Morphological and molecular responses of sunflower (*Helianthus annuus* L.) lines to drought stress

Mozhgan Sarvari[1], Reza Darvishzadeh[2*], Roghayeh Najafzadeh[3]

[1]*Department of Biology, Urmia University, Urmia, Iran.*
[2]*Department of Plant Breeding and Biotechnology, Urmia University, P. O. Box: 165, Urmia, Iran.*
[3]*Department of Medicinal Plants, Shahid Bakeri Higher Education Center, Urmia University, Miyandoab, Iran.*
[*]*Corresponding author, Email: r.darvishzadeh@urmia.ac.ir. Tel: +98-9149734458. Fax: +98-4432752746.*

Received: 21 Aug 2016; Accepted: 5 Apr 2017.

Abstract

Drought stress is a serious adverse factor limiting growth and productivity of sunflower. Evaluation of defense systems is important for producing resistant cultivars. In this study, some agro-morphological and molecular characteristics of six sunflower lines were evaluated under normal and irrigation at 40 and 60% of field capacity in randomized complete block design (RCBD). The results showed that drought stress affected the measured traits compared to the control. However, the lines showed different responses to the stress. According to the results, C100, C104 and LR55 lines had better growth, yield and drought tolerance indices in water stress conditions. In addition, drought stress increased total protein content of the lines especially resistant ones (C100, C104 and LR55). The protein SDS-PAGE electrophoresis pattern also showed that drought stress caused the accumulation of low molecular weight proteins specially the bands 69.3 and 70.5 kDa were produced in the resistant lines C100, C104 and LR55 under drought stress conditions. Moreover, more protein bands were observed in the resistant lines, C104 and LR55, under normal compared to the stress conditions. It seems that the protein bands 69.3 and 70.5 kDa are resistance-related proteins that were expressed in the tolerant lines. The findings of this study can be useful in sunflower breeding programs for producing resistant cultivars to drought stress.

Key words: Abiotic stress, Drought tolerance indices, Oil crops, SDS-PAGE, Seed yield.

INTRODUCTION

Drought stress known as one of the important adverse factors causesing different morphological, physiological and biochemical changes and limits plant growth and productivity (Reddy *et al*., 2004; Anjum *et al*., 2011; Andrade *et al*., 2013). The most important effects of drought are the reduction of photosynthesis which happens due to stomata closure, reduction of plant growth, lack of required photosynthetic compounds to fill the grains and reduction of grain filling period (Reddy *et al*., 2004; Nayyar and Gupta, 2006; Cao *et al*., 2011; Anjum *et al*., 2011). Sunflower (*Helianthus annuus* L.) is one of the most important oil seed crops (Hussain *et al*., 2015) that its production is greatly affected by drought stress (Pasda and Diepenbrock, 1990; Rauf, 2008; Hussain *et al*., 2014) if occurred at the critical stages of growth and development (Hussain *et al*., 2014).

Development of stress resistant crops is a major goal for improving food security. From an agronomic perspective, the most desirable type of stress resistance is represented by genotypes that produce acceptable yield under stress conditions, yet maintain high yield potential under non-stress conditions (Chimenti *et al*., 2002). Several different tolerance indices have been reported in the literature, including stress susceptibility index (SSI) (Fischer and Maurer, 1978), tolerance index (TOL) (Rosielle and Hamblin, 1981), stress tolerance index (STI) (Fernández, 1992), mean productivity

Table 1. Characteristics of the studied sunflower lines.

Line	Origin	Type	Characteristics Poormohammad Kiani *et al*. (2007, 2008, 2009)
C104	France	RIL	Good water status and osmotic adjustment as well as biomass and yield under drought stress
LR25	France	RIL	Good water status and osmotic adjustment as well as biomass but it lost grain weight under drought stress
LR4	France	RIL	Average water status and osmotic adjustment as well as biomass and yield under drought stress
C100	France	RIL	Good water status and osmotic adjustment but low in yield under both well-watered and drought stress
LR55	France	RIL	The lowest water status traits and osmotic adjustment as well as biomass and yield under drought stress
RHA266	USA	BL	Low water status traits and osmotic adjustment and average biomass and yield under drought stress

BL: Breeder's line; RIL: Recombinant inbred line.

(MP) (Rosielle and Hamblin, 1981), geometric mean productivity (GMP) (Fernández, 1992; Kristin *et al*., 1997), harmonic mean (HM) (Jafari *et al*., 2009), yield index (YI) (Gavuzzi *et al*., 1997) and yield stability index (YSI) (Bouslama and Schapaugh, 1984) which identify susceptible and tolerant genotypes based on their yield under stress and non-stress conditions. The best selection index must be able to distinguish genotypes with uniform superiority in both mentioned conditions.

Many biochemical and physiological changes occur in response to drought stress in a wide range of plant species. Changes in protein production or its destruction are basic metabolic processes that may be affected by drought stress (Ouvrard *et al*., 1996). There is profound evidence related to protein aggregation induced by drought and it has a relationship with physiological adaptations to water scarcity (Riccardi *et al*., 1998). Electrophoresis of proteins is a powerful tool for the identification of genetic variation and the SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is particularly considered as a reliable technology for the separation of proteins (Javid *et al*., 2004; Iqbal *et al*., 2005b). Researchers use electrophoresis techniques in a broad range as a useful method to separate plant proteins and other macromolecules to study their variability.

Analysis of morphological traits is useful for studying plant adaptations to environmental stresses such as water deficit (Andrade *et al*., 2013). Although there is a plenty of literature available on morphological traits, drought tolerance indices and protein content of sunflower under drought stress (Raymond *et al*., 1994, 1995; Petcu *et al*., 2001; Chimenti *et al*., 2002; Tahir *et al*., 2002; Agele, 2003; Valinezhad *et al*., 2004; Igbal *et al*., 2005a; Daneshian *et al*., 2005, 2006; Erdem *et al*., 2006; Jafarzadeh Bilvari *et al*., 2006; Safari, 2006; Khalilvand and Yarnia, 2007; Daneshian and Jonoubi, 2008; Rauf and Sadaqat, 2008; Nezami *et al*., 2008; Ahmad *et al*., 2009; Darvishzadeh *et al*., 2010; Rahimizadeh *et al*., 2010; Soleimanzadeh *et al*., 2010; Alahdadi *et al*., 2011; Mobasser and Tavassoli, 2013; Hemmati and Soleymani, 2014; Safavi *et al*., 2015; Parveen *et al*., 2015), but information regarding the effect of water deficit simultaneously on both morphological and molecular characteristics like protein electrophoresis pattern is scanty. Therefore, this study was mainly conducted to determine water deficit influence on agro-morphological and molecular characteristics of sunflower lines in order to use the information in breeding programs for producing resistant cultivars to drought stress.

MATERIALS AND METHODS

Plant material

Six oilseed sunflower lines were selected from 125 inbred lines based on their different responses to drought stress (Poormohammad Kiani *et al*., 2007, 2008, 2009). Prominent features of the studied lines are summarized in Table 1. The seeds of the lines were planted in plastic pots with a diameter of 12 and height of 14 cm, filled with a soil and peat moss (3:1). The experiment was conducted based on randomized complete block design (RCBD) with three replications. The plants grew in a greenhouse with the condition of 12 h light and the maximum and minimum temperatures of 28 and 12 ºC, respectively. Plants were irrigated identically for all treatments from the beginning of

Table 2. Drought tolerance indices of the studied sunflower lines.

