November 1 for other genotypes. The arils juice was extracted by pressing the arils using a Garlic press instrument. Physiological traits including RWC, chlorophyll *a,* chlorophyll *b*, total chlorophyll, proline, WSCs were measured once from mid leaves at the mid-fruit growth stage. Leaf water status was determined by estimating the RWC according to the method described by Ritchie *et al*. (1990). Chlorophyll *a* (Chl*a*), and chlorophyll *b* (Chl*b*) were measured by spectrophotometry, using 80% acetone as a solvent and afterwards, total chlorophyll (TChl) content was calculated (Lichtenthaler and Buschmann, 2001). Proline content was measured according to a method developed by Bates *et al*. (1973). WSCs content were extracted from leaves according to the method of Zhang *et al*. (2006). For enzyme extraction, fresh leaf samples (0.1 g) were ground with pre-chilled pestle and mortar in liquid nitrogen and homogenized in 50 mM potassium phosphate buffer (pH 7.8), which contained 0.5 mM EDTA. The homogenate was centrifuged at 15,000 rounds per minute (rpm) for 15 min and the supernatant was collected and used for enzyme activity assays. SOD activity was estimated according to Sairam *et al*. (2002) and CAT activity was calculated as the reduction of the absorbance at 240 nm for 1 min following the decomposition of H_2O_2 (Aebi, 1984). Enzyme activities were expressed on the basis of per unit protein weight. Quantification of Cyd-3-glu was determined in arils extracts using the pH-differential procedure with 2 buffer systems [*i.e.*, KCl (pH 1.0 and 0.025 M), and $CH_3CO_2Na.3\ H_2O$ (pH 4.5 and 0.4 M)] as described by Giusti and Wrolstad (2001). Briefly, two dilutions of each sample were prepared, one containing KCl (potassium chloride) and the other containing $CH_3CO_2Na.3\ H_2O$ (sodium acetate) buffer, the pH was adjusted with concentrated HCl (hydrochloric acid). Then, these dilutions were equilibrated for 15 min, and the absorbance of each dilution was measured at 530 and 700 nm against a blank cell filled with distilled water. Results were calculated according to the following equation:

A = (A530- A700) pH 1.0 – (A530-A700) pH 4.5

Cyanidin-3-glucoside content (mg L^{-1}) =

$(A \times MW \times DF \times 10^3)/ (\varepsilon \times 1)$.

Where, MW indicates molecular weight value of cyanidin-3-glucoside (Cyd-3-glu) (449.2 g mol^{-1}); DF indicates the dilution factor; ε is the molar extinction coefficient in L × mol^{-1} × cm^{-1} (26900); 10^3 is a factor for conversion of g to mg, and the equation presented above assumes a path length of 1 cm.

The total phenolic content (TPC) was determined colorimetrically using Folin-Ciocalteu reagent as described by Ghasemnezhad *et al*. (2013). In this regard, ten-fold diluted Folin-Ciocalteu reagent (1.5 mL), 7.5% sodium carbonate (1.2 mL), and diluted juice (0.3 mL) were mixed. The mixture was kept at 24 °C for 90 min. All the steps were performed in the dark. The absorbance was then measured at 760 nm against a blank using a spectrophotometer (Hitachi U1800, Japan). The estimation of the phenolic compounds was calculated using a calibration curve based on Gallic acid. The data were reported as mg of Gallic acid equivalents (GAE) per 100 mL juice (mg GAE 100 mL^{-1} J). The TSS content in the juice was measured by hand refractometer (Model N-10; Atago, Tokyo, Japan). Measurements were conducted at 20 °C, and results expressed as °Brix. Furthermore, the TA, expressed as g of citric acid equivalent per L of juice, was determined by acid-base titration of the fruit juice (10 mL) with NaOH 0.1 N to the end point of pH 8.2 using a digital pH meter (Model 2001, Crison, Barcelona, Spain).

Statistical analysis

Data were subjected to ANOVA using Statistical Analysis Software (v. 9.1; SAS Institute, Cary, NC). The mean values were compared via the LSD test (Steel and Torrie, 1980). Principal component analysis (PCA) was conducted to identify the interrelationships among the pomegranate genotypes and the measured traits. Multiple dimensions of the data

Table 2. Analysis of variance for measured traits in 10 pomegranate genotypes evaluated in three irrigation regimes.

Traits	Replication (df=2)	Irrigation (I) (df=2)	E_i (df=4)	Genotype (G) (df=9)	G×I (df=18)	Error (df=54)
FY	0.49^{ns}	833.41**	7.81	1057.81**	133.10**	4.96
RWC	8.23^{ns}	186.47**	30.07	134.05**	167.13**	39.87
WSC	0.27^{ns}	25.96**	0.56	1.09**	0.88**	0.72
CAT	0.003^{ns}	0.15**	0.003	0.02**	0.05**	0.00
SOD	18.12^{ns}	968.96**	7.14	56.66**	116.30**	6.74
TChl	0.01^{ns}	0.72**	0.009	0.14**	0.13**	0.02
Proline	4.22^{ns}	157.94**	4.19	154.84**	82.84**	3.67
Chl*a*	0.02^{ns}	77.95**	0.01	0.83**	0.83**	0.01
Chl*b*	0.01^{ns}	130.19**	0.03	14.77**	12.95**	0.14
TPC	18.51^{ns}	1634.98**	20.68	4330.67**	608.78**	41.35
Cyd-3-glu	1.58^{ns}	47.86**	0.26	159.31**	37.87**	2.24
TSS	7.71*	66.42**	1.90	9.51**	4.79**	2.39
TA	0.09^{ns}	0.92**	0.37	4.92**	0.45**	0.20

*$P<0.05$; **$P<0.01$; n.s., not significant ($P>0.05$).

Table 3. Effects of different irrigation regimes on measured traits of 10 pomegranate genotypes.

Irrigation regime	TSS (°Brix)	Cyd (mg L^{-1} J)	TPC (mg GAE 100 mL^{-1} J)	Chl*b* (mg g^{-1} leaf)	Chl*a* (mg g^{-1} leaf)	Proline (µm g^{-1} leaf)	TChl (mg g^{-1} leaf)	SOD (µmol min^{-1} mg^{-1} protein)	CAT (µmol min^{-1} mg^{-1} protein)	WSC (mg mL^{-1})	RWC (%)	FY (kg $tree^{-1}$)	TA (%)
Control	18.39[a]	128[c]	117.57[b]	0.46[a]	0.52[c]	10.50[b]	0.98[a]	9.46[c]	0.08[b]	10.47[b]	79.35[a]	39.38[a]	1.7[b]
Mild stress	16.42[b]	133[b]	131.03[a]	0.27[b]	0.61[b]	12.74[a]	0.88[b]	12.76[b]	0.17[a]	11.84[a]	71.63[b]	30.04[b]	1.8[b]
Severe stress	15.47[b]	160[a]	133.36[a]	0.09[c]	0.70[a]	8.15[c]	0.79[c]	20.25[a]	0.23[a]	12.33[a]	67.17[c]	23.47[c]	2.84[a]

Means followed by the same letter in each column are not significantly different according LSD test ($P < 0.05$).

space were reduced and the biplot was drawn using the stat graphics software (ver. 16.1.11). A cluster analysis was performed in order to distinguish among the genotypes based on the arithmetic mean method (UPGMA). The analysis was performed by the SPSS software on Windows 20.0 (SPSS Inc., Chicago, IL).

Results

There were significant differences among irrigation regimes for all of the measured traits ($P < 0.01$; Table 2). The effect of genotype was also significant for the traits, indicating a high level of variation among the genotypes ($P < 0.01$). The results of ANOVA revealed that the interaction effect of genotype with irrigation was significant for all measured characters ($P < 0.01$). Fruit yield (FY) was significantly decreased in mild and severe drought stress conditions by 23.71 and 40.40%, respectively compared to the control (Table 3). The results showed that the highest and lowest FY were obtained in TT (55.22 kg $tree^{-1}$) and 'Post Sefide Bihaste Shomal' (PSBS) (9 kg $tree^{-1}$) genotypes at control and severe drought stress, respectively (Table 4). In both irrigation regimes, the TT and 'Post Sefide Shirin' (PSS) genotypes produced the higher FY, compared to other genotypes. The FY trait was constant in PSS genotype at the three irrigation regimes, however under severe drought stress, the FY was dramatically decreased in all other genotypes compared to their controls. RWC decreased by 9.72 and 15.34% under mild and severe drought stress levels, respectively, compared to the control. The result showed that with increasing the stress levels from control to severe drought stress, RWC was decreased in most of the genotypes, however in 'Alake Shirin' (AS) and 'Post Sefide Torsh' (PST) genotypes it was increased. There was no significant difference between the mean values of mild and severe drought stresses for WSC, however, in both of them it was greater than control. The maximum and minimum of this character were observed in 'Agha Mohamad Ali' (AMA) and 'Malas Shirin Saveh' (MSS) genotypes at normal and severe drought stress, respectively. According to the results, CAT and SOD activities were increased in severe drought stress compared to the control in most of the genotypes, however the genotypes showed different responses to these enzymes at mild drought stress compared to the control.

Total chlorophyll (TChl) and chlorophyll *b* (Chl*b*) contents decreased under severe drought stress, in contrast, it significantly increased chlorophyll *a* (Chl*a*) compared to the control. The results demonstrated that the PST genotype had the highest mean values of TChl in the three irrigation levels compared to the other genotypes. The results showed that response of the genotypes in terms of proline content in the three irrigation regimes depended on the genotype. For example in 'Malase Torshe Saveh' (MTS) and PSBS genotypes proline contents were opposite response under mild and severe drought stress conditions compared to the control. The proline content in the mild and severe drought stresses was increased and decreased compared to the control, respectively. The amounts of both of the phytochemical characteristics of aril extracts including TPC and Cyd-3-glu depended on degree of drought stress. The TPC was significantly increased in mild and severe drought stresses compared to the control, however, it was not significantly different between the mild and severe drought stresses. The highest and lowest TPCs were observed in PS and PSBS genotypes at severe drought stress and normal conditions, respectively. By increasing the drought stress levels Cyd-3-glu was increased. In the most of the genotypes the highest Cyd-3-glu were observed at se-

Table 4. Interaction effects of genotype and water irrigation regimes on measured characters of 10 pomegranate genotypes.