Drought tolerance indices	Equation	Reference
Stress susceptibility index (SSI)	$SSI = \frac{1-(\frac{Y_S}{Y_P})}{1-(\frac{\bar{Y}_S}{\bar{Y}_P})}$	Fischer and Maurer (1978)
Tolerance index (TOL)	$TOL = Y_P - Y_S$	Rosielle and Hamblin (1981)
Stress tolerance index (STI)	$STI = \frac{(Y_S)(Y_P)}{(\bar{Y}_P)^2}$	Fernández (1992)
Mean productivity (MP)	$MP = \frac{Y_S + Y_P}{2}$	Rosielle and Hamblin (1981)
Geometric mean productivity (GMP)	$GMP = \sqrt{(Y_S)(Y_P)}$	Fernández (1992) and Kristin *et al*. (1997)
Harmonic mean (HM)	$HM = \frac{2(Yp \times Ys)}{Yp + Ys}$	Jafari *et al*. (2009)
(YI) Yield index	$YI = \frac{Y_S}{\bar{Y}_S}$	Gavuzzi *et al*. (1997)
Yield stability index (YSI)	$YSI = \frac{Y_S}{Y_P}$	Bouslama and Schapaugh (1984)

Y_P: Yield in non-stress conditions; Y_S: Yield in stress conditions; $\bar{Y}_P$: Average yield of all lines in non-stress condition; $\bar{Y}_S$: Average yield of all lines in stress condition.

the planting time until the complete establishment of sunflower plants (eight-leaf (V8) stage). When plants grew into the 8 leaf stage, control pots were maintained at the field capacity, and other pots were irrigated at the 40% of field capacity (Poormohammad Kiani *et al*., 2007) until the end of growth period. It should be noted that in the drought stress experiment, firstly plants were irrigated at 60% of field capacity during 5 days and leaf samples was taken from each pot. Then, the pots were irrigated at 40% of field capacity until the end of the growth period.

Evaluation of morphological and molecular characteristics

Some morphological traits such as plant height (cm), stem diameter (cm), number of leaves, leaf length and width (cm), petiole length (cm), aerial plant dry weight (g), capitulum diameter (cm) and weight (g) were measured in full bloom. To obtain the dry weight of foliage, the materials were placed in an oven at 70 ºC for 72 h to dry completely and then weighed. In addition, seed traits such as the number of seeds, seed weight, 100 seed and kernel weight (g) were measured after harvesting. Moreover, the seeds of each capitulum were collected after ripening, then dried and weighed and quantitative drought tolerance indicators for each line were calculated according to the formulas presented in Table 2. In order to evaluate protein electrophoresis pattern, the leaf soluble protein content was measured by Lowry *et al*. (1951) method. About 0.25 g of leaves was homogenized in liquid nitrogen and 4 ml Tris buffer 0.2 M was added. Then the whole volume was brought to 100 ml with distilled water and acidity was set in pH=8. The extractions were held at 4 ºC for 24 h and then centrifuged at 4000 rpm for 20 min. An aliquot of 4 ml of the solution containing (5 ml of the solution containing 2 g Na_2CO_3, 0.4 g NaOH and 0.02 g potassium sodium tartrate tetrahydrate cryst dissolved in 100 ml distilled water plus 1 ml of the solution containing 0.5 g $CuSO_4.5H_2O$ dissolved in 100 ml distilled water) were added to 1 ml of supernatant. After 10 min, 1.5 ml of Folin-phenol reagent diluted 1:9 was added to the extracts. The samples were held in dark for 30 min. After the absorption of samples was read at 660 nm wave length with the spectrophotometer, the protein content was calculated. Finally, the protein banding pattern was evaluated using SDS-PAGE and silver staining method (Kyte, 1995; Wester *et al*., 1999).

The protein band scoring were done as presence (1) or absence (0) for each band row. The RM of the protein bands was measured as the following formula:

$$Rm = \frac{\text{Migration distance of the protein band}}{\text{Migration distance of the dye front}} \quad (1)$$

Statistical analysis

All data were statistically analyzed using GLM (General linear model) and mean comparisons were made using Tukey test using SAS software. Type I error of Tukey's test is lower than other tests such as Duncan's multiple test (Einot and Gabriel, 1975). Also, correlation among factors was calculated by SPSS software. Three-dimensional diagram, biplot graph and cluster analysis was performed in Stargraphic and Minitab 17.

RESULTS AND DISCUSSION

Morphological traits

Analysis of variance revealed significant differences ($P \leq 0.05$) among the lines in most studied traits in both non-stress and water-stress conditions (Table 3). As shown in Table 4, the mean value of the studied traits of the lines decreased significantly in drought stress compared to non-stress conditions. In non-stress conditions, the highest plant height, stem diameter, leaf length and width, petiole length, aerial plant dry weight, capitulum or head diameter and weight, capitulum dry weight, 100 seed weight and seed kernel weight was observed in C104 line. The maximum number of seeds per capitulum and seed weight per capitulum was obtained in C100 and LR55 lines, respectively. In water stress conditions, the maximum values for plant height, number of leaves, leaf width, capitulum diameter and weight, seed weight per capitulum, 100 seed weight and seed kernel weight was observed in lines C104 and then in C100. According to the results, lines C100, C104 and LR55 had better growths and performances. These lines particularity had the highest plant height and weight, leaf characteristics, number of seeds, capitulum and seed weight than other lines in both non-stress and water stress conditions.

Drought stress affects growth, dry mater and harvestable yield in plants (Anjum *et al*., 2011). Shoot internode shortening and subsequently lower height of shoot are important adaptation mechanisms in plants to drought stress (Gupta, 1997). Cell growth is the most sensitive process affected by water stress. With decreasing cell growth, organ size is limited and causes a reduction in plant height and leaf size (Hsiao, 1973). Goksoy *et al*. (2004) reported that plant height was limited in sunflower under drought stress. Water stress also reduces the number of leaves significantly that consequently, reduces the number of photosynthetically active leaves (Goksoy *et al*., 2004). Due to drought stress and probably stomata closure, plant photosynthesis is reduced leading to a reduction in vegetative growth and low stem diameter.

In addition, a common adverse effect of water stress on plants is reduction in fresh and dry biomass production (Zhao *et al*., 2006). Increasing grain weight can be obtained by increasing the rate and duration of grain filling (Zaffaroni and Schneiter, 1989) which is affected by drought stress. D'Andria *et al*. (1995) reported that yield components such as seed weight in sunflower is high under well-watered condition compared to drought-stressed one. Petcu *et al*. (2001) showed that grain yield of sunflower hybrids was affected by drought stress. In addition, Chimenti *et al.* (2002) and Erdem *et al*. (2006) indicated that grain yield and 1000 grain weight decreased with increasing drought stress. Also, Khalilvand and Yarnia (2007) found that drought stress reduced morphological characteristics, grain weight and yield of sunflower. Nezami *et al*. (2008) have indicated that plant height, dry matter; stem diameter, capitulum or head size, seed number per head, weight of 100 grains and head weight declined in dry and semi-dry conditions. Moreover, Ahmad *et al.* (2009) reported that plant height and dry matter decreased with increasing water stress severity. Also, Soleimanzadeh *et al*. (2010) reported that drought stress significantly decreased plant height, head diameter, seed number, weight of 100 grain and yield of sunflower plants. Nevertheless, in a study performed to investigate the effect of drought on sunflower yield, it was revealed that irrigation cycle had a significant effect on yield and it decreased the number of seeds per capitulum (Rahimizadeh *et al*., 2010). In other studies decreasing growth characteristics in sunflower has also been reported under drought stress (Vivek and Chakor, 1992; Tahir *et al*., 2002; Agele, 2003; Goksoy *et al*., 2004; Turhan and Baser, 2004; Valinezhad *et al*., 2004; Daneshian *et al*., 2005, 2006; Jafarzadeh Bilvari *et al*., 2006; Safari, 2006; Poormohammad Kiani *et al*., 2009; Daneshian and Jonoubi, 2008; Pirzad and Shokrani, 2012; Parveen *et al*., 2015). Totally, drought stress reduces plant growth and the number of leaves that it significantly reduces photosynthesis and productivity.