Genotype	Level	FY (kg tree^{-1})	RWC (%)	WSC (mg mL^{-1})	CAT (µmol min^{-1} mg^{-1} protein)	SOD (µmol min^{-1} mg^{-1} protein)	TChl (mg g^{-1} leaf)	Proline (µm g^{-1} leaf)	Chl*a* (mg g^{-1} leaf)	Chl*b* (mg g^{-1} leaf)	TPC (mg GAE 100 mL^{-1} J)	Cyd (mg L^{-1} J)	TSS (°Brix)	TA (%)
MTS	I1	51.33±1.1	73.74±1.04	11.1±0.38	0.04±0.01	6.63±1.7	0.51±0.38	19.55±1.58	0.11±0.08	0.34±0.25	137.06±3.3	133.25±4.58	18.33±1.5	1.5±0.4
	I2	51.33±3.21	72.7±1.1	11.13±0.23	0.25±0.01	5.07±0.46	0.49±0.2	32.32±2.3	0.1±0.04	0.33±0.13	139.76±8.35	133.53±2.71	16.9±1.15	1.52±0.55
	I3	18.47±0.76	62.12±2.14	12.45±0.16	0.24±0.03	24.97±2.51	0.25±0.1	7.82±1.27	0.15±0.02	0.17±0.07	115.42±5.06	149.41±1.88	18.1±2.54	2.14±0.23
MSS	I1	29.58±1.1	68.49±9.86	10.97±0.3	0.05±0.01	12.23±1.24	1.07±0.33	4.64±1.06	0.1±0.07	0.66±0.07	114±1.26	131.61±3.57	18.5±1.8	0.96±0.04
	I2	32.5±3.9	65.63±2.68	11.09±0.42	0.03±0.01	6.13±1.21	0.89±0.08	10.61±1.67	0.07±0.01	0.37±0.06	133.74±7.52	133.09±5.03	14.07±1.01	1.04±0.14
	I3	24.96±2.8	61.15±13.59	13.7±2.44	0.25±0.03	17.77±1.31	0.26±0.1	3.13±0.65	0.27±0.07	0.34±0.22	122.17±4.92	164.37±29.25	14.67±0.58	1.44±0.42
AMA	I1	25.18±0.91	75±8.33	9.67±0.92	0.05±0.01	10.88±0.96	0.54±0.05	11.59±0.67	0.02±0.01	0.37±0.03	108.45±0.18	116.02±11	18.67±1.04	1.5±0.14
	I2	22.68±0.75	74.53±2.07	10.78±0.22	0.26±0.04	26.83±4.88	0.39±0.36	10.46±0.34	0.08±0.08	0.26±0.24	120.29±1.37	129.33±2.65	14.33±2.18	0.96±0
	I3	18±1	70.39±0.95	12.21±0.13	0.24±0.01	15.43±1.48	0.14±0.51	4.4±0.76	0.11±0.11	0.16±0.34	136.84±1.29	146.54±1.61	15.7±0.61	1.82±0.6
AT	I1	23.93±0.95	69.29±6.72	10.85±0.14	0.38±0.04	8.82±2.48	0.48±0.3	3.89±0.76	0.07±0.06	0.41±0.2	111.77±2.18	130.15±1.66	18.5±0.87	2.99±0.02
	I2	16.43±1.81	71.6±2.12	10.93±0.36	0.22±0.01	17.53±1.75	0.32±0.31	13.66±1.48	0.1±0.07	0.32±0.21	106.71±0.73	131.2±4.36	15.87±0.32	3.84±0.87
	I3	11.38±1.2	58.89±8.39	12.54±0.69	0.04±0.01	20.58±4.77	0.36±0.22	8.87±0.78	0.08±0.05	0.24±0.15	137.83±2.95	150.53±8.28	18.5±1.5	3.84±0.38
AS	I1	17±0	74.66±7.59	11.03±0.13	0.24±0.01	10.75±1.39	0.8±0.14	14.87±1.82	0.1±0.03	0.53±0.09	113.82±0.77	132.36±1.52	19.5±0.87	1.2±0
	I2	12.33±1.53	83.08±2.66	10.77±0.19	0.05±0.01	3.27±1.97	0.52±0.24	4.16±0.97	0.11±0.05	0.34±0.16	138.43±1.27	129.24±2.28	15.33±1.24	1.12±0.14
	I3	10.5±1.32	84.2±7.37	12.34±0.26	0.22±0.04	26.7±3.06	0.3±0.06	9.64±0.54	0.16±0.01	0.2±0.04	136.39±15.03	148.11±3.1	18.67±2.31	1.24±0.48
PSS	I1	40.1±1	72.22±5.99	11.15±0.26	0.03±0	7.57±1.83	0.67±0.03	11.91±0.88	0.04±0.01	0.11±0.02	105.96±3.62	133.85±3.07	16±1	1.44±0.24
	I2	38.43±2.55	73.54±2.28	12.89±3.08	0.04±0	0.57±0.43	0.29±0.25	11.87±0.55	0.06±0.05	0.1±0.17	131.08±8.74	154.7±36.93	14.83±2.57	1.44±0.24
	I3	40±2	70.78±9.76	11.97±0.19	0.2±0.05	17.22±3.56	0.17±0.71	9.61±0.63	0.14±0.15	0.09±0.47	129.53±1.77	143.6±2.3	16.63±0.55	2.06±1.09
PST	I1	36.43±5.39	77.99±13.17	10.89±0.27	0.18±0.02	10.2±1.04	4.33±0.2	6.87±0.65	0.07±0.04	0.82±0.14	116.72±1.2	130.68±3.29	18±1	2.18±0.03
	I2	27.7±2.45	72.21±1.89	10.74±0.13	0.04±0.01	6.33±0.58	1.32±1.73	12.78±1.02	0.91±1.21	0.86±3.79	140.72±8.76	128.85±1.56	15.73±2.83	2.4±0.72
	I3	24.67±0.58	83.98±0.64	12.67±0.53	0.03±0.01	26.52±3.61	1.1±1.06	3.99±0.85	0.21±0.22	0.67±0.7	130.12±4.17	152.03±6.41	15.33±0.58	3.52±0.5
PS	I1	26.93±5.94	74.06±5.64	11.05±0.12	0.12±0.02	7.62±1.16	0.57±0.11	7.08±1.51	0.12±0.02	0.38±0.07	127.96±8.13	132.57±1.45	16.67±0.58	1.44±0
	I2	25.33±0.58	72.3±1.47	10.25±0.94	0.03±0.01	16.38±5.1	0.47±0.12	9.85±1.01	0.1±0.03	0.31±0.08	141.97±1.4	123.03±11.28	16.83±1.44	2±0.84
	I3	18.95±0.36	63.43±3.34	12.22±0.08	0.55±0.16	18.72±0.63	0.39±1.06	4.2±0.82	0.17±0.22	0.22±0.7	156.1±0.56	146.68±1	16±1	1.26±0.25
TT	I1	55.22±2.59	69.18±4.31	10.43±1.26	0.13±0.01	7.67±1.53	0.67±0.07	11.28±0.48	0.16±0.06	0.52±0.06	137.59±3.15	125.14±15.14	19.33±1.53	1.69±0.09
	I2	36.87±1.33	72.27±0.96	10.99±0.16	0.04±0.01	19.5±0.5	0.53±0.14	10.59±0.5	0.11±0.03	0.35±0.09	142.41±18.99	131.84±1.92	13.83±3.4	2.16±0.87
	I3	30.37±1.46	66.92±7.69	12.1±0.01	0.35±0.09	18.88±5.22	0.52±0.41	8.07±1.09	0.17±0.09	0.31±0.27	131.81±10.13	145.24±0.08	13.1±1.39	1.68±0
PSBS	I1	17±1.73	76.66±4.77	10.28±0.51	0.21±0.01	12.3±1.13	0.7±0.06	12.9±1.19	0.1±0.01	0.54±0.04	94.25±3.42	123.36±6.09	20.4±0.53	2.56±0.24
	I2	12.33±2.08	71.97±1.93	10.63±0.36	0.02±0.01	16±3.61	0.64±0.37	10.6±1.79	0.15±0.08	0.46±0.24	152.53±0.53	127.53±4.34	17±1.73	1.84±0.5
	I3	9±1	64.29±7.14	12.53±0.27	0.24±0.02	15.77±4.9	0.19±0.69	21.47±0.51	0.25±0.15	0.39±0.46	128.31±0.67	150.35±3.29	17.5±2.18	1.84±0.6