Drought tolerance indices

In order to screen drought-tolerant lines, different related indices were calculated, based on the performance of

Table 3. Variance analysis of morphological traits of the studied sunflower lines under non-stress and water-stress conditions.

Source of variations	df	PH		SD		LL		LW		PL		CD	
		WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS
line	5	1696.02*	381.55*	1.99*	0.06^{ns}	7.66*	2.21^{ns}	9.71*	1.9*	3.87*	0.48^{ns}	9.06*	1.96*
Error	12	261.58	37.12	0.17	0.08	1.02	0.91	1.16	0.42	0.29	0.17	0.85	0.21
CV		12.96	9.39	10.1	9.16	7.52	9.95	12.89	11.04	8.82	11.53	9.78	7.57

Source of variations	Item	NL		APDW		CW		NS		SW		100SW		SKW	
		WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS
line	df_N	2.53	2.64	3.39	3.46	3.54	3.92	3.76	2.81	2.38	2.97	3.42	2.81	3.35	2.85
	df_D	5.39	5.67	7.52	7.71	7.92	8.94	5.27	6.06	5.05	6.48	7.62	6.08	7.42	6.17
	F-value	0.28^{ns}	0.504^{ns}	0.023*	0.048*	0.024*	0.002*	8.06*	3.10^{ns}	4.22*	2.22^{ns}	0.04*	0.14*	0.2^{ns}	0.15^{ns}

PH: Plant height; SD: Stem diameter; NL: Number of leaves; LL: Leaf length; LW: Leaf width; PL: Petiole length; APDW: Aerial plant dry weight; CD: Capitulum diameter; CW: Capitulum weight; NS: Number of seeds; SW: Seed weight; 100SW: 100 seed weight; SKW: Seed kernel weight; Well-water (WW); Water-stress (WS).
df: Degrees of freedom, cv: Coefficient of variation, df_N: Numerator degree of freedom, df_D: Denominator degree of freedom.
^{ns}: Not significant; *: Significant at probability level of 0.05.

Table 4. Morphological traits of the studied sunflower lines under non-stress and water-stress conditions.

Line	PH (cm)	SD (cm)	NL	LL (cm)	LW (cm)	PL (cm)	APDW (g)	CD (cm)	CW (g)	NS	SW (g)	100SW (g)	SKW (g)
Well-water (WW)													
LR4	118.67±2.06	3.5±0	23.3±1.36	11.4±0.44	6.7±0.26	4.5±0.43	16.3±3.44	9.5±0.44	8.3±2.16	219.5±39.50	5.42±1.24	3.3±0.04	2.6±0.03
LR25	89.67±25.8	3.2±0.25	21±0.89	12±1.24	7.1±1.29	5.7±0.44	12.4±2.46	7.9±0.56	6.0±2.15	144±54.0	3.98±1.33	4±0.06	3.1±0.05
LR55	147.8±7.62	4.1±0.10	23.7±1.36	14.0±0.47	8.5±0.36	5.7±0.46	21.5±3.11	9.5±0.44	9.0±0.90	381.33±32.38	15.15±1.11	4.3±0.03	3.3±0.02
RHA266	109.8±4.94	4.3±0.51	25.7±2.73	13.2±1.43	7.5±0.89	6±0.62	29.5±5.34	7.4±1.14	5.2±1.61	15±6.99	0.57±0.31	2.8±0.11	2.2±0.09
C100	129.67±21.47	3.8±0.51	21±5.58	14±0.80	8.7±1.39	6.6±0.45	18.5±7.96	10±1.34	9.2±2.23	478.5±127.51	10.4±6.58	3.7±0.07	2.7±0.03
C104	152.67±6.34	5.5±0.44	25.3±1.36	15.9±0.47	11.6±0.95	7.9±0.43	29.8±2.23	12.3±0.51	16.1±3.30	202±68.38	8.99±2.52	5.2±0.04	3.5±0.04
Water-stress (WS)													
LR4	96.33±2.87	2.8±0.25	22.3±1.36	9.36±0.88	5.6±0.71	3.5±0.34	4.5±0.48	5.6±0.25	2.2±0.18	191±27.73	2.91±0.36	1.6±0.008	1.2±0.005
LR25	78.83±6.99	3.1±0.10	21.3±0.51	8±1.13	4.8±0.66	3.1±0.65	4.7±0.88	6.2±0.34	3.9±0.20	103.5±37.50	2.48±0.37	2.3±0.04	1.8±0.03
LR55	92.67±5.24	3±0	23.7±1.36	10.1±0.49	5.8±0.42	3.2±0.26	5.9±0.45	5.5±0.22	3.1±0.25	186.33±15.90	3.48±0.37	2.1±0.01	1.6±0.01
RHA266	75.67±4.03	3.2±0.25	23.3±3.14	9.47±0.72	5.3±0.03	3.8±0.22	7.79±1.73	5.7±0.25	2.8±0.44	9±8.00	0.23±0.21	1.6±0.11	1.3±0.07
C100	99.33±11.67	3.2±0.41	24.3±5.75	10.4±0.71	6.7±0.56	4.1±0.04	7.4±2.12	7.7±0.68	4.6±0.61	161.67±64.77	3.41±1.35	2±0.03	1.6±0.01
C104	103.33±10.67	3.2±0.25	25±1.54	10.0±0.98	6.9±0.74	4±0.41	6.7±2.33	6.2±0.51	3.7±0.51	160±31.43	3.98±1.18	2.7±0.03	2±0.02

Traits: see the Table 3.

Table 5. Drought tolerance indices of the studied sunflower lines under water-stress conditions.

Line	Yp	Ys	SSI	TOL	STI	MP	GMP	HM	YI	YSI
LR4	5.43	2.92	0.73	2.51	0.29	4.18	3.98	3.80	1.06	0.54
LR25	3.98	2.48	0.60	1.50	0.18	3.23	3.14	3.06	0.90	0.62
LR55	15.15	3.48	1.22	11.67	0.96	9.32	7.26	5.66	1.26	0.23
RHA266	0.58	0.24	0.93	0.34	0.00	0.41	0.37	0.34	0.09	0.41
C100	10.40	3.41	1.07	6.99	0.64	6.91	5.96	5.14	1.24	0.33
C104	8.99	3.98	0.89	5.01	0.65	6.49	5.98	5.52	1.45	0.44

Indices: see the Table 2.

the lines in non-stress and stress conditions (Table 5). The maximum yield in non-stress (Yp) and water stress conditions (Ys) was observed in LR55, C100 and C104 lines. Totally, high yield and productivity of these lines in both non-stress and stress conditions indicate their greater tolerance to the stress. Furthermore, the least stress susceptibility index (SSI) was observed in LR25 line. The highest tolerance index (TOL) and stress tolerance index (STI) were obtained in LR55 line. In addition, LR55 and then C100 and C104 lines had the highest mean productivity (MP), geometric mean productivity (GMP) and harmonic mean (HM). In terms of yield index (YI) and yield stability indicator (YSI), the maximum value was related to lines C104 and LR25, respectively.