I1: Control, I2: Mild drought stress, I3: Severe drought stress.

vere drought stress. Moreover, the lowest and the highest mean values of TSS were obtained from the genotypes under drought stress and control, respectively. To explore the interrelationships among the studied genotypes and traits, PCA was also performed. Afterwards, to classify the genotypes based on the PCs, the biplot of PC1 and PC2 was constructed. The results revealed that the first two components explained more than 57, 49, and 52% of the total variation in control, mild and severe drought stress, respectively (Table 5).

Under normal condition the PC1 had significant negative correlations with RWC and TA and significant positive correlations with WSC, TChl, proline, Chl*a*, Chl*b*, and TPC. Also, the second principal component had significant positive correlations with CAT, SOD, and TSS and a significant negative correlation with FY. Similar to PC1 in control conditions, PC1 in mild and severe drought stresses had significant positive correlations with TChl, proline, Chl*a*, and Chl*b* characters. According to the biplot analysis of PC1 and PC2, under normal irrigation regime, genotypes TT, MTS, and PSS were in a group. These genotypes had higher FY than the other genotypes under control conditions. In contrast, PSBS genotype formed a distinct group characterized by low value of FY. On the other hand, genotypes 'Post Siyah' PS, MSS, and AS exhibiting a high photosynthetic capacity and low value of FY, formed a distinct group (Figure 1). Under mild drought-stress, cultivar PST had low PC1 and high PC2 and also genotypes MTS and PSS with high FY, proline, WSC, and CAT were hence identified as preferable and superior genotypes for

Table 5. PCA based on the measured traits in 10 pomegranate genotypes.