Crop yield is influenced by environmental conditions, genetic structure and their interaction. Among environmental stresses, water deficit is a main factor, limiting sunflower production in arid and semi-arid regions (Chimenti and Hall, 2002). Tahir *et al*. (2002) and Rauf and Sadaqat (2008) reported that sunflower yield is reduced in water stress conditions. Igbal *et al*. (2005a) reported a trend in yield decline and reduction of yield components due to water stress treatments. In a experiment conducted by Daneshian and Jonoubi (2008), dehydration stress caused a reduction in yield and yield components in sunflower hybrids. Alahdadi *et al*. (2011) and Hemmati and Soleymani (2014) observed that water stress significantly decreased seed yield and yield components. In the experiment of Mobasser and Tavassoli (2013) water stress decreased yield components in sunflower, however, the studied cultivars showed different reactions to the stress. Different strategies have been proposed for the selection of the resistant genotypes, as Fisher and Maurer (1978) reported that achene yield in water stresseconditions could be considered as a drought-resistance index. Darvishzadeh *et al*. (2010) showed that there were significant differences among sunflower genotypes for all drought tolerance indices, except for SSI and YSI. They suggested that tolerance indices like MP, GMP and HM had significant and positive correlations with performance at non-stress and stress conditions. Thus, these indices are suitable for screening sunflower drought tolerant genotypes. Moreover, Safavi *et al*. (2015) reported that STI, MP, GMP, HAR and YI exhibited a high correlation with seed yield. These indices were recognized best for selecting cultivars with high yield potential in both the non-stress and stress conditions. In their study, some genotypes had the highest drought tolerance indices and yield. According to the results, C100, C104 and LR55 genotypes had the highest yield and were more tolerant to drought stress than other lines

Correlation analysis

In non-stress conditions, there were positive significant correlations ($P \leq 0.05$, r≥0.81) between plant height and capitulum diameter, leaf length and width and aerial plant dry weight with stem diameter, leaf number and shoot dry weight, capitulum diameter and 100 seed weight with leaf width, capitulum dry weight and 100 seed weight, the number of seeds and seed weight. In addition, there were positive significant correlations ($P \leq 0.01$, r≥0.91) between leaf width and petiole length with leaf length and capitulum diameter, capitulum diameter and capitulum dry weight, 100 seed weight and seed kernel weight. In stress conditions, leaf width and the number of seeds and seed weight with plant height, leaf length and width with the number of leaves, leaf length and leaf width, capitulum diameter and capitulum dry weight, the number of seeds and seed weight showed significant positive correlations ($P \leq 0.05$, r≥0.81). Also, 100 seed weight showed a significant positive correlation with seed kernel weight ($P \leq 0.01$, r≥0.91) (Table 6).

Correlation analysis between yield in non-stress and water-stress conditions and quantitative stress-tolerance indices were calculated and summarized in Table 7. Indicators that are significantly correlated

Table 6. Correlation coefficients between morphological traits of the studied sunflower lines under non-stress and water-stress conditions.

Trait	PH	SD	NL	LL	LW	PL	APDW	CD	CW	NS	SW	100SW	SKW
Well-water (WW)													
PH	1												
SD	0.728^{ns}	1											
NL	0.410^{ns}	0.741^{ns}	1										
LL	0.788^{ns}	0.884*	0.405^{ns}	1									
LW	0.771^{ns}	0.894*	0.367^{ns}	0.956**	1								
PL	0.512^{ns}	0.805^{ns}	0.260^{ns}	0.918**	0.918**	1							
APDW	0.532^{ns}	0.888*	0.887*	0.709^{ns}	0.622^{ns}	0.614^{ns}	1						
CD	0.816*	0.683^{ns}	0.177^{ns}	0.714^{ns}	0.850*	0.626^{ns}	0.316^{ns}	1					
CW	0.797^{ns}	0.786^{ns}	0.295^{ns}	0.780^{ns}	0.915**	0.715^{ns}	0.430^{ns}	0.979**	1				
NS	0.517^{ns}	-0.106^{ns}	-0.527^{ns}	0.264^{ns}	0.221^{ns}	0.094^{ns}	-0.313^{ns}	0.443^{ns}	0.291^{ns}	1			
SW	0.767^{ns}	0.203^{ns}	-0.175^{ns}	0.493^{ns}	0.452^{ns}	0.222^{ns}	-0.049^{ns}	0.575^{ns}	0.496^{ns}	0.849*	1		
100SW	0.617^{ns}	0.574^{ns}	0.042^{ns}	0.694^{ns}	0.815*	0.661^{ns}	0.162^{ns}	0.787^{ns}	0.840*	0.302^{ns}	0.600^{ns}	1	
SKW	0.524^{ns}	0.388^{ns}	-0.065^{ns}	0.532^{ns}	0.646^{ns}	0.470^{ns}	0.016^{ns}	0.648^{ns}	0.693^{ns}	0.323^{ns}	0.648^{ns}	0.964**	1
Water-stress (WS)													
PH	1												
SD	-0.136^{ns}	1											
NL	0.646^{ns}	0.493^{ns}	1										
LL	0.675^{ns}	0.181^{ns}	0.889*	1									
LW	0.870*	0.331^{ns}	0.909*	0.830*	1								
PL	0.464^{ns}	0.544^{ns}	0.756^{ns}	0.651^{ns}	0.772^{ns}	1							
APDW	0.006^{ns}	0.786^{ns}	0.718^{ns}	0.612^{ns}	0.48`^{ns}	0.755^{ns}	1						
CD	0.343^{ns}	0.527^{ns}	0.332^{ns}	0.299^{ns}	0.523^{ns}	0.585^{ns}	0.396^{ns}	1					
CW	0.205^{ns}	0.697^{ns}	0.311^{ns}	0.134^{ns}	0.419^{ns}	0.332^{ns}	0.342^{ns}	0.865*	1				
NS	0.827*	-0.529^{ns}	0.208^{ns}	0.372^{ns}	0.479^{ns}	-0.085^{ns}	-0.461^{ns}	0.119^{ns}	0.057^{ns}	1			
SW	0.858*	-0.172^{ns}	0.381^{ns}	0.368^{ns}	0.635^{ns}	0.052^{ns}	-0.293^{ns}	0.301^{ns}	0.372^{ns}	0.909*	1		
100SW	0.383^{ns}	0.428^{ns}	0.360^{ns}	0.006^{ns}	0.428^{ns}	0.051^{ns}	-0.026^{ns}	0.223^{ns}	0.582^{ns}	0.281^{ns}	0.636^{ns}	1	
SKW	0.294^{ns}	0.535^{ns}	0.338^{ns}	-0.023^{ns}	0.391^{ns}	0.067^{ns}	0.048^{ns}	0.315^{ns}	0.685^{ns}	0.184^{ns}	0.565^{ns}	0.987**	1

Traits: see the Table 3.

^{ns}: Not significant; *: Significant at probability level of 0.05, **: Significant at probability level of 0.01.

Table 7. Correlation coefficients between yield and drought tolerance indices of the studied sunflower lines under water-stress conditions.

Trait	Y_P	Y_S	SSI	GMP	MP	HM	TOL	STI	YI	YSI
Y_P	1									
Y_S	0.792^{ns}	1								
SSI	0.713^{ns}	0.208^{ns}	1							
GMP	0.961^{**}	0.929^{**}	0.534^{ns}	1						
MP	0.992^{**}	0.865^{*}	0.631^{ns}	0.989^{**}	1					
HM	0.90^{*}	0.978^{**}	-0.404^{ns}	0.985^{**}	0.949^{**}	1				
TOL	0.981^{**}	0.659^{ns}	0.813^{*}	0.890^{*}	0.948^{**}	0.799^{ns}	1			
STI	0.991^{**}	0.807^{ns}	0.720^{ns}	0.964^{**}	0.987^{**}	0.910^{*}	0.966^{**}	1		
YI	0.790^{ns}	1.00^{**}	0.207^{ns}	0.928^{**}	0.863^{*}	0.977^{**}	0.656^{ns}	0.805^{ns}	1	
YSI	-0.706^{ns}	-0.194^{ns}	-1.00^{**}	-0.523^{ns}	-0.623^{ns}	-0.392^{ns}	-0.808^{ns}	-0.713^{ns}	-0.193^{ns}	1

Indices: see the Table 2.
^{ns}: Not significant; *: Significant at probability level of 0.05, **: Significant at probability level of 0.01.

with yield in desired and water stressed environments are considered as the best tolerance indices. These indicators will be able to separate genotypes with high yield in both conditions (Imam Jomeh, 1999; Maroufi, 1999). The results of correlation analysis showed that there was a significant and positive correlation between GMP, MP and HM indices with performance at non-stress and water-stress conditions. Therefore, we can consider these indices as the best indicators for screening drought tolerant genotypes with high yield. In the studies conducted by Imam Jomeh (1999) and Farshadfar *et al*. (2002) on chickpea lines, MP, HM, GMP and STI indices were introduced as the most appropriate drought tolerance indices. In addition, a significant correlation was observed between TOL and STI with yield only in non-stress conditions. Also, SSI showed a negative and significant correlation with YSI while its correlation was not significant with yield in stress and non-stress conditions. Fischer and Maurer (1978) showed that the evaluation of genotypes using SSI alone could classify the test materials according to their resistance and susceptibility to the stress. In other words, this indicator can identify the susceptible and resistant cultivars regardless of their potential performances. Moreover, correlation between SSI and TOL was not significant. YI index showed a significant correlation with yield in stress conditions but its correlation was not significant with yield in non-stress conditions. Totally, there was a significant correlation between some characteristics such as MP, HM and GMP indices with performance. Hence, these indices can be considered as the best indicators for screening drought tolerant genotypes with high yield.

Cluster analysis

After identifying the best drought tolerance indicators (MP, HM and GMP), in order to select drought tolerant lines with high yield in non-stress and water-stress conditions, a three-dimensional diagram was drawn (Figure 1). The diagram demonstrated the relationship between three variables: Yp and Ys and one of the selected indicators for tolerance, in which X axis displays yield in water stress conditions (Ys), Y-axis displays yield in non-stress condition (Yp), and Z-axis shows the tolerance indicator. According to the three-dimensional graphs the lines are divided into four groups: Group A comprises of lines with the same performance in both conditions. Group B comprise of lines with good performance in non stress condition. Group C contains lines with good performance in stress conditions. Group D contain lines with poor performance in both states.

The three-dimensional scattering plot has been used to select drought tolerant genotypes in different studies (Imam Jomeh, 1999; Darvishzadeh *et al*., 2010). According to the results of Fernández (1992), the best indicator is the one that can distinguish group A from the other groups. To identify group A from other groups, Shirinzadeh *et al*. (2009) and Ghaffari (2008) used the three-dimensional diagram. In our study, it was observed that C100, C104 and LR55 lines were placed in Fernández (1992) group A. To investigate the relationship between more than 3 variables simultaneously, a multivariate graph called biplot was also used (Figure 2). Biplot is a useful tool for analyzing and theoretically evaluating structure information of a large two-sided matrix (Gabriel, 1971). In the biplot space, lines were placed into certain groups associated with their yield and tolerance to water deficit. In other

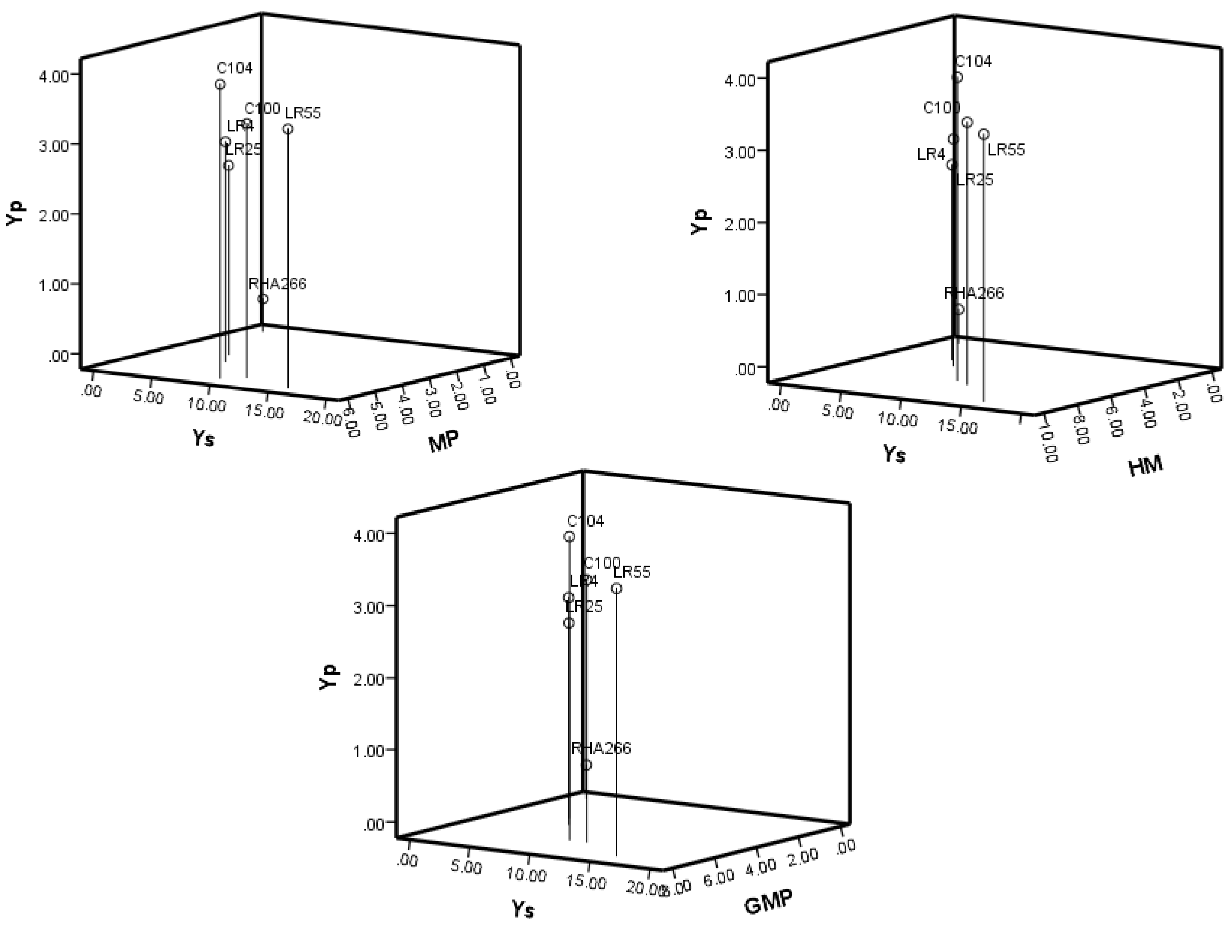

Figure 1. Three-dimensional scatter plot to determine drought tolerant lines based on drought tolerance indices. X-axis: Yield in stress conditions (Ys); Y-axis: Yield in non-stress conditions (Yp); Z-axis: Drought tolerance indicators including MP, HM and GMP.