Traits	Control		Mild		Severe	
	PC1	PC2	PC1	PC2	PC1	PC2
FY	0.40	**-0.76**	0.11	**-0.83**	0.26	-0.25
RWC	**-0.60**	0.35	-0.22	0.24	0.003	-0.21
WSC	**0.62**	0.051	-0.24	**-0.79**	**0.54**	0.48
CAT	-0.29	**0.63**	0.31	-0.07	0.27	**-0.82**
SOD	-0.27	**0.68**	-0.27	**0.56**	**-0.49**	0.28
TChl	**0.9**	0.41	**0.98**	-0.08	**0.97**	0.2
Proline	**0.9**	0.43	**0.96**	0.03	**0.96**	0.21
Chl*a*	**0.81**	0.16	**0.98**	-0.08	**0.97**	0.20
Chl*b*	**0.88**	0.42	**0.97**	-0.07	**0.97**	0.21
TPC	**0.89**	-0.16	0.32	**0.79**	-0.44	0.31
Cyd-3-glu	-0.02	**-0.78**	**0.65**	-0.01	-0.09	**0.85**
TSS	-0.18	**0.53**	0.19	0.29	**-0.60**	0.34
TA	**-0.71**	0.22	0.27	0.36	-0.28	**0.78**
% of variance	32.48	25.22	29.17	20.59	32.28	20.43
Cumulative %	32.48	57.70	29.17	49.76	32.28	52.71

The values higher than 0.5 are presented as bold significant.

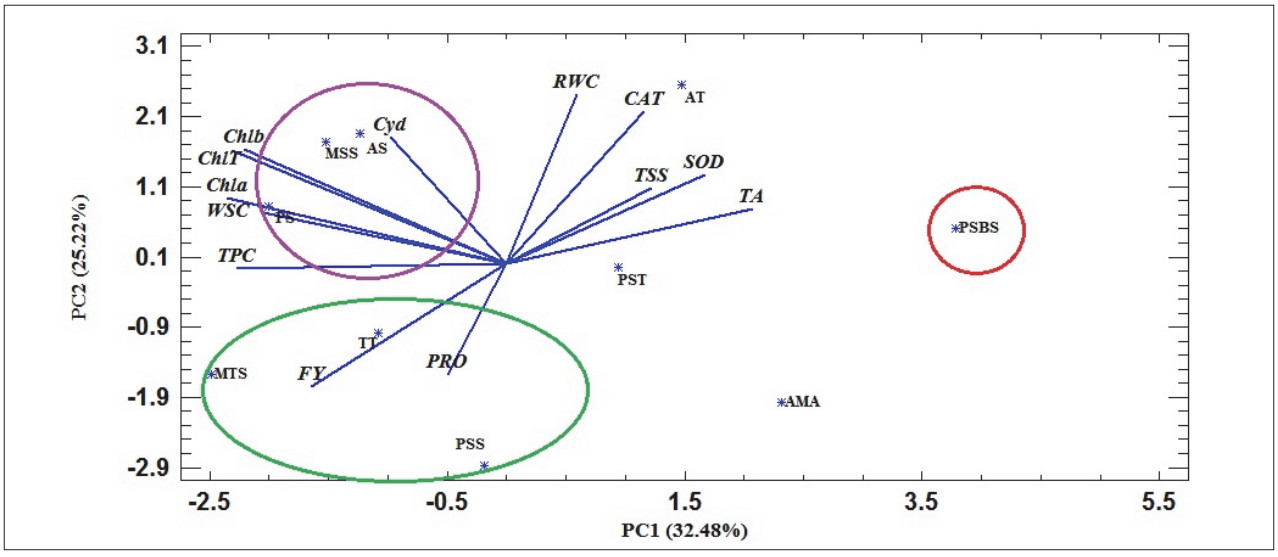

Figure 1. PCA of ten pomegranate genotypes under normal conditions. FY (fruit yield), CAT (catalase activity), Pro (proline), SOD (superoxide dismutase enzyme), RWC (relative water content), WSC (water-soluble carbohydrates), TSS (total soluble solids), TA (titratable acidity), ChlT (total chlorophyll), Chl*a* (chlorophyll *a*), Chl*b* (chlorophyll *b*), TPC (total phenolic content), Cyd (cyanidin-3-glucoside content).

this level of irrigation regime (Figure 2). Clearly, genotype PSS, TT and PST formed a distinct group characterized by high chlorophyll content (ChlT, Chl*a*, Chl*b*), FY, CAT and low WSC. Hence, three of the above-mentioned genotypes can be possibly preferable for severe stress condition (Figure 3).

Discussion

Drought tolerance mechanisms depend on a variety of factors, including physiological functions (such as osmoregulation) and secondary plant products (such as flavonoid and phenolic content); hence, identification of factors relevant to drought tolerance for screening and selection of drought tolerant genotypes are an essential prerequisite in breeding of plants/tress. The present study assessed a set of cultivars of Iranian local pomegranate in terms of field drought tolerance based on physiological and phytochemical traits. Significant differences were indicated among evaluated cultivars for physiological and phytochemical characters and FY, suggesting the presence of considerable genotypic variation in the studied germplasm collection. Therefore, this variation can be used for selecting high potential-FY and drought -tolerant cultivars.

Drought stress had significant effect on most of the traits, as it decreased FY, RWC and chlorophyll content (ChlT and Chl*b*) in both irrigation stress compared with non-stressed conditions. Meanwhile, severe and mild drought stress con-

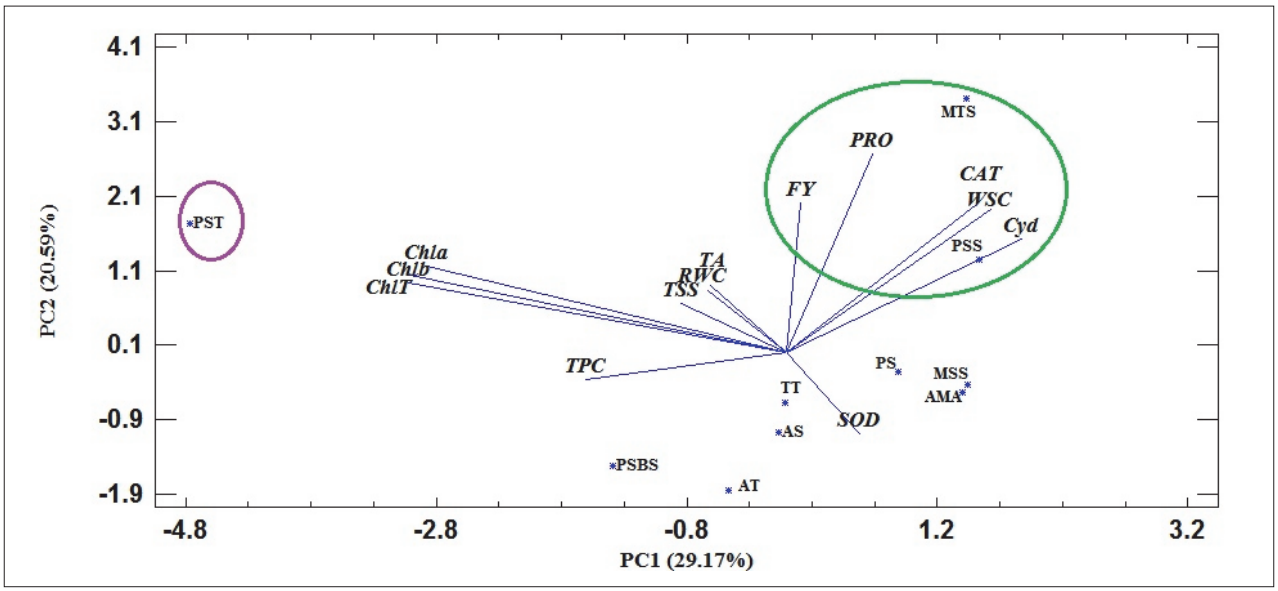

Figure 2. PCA of ten pomegranate genotypes under mild drought-stress. FY (fruit yield), CAT (catalase activity), Pro (proline), SOD (superoxide dismutase enzyme), RWC (relative water content), WSC (water-soluble carbohydrates), TSS (total soluble solids), TA (titratable acidity), ChlT (total chlorophyll), Chl*a* (chlorophyll *a*), Chl*b* (chlorophyll *b*), TPC (total phenolic content), Cyd (cyanidin-3-glucoside content).