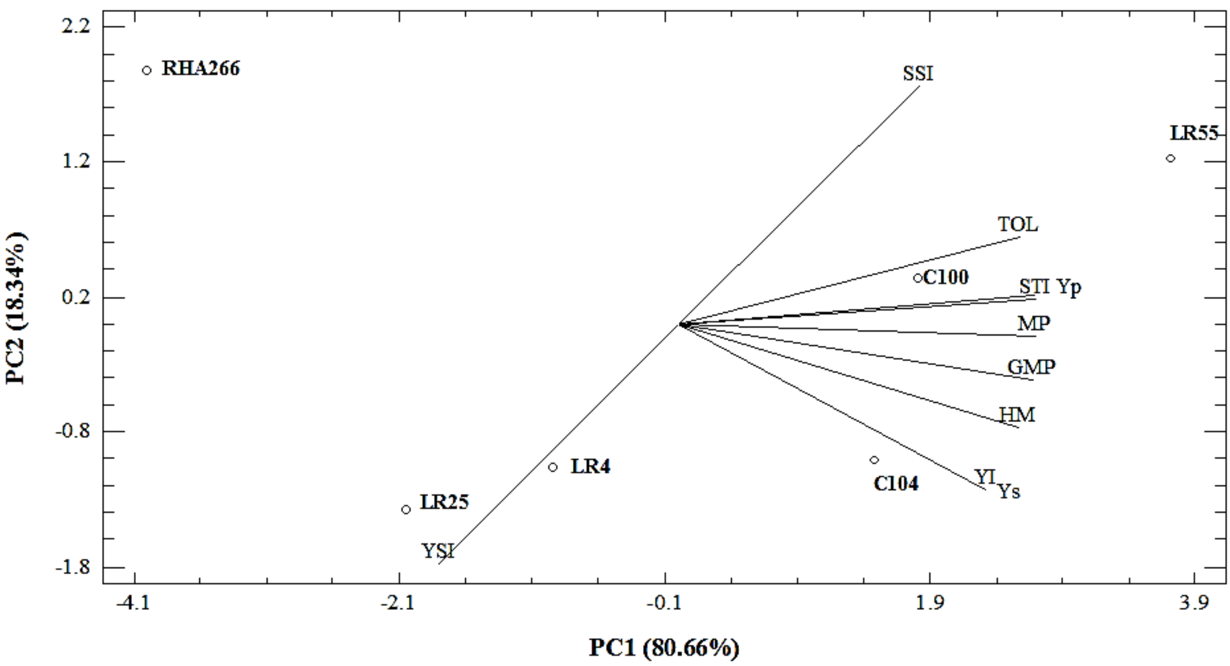

Figure 2. Biplot of the studied sunflower lines for all drought tolerance indices.

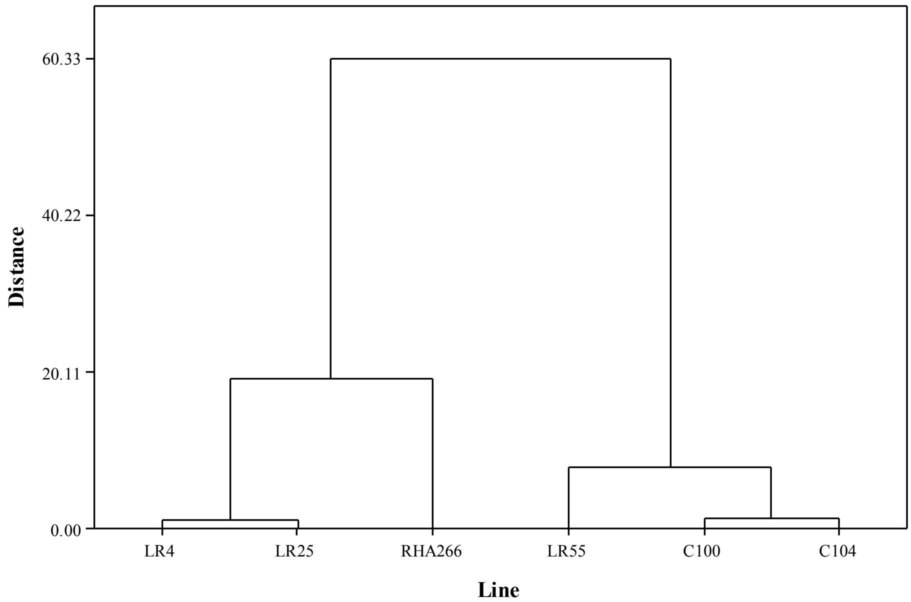

Figure 3. Dendrogram of the studied sunflower lines based on all drought tolerance indices.

studies to investigate the relationship between the variables, biplot was also used (Nourmand Moayed, 1997; Fischer and Maurer, 1978; Imam Jomeh, 1999; Maroufi, 1999).

By cluster analysis based on drought tolerance indices, the lines were sub divided into three main groups (Figure 3). LR4 and LR25 lines were placed in the first group with a low performance in water stress but suitable performance in non-stress conditions. These lines belong to B class according to Fernández (1992) classification. RHA266 line was assigned to the second group with a low yield in both non-stress and stress conditions. This line belongs to Group D according to Fernández (1992) classification. This line had high levels of TOL and SSI. Lines C100, C104 and LR55 were placed in the third group. These lines had high levels of STI, HM and GMP compared to the other lines; and their performances were higher in both environmental conditions than others.

Totally, based on the three-dimensional scatter plots, biplot and cluster analysis, the lines LR4 and LR25 were classified in group B, RHA266 in group D and LR55, C100 and C104 in group A, according to Fernández (1992) classification. Therefore, LR55, C100 and C104 lines with high levels of drought tolerance indicators are the best lines for drought stress conditions. In a study, Darvishzadeh *et al.* (2010) identified ‘LR4×LR25’ hybrid as most tolerant genotype to drought, using three dimensional scatter plot and cluster analysis.

Molecular traits

Evaluation of soluble protein changes showed that the protein content of the lines increased in water stress, compared to non-stress conditions, the exceptions were LR25 and RHA266 lines irrigated at 40% of field capacity. The highest increase in protein content was observed in LR25 and LR4 lines irrigated at 60% and 40% of field capacities, respectively. According to the results, the highest protein content in irrigation at 60% of field capacity was observed in LR25 and LR55 lines. In irrigation at 40% of field capacity, the highest protein content was observed in LR4, C100 and C104 lines compared to control plants (Figure 4).

The stress stimulates synthesis of new proteins and increases their content (Dubey and Rani, 1989). Rajendra *et al*. (1991) and Genadii *et al*. (2002) reported that protein content increased under drought stress. It can be mentioned that increasing in antioxidant enzyme activity in water deficit possibly prevents the decomposition of plant protein under drought stress. Other researchers have also reported an increase in soluble protein content in roots and endosperm of wheat under drought stress (Konopka *et al*., 2007; Bakalova *et al*., 2008). Saint Pierre *et al.* (2007) observed that water deficit caused an increase in protein content in different wheat cultivars. Also, Alahdadi *et al*. (2011) reported that water stress significantly increased seed proteins in some sunflower hybrids.