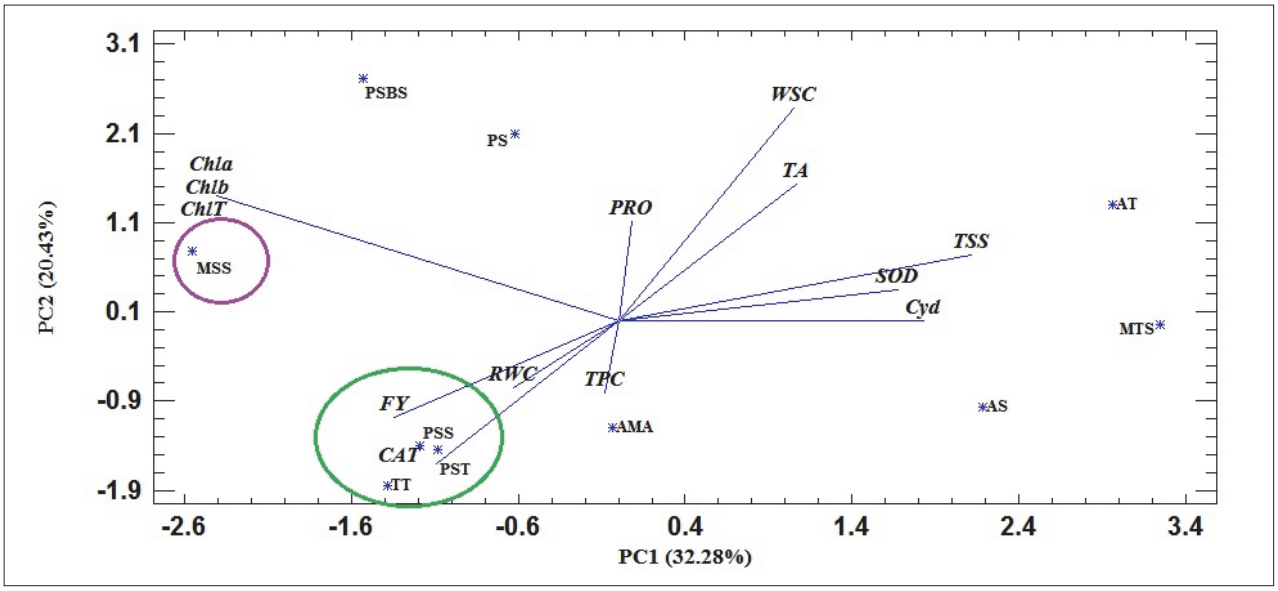

Figure 3. PCA of ten pomegranate genotypes under severe drought-stress. FY (fruit yield), CAT (catalase activity), Pro (proline), SOD (superoxide dismutase enzyme), RWC (relative water content), WSC (water-soluble carbohydrates), TSS (total soluble solids), TA (titratable acidity), ChlT (total chlorophyll), Chl*a* (chlorophyll *a*), Chl*b* (chlorophyll *b*), TPC (total phenolic content), Cyd (cyanidin-3-glucoside content).

ditions increased proline, WSC, SOD, and CAT. Consistent with our findings, several researchers reported that drought stress reduced the plant biomass in different plant species (Irani *et al*., 2015; Khan *et al*., 2015). Decreased yield may be due to a variety of factors, including considerable decrease in plant growth, photosynthesis and canopy structure, as indicated by leaf senescence during drought stress (Sankar *et al*., 2007). In addition, this can be evidently due to stomatal closure in response to low water potential, which decreases the intake of CO_2, and the consequent decline in net assimilation and photosynthesis by the leaves (Khalid, 2006; Merewitz *et al*., 2010).

RWC can be used as one of the considerable indicators of equilibrium water in plant breeding approaches under stress (Vaezi *et al*., 2010). Indeed, osmotic regulation is an indication of response to osmotic stress and when there is a water limitation caused by abiotic stress such as drought stress, osmotic potential declines and this in turn causes the reduction of RWC of the leaves (Bybordi, 2012). The decrease of osmotic potential under drought-induced stress may be accounted for by status of stomata, increased transpiration rate of the leaves and capacity of plants to better absorb soil water and to prevent water loss through stomata (Bybordi, 2012; Keyvan, 2010). The decrease of RWC under drought stress and continued to decrease with increasing drought stress in our study, was in agreement to the results reported in different species under stress condition (Geravandi *et al*., 2011; Maghsoodi and Razmjoo, 2015; Wang *et al*., 2012).

The decrease in chlorophyll content (Chl*b*, and ChlT) under drought stress has been discussed as an index of damage to chloroplasts by ROS and may be due to pigment photo-oxidation, chlorophyll degradation, reduction of Calvin cycle enzyme activity and damaged photosynthetic apparatus (Anjum *et al*., 2011). Additionally, Bota *et al*. (2004) reported that severe drought conditions limit photosynthesis due to a decline in Rubisco activity, as the activity of the photosynthetic electron transport chain is finely tuned to the availability of CO_2 in the chloroplast and change in photosystem II under drought conditions (Loreto *et al*., 1995). On the other hand, the Chl*a* content was higher than the Chl*b* content, which can be justified by faster injury to Chl*b* compared to Chl*a* under drought stress condition. Indeed, the decrease shown in Chl*b* content may suggest a structural modification of antenna under drought stress condition (Netondo *et al*., 2004); because Chl*b* is mainly associated with photosystems II (PSII) antenna, and Chl*a* is found in both the reaction centers of photosystems I (PSI) and II and photosynthetic antennas (Lichtenthaler and Buschmann, 2001). Consistent with our findings, many studies indicated that drought stress could lead to lower photosynthesis efficiency, injury to the photosynthetic apparatus particularly PSII and diminished chlorophyll content (Dhanda *et al*., 2004; Munns, 2002).

Proline content increased under drought stress is caused by a combination of increased biosynthesis and slow oxidation in mitochondria, through decreased activity of proline oxidase and increased activity of glutamate pathway enzymes such as γ-glutamyl kinase (Fujita *et al*., 2003; Manivannan *et al*., 2007). The accumulation of proline in the stressed plants may be an adaptation to dominate the stress conditions (Pirnajmedin *et al*., 2015). Proline accumulated under oxidative stress supplies energy for growth and survival and by the suppressed catabolic pathway thereby supports plants to decrease oxidative damage and tolerate stress (Pirnajmedin *et al*., 2015). This compound also improved plant stress tolerance by maintaining osmotic turgor, preventing electrolyte leakage, protecting and stabilizing membranes and enzymes during stress conditions (Hayat *et al*., 2012). Similar to our findings, some studies have documented elevated proline content under drought stress in walnut (*Juglans regia* L.) (Lotfi *et al*., 2010; Sheikh Beig Goharrizi *et al*., 2016), orchardgrass (*Dactylis glomerata*) (Saeidnia *et al*., 2018), wheat (Keyvan, 2010) and broad bean (*Vicia faba* L.) (El-Tayeb, 2006). In addition to proline, WSCs is one of the main osmolytes involved in drought stress, which could thus be considered as a physiological index for dehydration tolerance in plants (Irani *et al*., 2015). Research has indicated that concentrations of WSC were higher in drought-tolerant genotypes than in sensitive ones. The increase of WSC under drought stress is caused by the prevention of growth and hydrolysis of complex carbohydrates such as starch, thereby reducing the water potential (Yang *et al*., 2007).

The effects of stresses on antioxidant enzyme activity depend on crop species and type, duration, and intensity of drought stress (Pirnajmedin *et al*., 2015). In the present study, drought stress increased CAT and SOD activities; similar results were reported in other fruit trees (Sofo *et al*., 2005). The effect of abscisic acid (ABA) as a crucial aspect of the plant response to drought is well known (Boroomand *et al*., 2018). Plants must constantly adjust ABA levels to respond to changing physiological and environmental conditions (El-Tayeb, 2006). ABA induces up-regulation in the activities of ROS scavenging enzymes such as SOD and CAT, which protect plants cells against oxidative damage (Ye *et al*., 2011). Also, ABA enhances CAT enzyme activity under drought stress by increasing the expression and the activity of ROS network genes (Ma *et al*., 2014).

In the present study, with increasing the drought stress level, the Cyd-3-glu content increased; while TPC increased under mild drought stress, and it had no significant difference with severe drought stress. It seems that severe drought stress might inhibit the synthesis of phenolic compounds of aril extract which was in agreement with the results obtained in grapevine (Król *et al*., 2014) and *Achillea* (Gharibi *et al*., 2016), respectively. The plant phenylpropanoid pathway genes expression and following accumulation of flavonoids and phenolic compounds may be closely related to drought tolerance which needs high energy inputs (Ma *et al*., 2014). However, under low to moderate drought stress condition, it seems that the amount of energy inputs are sufficient to induce and up-regulate genes expression which involved in polyphenols pathway, but these energy-intensive processes are limited under severe stress condition (Król *et al*., 2014).

Our results showed that TA was enhanced by drought stress that agree with various reports in pomegranate, apple, grape berries and peach (Laribi *et al*., 2013; Mpelasoka *et al*., 2001). It may thus be argued that the decrease in TSS levels under drought stress may be due to the limited carbohydrate availability as a consequence of photosynthesis decline (Goicoechea *et al*., 2005). Citric and malic acids are the major organic acids in pomegranate fruit which accounts for most of the TA (Hasnaoui *et al*., 2011). Despite some studies indicated a reduction or increase of TA in response to drought stress (Laribi *et al*., 2013), whereas others found no effect on this parameter (Intrigliolo *et al*., 2011; Marsal *et al*., 2012). Although this study showed that TA in fruits was different among irrigation treatments, no difference in TA was discernible between control and mild drought stress.

The distribution of genotypes on the biplot of PCA under control condition revealed a high genetic variation among the Iranian pomegranate genotypes; this diversity could be due

to genetic and interaction between genetic and environmental factors (Boroomand *et al*., 2018; Farajpour *et al*., 2017; Hassanabadi *et al*., 2019). According to the results, similar traits had significant correlation with PC1 in the three irrigation regimes, indicating that the PC1 could be attributed to the genetic factors. Based on the clustering by PCA under control condition TT, MTS and PSS genotypes classified in a group; these genotypes had higher proline and chlorophyll contents, and WSC in their leaves. It is worth mentioning that the more productive genotypes in the control condition also tended to be more productive in the drought treatments. So, MTS and PSS genotypes, with high FY, proline content, WSCs content and CAT activity, were identified as preferable and suitable genotypes for both normal and mild stress conditions. In addition, PSS genotype maintained its FY in mild and severe drought stresses compared to the control, which suggested as a preferable and superior genotype for cultivation in areas under drought stress.

Conclusions

The present research conducted a comparison among ten Iranian pomegranate genotypes under different irrigation regimes in terms of physiological, phytochemical and phytonutrients characters. FY was significantly decreased in mild and severe drought stress conditions by 23.71 and 40.40%, respectively, compared to the control. The development of genotypes with high yield under normal conditions and maintaining its yield under biotic and abiotic stress conditions is the main purpose of breeding programs. Hence, results of the present study suggest that proline content, WSC and CAT can be used for discriminating pomegranate drought-tolerant genotypes with high yield potential. Accordingly, MTS and PSS genotypes were found to be more drought-tolerant with high yield potential; so, these genotypes suggested as a preferable and superior genotypes for cultivation in areas under drought stress.

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