Protein SDS-PAGE electrophoresis pattern showed that different bands appeared on gels loaded with proteins extracted from drought treated plants (Table

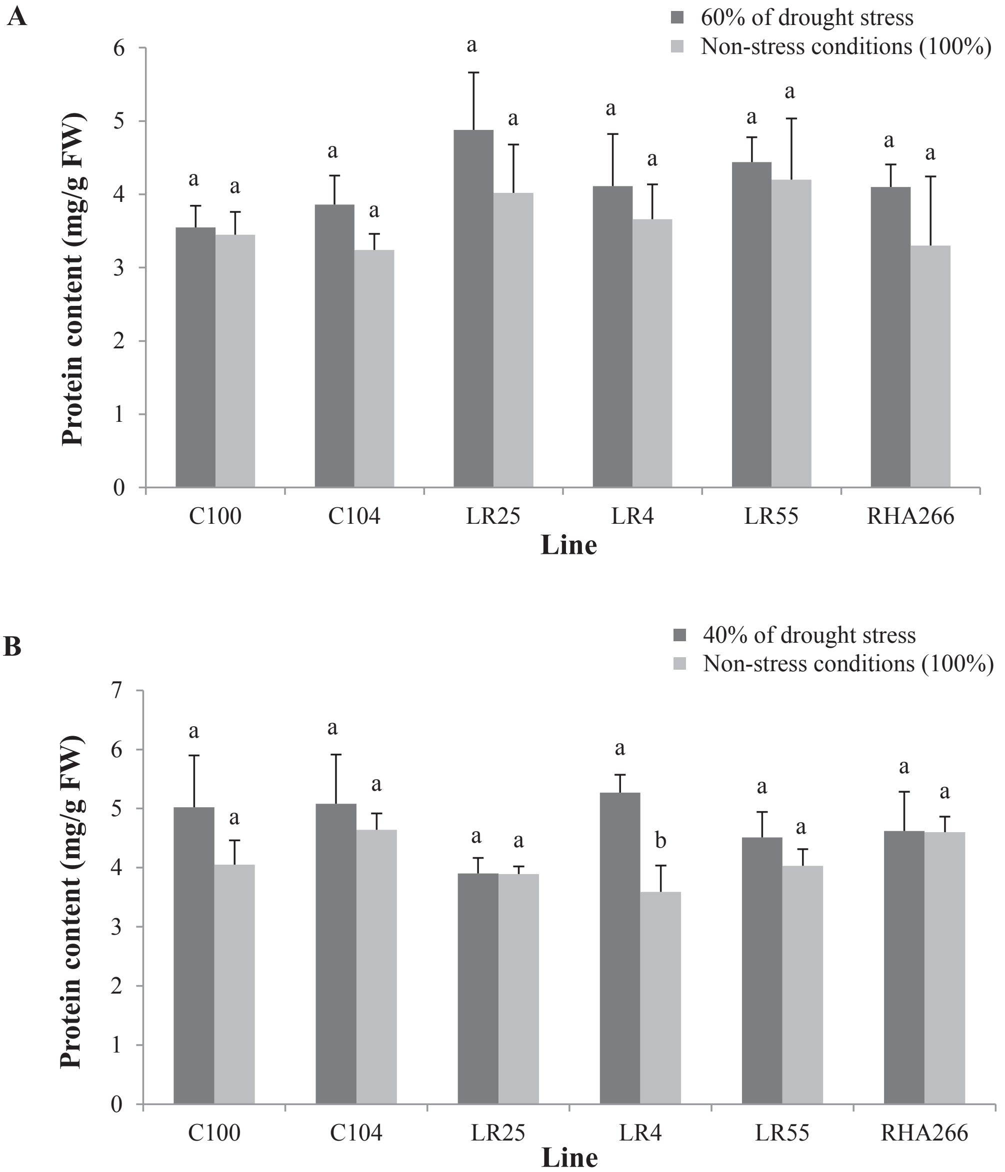

Figure 4. Protein content of the studied sunflower lines in **A:** 60% and **B:** 40% drought stress as compared to the non-stress conditions (100%).

8). Figure 5 indicates the separation of proteins of lines irrigated at 60% of field capacity. According to the results, a protein with a molecular weight of 80.5 kDa was expressed only in samples grown under non-stress conditions (Figure 5). A protein with a molecular weight of 70 kDa was expressed in LR25, LR55 and C104 lines grown under non-stress conditions. However, any band was not observed in these lines under drought-stressed conditions. Also, the band with a weight of 52.8 kDa disappeared in C104 and LR55 lines grown under drought-stressed conditions; while it was expressed in all other samples (Figure 5). Moreover, two protein bands with molecular weights of 25.7 and 15.1 kDa were observed in all lines under both non-stress and drought stressed conditions. Nevertheless, the expression of the 25.7 kDa protein decreased in drought treated LR4, RHA266 and C104 lines (Figure 5).

In addition, protein pattern of lines irrigated at 40% of field capacity showed that a protein with a molecular weight of 70.5 kDa was present in drought treated RHA266, C100 and C104; and the control plants of RHA266, C104 and LR55 lines (Figure 6). In the control plants of LR55 genotype, protein bands were

Table 8. Absence and presence of protein bands in the studied sunflower lines in 60 and 40% drought stress conditions.

Band No.	LR4		LR25		LR55		RHA266		C100		C104		MW KDa
	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	WW	WS	
In 60%													
1	1	0	1	0	1	0	1	0	1	0	1	0	80.5
2	1	1	1	0	1	0	1	1	1	0	1	1	78.7
3	0	0	1	0	1	0	0	1	0	0	1	0	70
4	**0**	**0**	**0**	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**1**	**1**	**69.3**
5	1	1	1	1	1	1	1	1	1	1	1	1	58.7
6	1	1	1	1	1	0	1	1	1	1	1	0	52.8
7	0	0	1	0	1	0	1	1	0	0	1	0	50.5
8	1	1	1	1	1	1	1	1	1	1	1	1	37.5
9	1	1	1	1	1	1	1	1	1	1	1	1	25.7
10	1	1	1	1	1	1	1	1	1	1	1	1	15.1
Total	7	6	9	6	10	5	9	8	8	6	10	6	
In 40%													
1	0	1	1	1	1	0	0	1	1	0	1	0	82.3
2	0	1	1	1	1	1	1	0	0	1	1	1	72.8
3	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**1**	**0**	**1**	**1**	**1**	**70.5**
4	1	0	1	1	1	0	1	1	1	0	1	0	65.8
5	0	1	0	1	0	1	1	0	0	1	0	1	56.3
6	0	1	0	1	1	1	0	1	0	1	1	1	47
7	1	1	1	1	1	1	1	1	1	1	1	1	44.6
8	0	1	1	1	1	1	1	1	0	0	1	0	38.7
9	1	1	1	1	1	1	0	1	1	1	1	1	29.3
10	1	1	1	1	1	1	1	1	1	1	1	1	24.5
Total	4	8	7	9	9	7	7	8	5	7	9	7	

Well-water (WW); Water-stress (WS).
Bold numbers refer to distinguished bands.

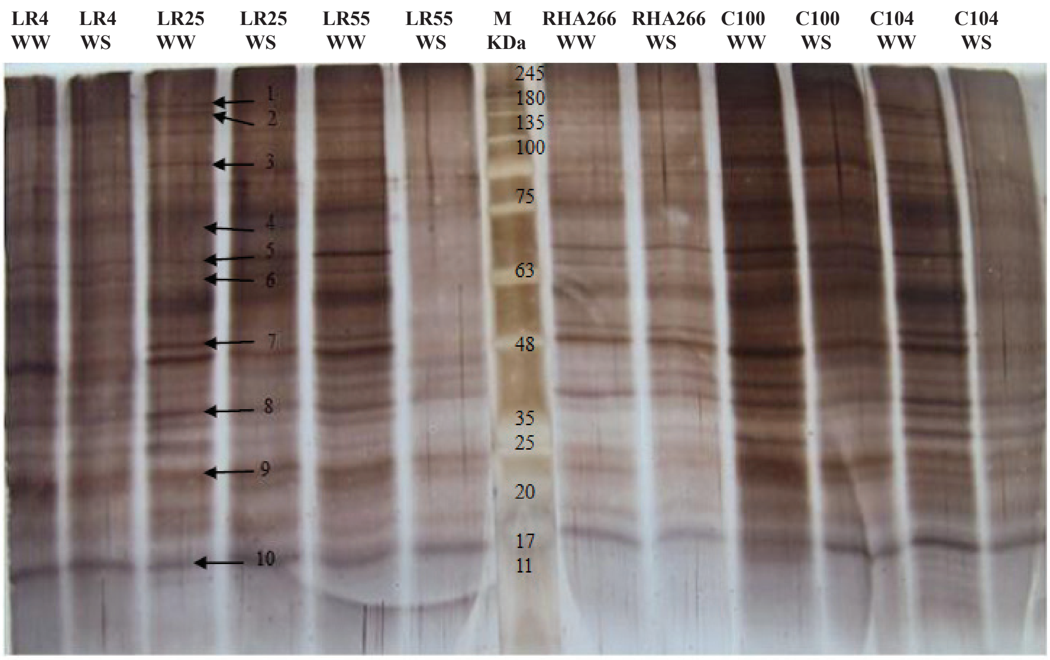

Figure 5. Protein electrophoresis pattern of the studied sunflower lines in 60% drought stress and non-stress conditions. Well-water (WW); Water-stress (WS); Protein marker (M).

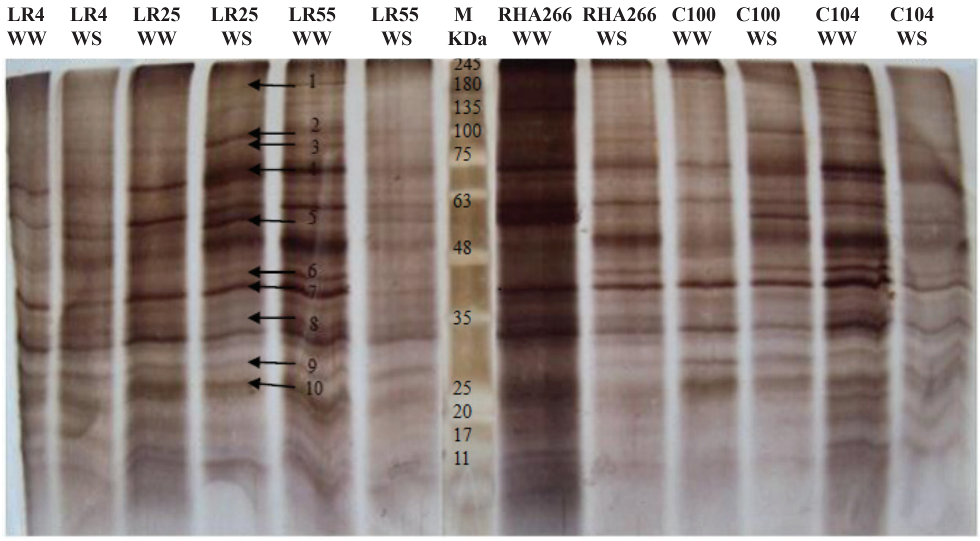

Figure 6. Protein electrophoresis pattern of the studied sunflower lines in 40% drought stress and non-stress conditions. Well-water (WW); Water-stress (WS); Protein marker (M).

sharper than those in others genotypes which indicate a higher expression of the proteins in this line. Also, the protein band with a molecular weight of 44.6 kDa was observed in all samples (Figure 6). Nonetheless, the expression of this protein in the drought treated LR55 was lower than that in others and also the intensity of this protein increased in the control plants of C104 line (Figure 6). A protein with a molecular weight of 29.3 kDa was also expressed in all samples except in control plants of RHA266. In addition, the protein with a molecular weight of 24.5 kDa was observed in all samples (Figure 6).

Drought stress induces expression of the proteins that are directly or indirectly related to stress. Some functions have been assigned to these proteins (Neslihan Ozturk *et al*., 2002). Among the stress induced proteins identified so far, are those implicated in the biosynthesis of osmolytes (Ishitani *et al*., 1995), uptake and compartmentation of ions (Lisse *et al*., 1996), hydroxyl-radical scavenging (Ingram and Bartels, 1996) and protection of cellular structure (Neslihan Ozturk *et al*., 2002). In overall, the proteins that have shown significant down regulation under drought stress have photosynthesis-related functions (Neslihan Ozturk *et al*., 2002). Changes in protein patterns induced due to drought stress play a pivotal role in the adaptive response of plants to the stress (Riccardi *et al*., 1998). The obtained differences in zymography of the enzymes are useful for the estimation of the genetic purity of sunflower hybrids. Similar results to our findings have been reported by Chikkadevaiah and Nandini, (2003) and Shabani and Rajaee, (2013).

Generally, drought stress can cause a decrease or increase in protein expression. It was revealed that the protein banding patterns of the studied lines was different in two irrigation regimes. According to these results, drought stress caused the accumulation of low molecular weight proteins in the studied lines, so that some protein bands were produced and some disappeared. In irrigation at 60% of field capacity, the protein band with a molecular weight of 70 kDa was expressed in the control plants of LR25, LR55 and C104 lines, while the intensity of this band was reduced in the control samples of RHA266 line. In addition, in irrigation at 40% of field capacity, there was a protein band of 70.5 kDa in drought treated RHA266, C104 and C100; and also in control plants of RHA266, C104 and LR55 lines. However, in control plants of the LR55, the protein band was sharp which represents its high expression in this line. In other cases, the 70.5 kDa protein had almost disappeared. Hu *et al*. (2010) reported that the 70 kDa heat shock protein had a vital role in the antioxidant defense system of maize, in response to a combination of drought and heat stresses. Therefore, it seems that the emergence of new protein bands or deletion of some bands at different levels of drought stress can be considered as biochemical markers of response to drought stress. In addition, the protein band of 52.8 kDa disappeared in drought treated LR55 and C104 lines and its expression was reduced in drought treated RHA266 line. Borovskii *et al*. (2000) investigated a group of proteins of maize under different abiotic stresses. They identified 5 dehydrin-like proteins in the mitochondria of plant cells. Also, maize adaptation to the low temperature caused a significant accumulation of polypeptides with molecular weights of 63 and 52 kDa. Moreover, the protein bands of 25.7 and 24.5 kDa were observed in control and the drought stress treated samples, respectively. It has been reported that the 25 kDa dehydrin protein has a protective role against stress (Bakalova *et al*., 2008). Fazeli *et al*. (2007) observed the emergence of a strong band in the range of 20-24 kDa under drought stress in a study on the drought resistant and sensitive varieties of sesame. Also, in a gel electrophoresis of protein samples obtained from 60% drought stress plants and their controls, the protein band of 15 kDa which belongs to the small subunit of rubisco was present in the control and drought-treated samples. Decreasing of synthesis of rubisco under drought stress has been reported as a result of sharp reduction in the frequency of smaller subunits (Jabari *et al*., 2009). Differences in SDS-PAGE patterns of helianthinin components have also been reported between different cultivars (Raymond *et al*., 1994, 1995).

Based on the findings of this study, C100, C104 and LR55 lines had better growth, yield and drought tolerance indices under drought stressed conditions. These resistant lines especially (C104 and LR55) had the highest protein band intensities in conditions that irrigation was done at 60 and 40% of field capacities, respectively. Therefore, LR55 in 60% and C100 and C104 in irrigation at 40% of field capacity had the highest protein contents. In addition, in 60% drought stress, the protein band of 69.3 kDa was observed in the tolerant lines C104, C100 and LR55. Moreover, in 40% drought stress, the protein band of 70.5 kDa was observed in the tolerant lines C104 and C100. It seems that the protein bands 69.3 and 70.5 kDa are resistant-related proteins that can be attributed to changes in gene expression under drought stress. An ongoing study is being carried out to identify the drought resistant gene loci which can be useful in sunflower breeding programs for producing resistant cultivars to drought stress.

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