Journal of Plant Physiology and Breeding
2017, 7(1): 105-119 ISSN: 2008-5168

Physio-biochemical and Enzymatic Responses of Sunflower to Drought Stress

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Received: August 14, 2016 Accepted: April 6, 2017
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Abstract
Drought stress is a serious adverse factor that limits sunflower growth and productivity. The stress induces a range of physiological and biochemical responses in sunflower. So, evaluation of defense systems is important for producing resistant cultivars. In this study, physio-biochemical changes and antioxidant enzymes activities of six sunflower lines were evaluated under normal and irrigation at 60 and 40% of the field capacity using a randomized complete block design with three replications. Different characteristics such as relative water content (RWC), chlorophyll a and b contents, carotenoid and proline contents, lipids peroxidation and accumulation of malon dialdehyde (MDA), as well as activity of antioxidative enzymes like guaiacol peroxidase (GPX), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) were studied. The results showed significant differences among sunflower lines for physio-biochemical and enzymes activity under drought stress. According to the results, the lines C104 and RHA266 showed the better tolerance to drought stress. The founding of this study can be useful in sunflower breeding programs for producing resistant cultivars to drought stress.

Keywords: Drought tolerance; Enzyme activity; Lipids peroxidation; Physiological characters; Sunflower

Introduction

Water resources are limited and need to be used efficiently in agricultural consumption. Drought stress has been a threat to agricultural activities in many parts of the world (Wang 2004; Vinocur and Altman 2005; Berenguer 2009). The stress affects different morphological, physiological and biochemical characteristics (Reddy *et al*. 2004; Anjum *et al*. 2011; Andrade *et al*. 2013) such as changes in chlorophyll contents (Nayyar and Gupta 2006; Cao *et al*. 2011), inhibition of photochemical activities and decrease the activities of enzymes in the Calvin cycle of photosynthesis (Monakhova and Chernyadev 2002). These changes cause retardation of plant growth and developmental processes. However, the effects of changes are dependent on the time, stage and severity of water stress (Cao *et al*. 2011). Consequently, the genetic improvement of plant cultivars for water stress tolerance and ability of a crop plant to produce maximum harvest over a wide range of stress and non-stress conditions, has been a principal objective of breeding programs for a long time (Moustafa *et al*. 1996; Chachar *et al*. 2016).

Drought stress induces oxidative stress in plants by generation of reactive oxygen species (ROS) (Farooq *et al*. 2009). The generation of ROS is one of the earliest biochemical responses of cells to the stress. Exceeding of ROS level from removing capacity, leads to oxidative damages including peroxidation of membrane lipids and acclimation of malon dialdehyde (MDA), destruction of photosynthetic pigments

and inactivation of photosynthetic enzymes (Smirnoff 1993). Acclimation of plants to water deficit is the result of different events, which lead to adaptive changes in plant growth and physio-biochemical processes such as changes in plant structure, growth rate, tissue osmotic potential and antioxidant defenses (Duan *et al*. 2007). Plant antioxidant defense systems including enzymatic and non-enzymatic protection have co-evolved with aerobic metabolism to counteract oxidative damage due to ROS (Davar *et al*. 2013). Scavenging of ROS and reducing their damaging effects may correlate with drought tolerance of plants. Efficient scavenging of ROS produced during drought stress requires the action of several enzymatic (APX[1], CAT[2], SOD[3], POX[4], MDHAR[5], DHAR[6], GR[7]) as well as non-enzymatic (ascorbate, phenolic compounds, carotenoids, glutathione, glycine betaine, proline, sugar, polyamines) antioxidants present in tissues (Gill and Tuteja 2010; Karuppanapandian *et al*. 2011).

Sunflower (*Helianthus annuus* L.) is one of the most important oil seed crops (Hussain *et al*. 2015). Although sunflower is moderately tolerant to water stress, its production is greatly affected by drought stress (Pasda and Diepenbrock 1990). Sunflower exhibits a large varietal difference for osmotic adjustment in response to water shortage (Hussain *et al*. 2014). This is proven that sunflower plants exposed to drought is bearing oxidative stress by overproduction of ROS (Rao 2006). Hence, evaluation of enzymatic and non-enzymatic antioxidant defense systems is important in producing resistant sunflower hybrids. Despite several studies concerning drought stress in sunflower (Terbea *et al*. 1995; Baldini *et al*. 1997; Baldini and Vannozzi 1998; Cellier *et al*. 1998; Maury *et al*. 2000; Tahir *et al*. 2002; Poormohammad Kiani *et al*. 2007; Rauf and Sadaqat 2008; Darvishzadeh *et al*. 2010; Safavi *et al*. 2015; Hussein *et al*. 2015), there are fewer reports about simultaneous study of both enzymatic and non-enzymatic antioxidant systems in diverged genotypes of sunflower. The relation between increasing the resistance to drought stress and enhancing the enzymatic antioxidant systems has been studied in some plants species such as rice (Guo *et al*. 2006), sugar beet (Bor *et al*. 2003), wheat (Khanna Chopra and Selote 2007), barley (Acar *et al*. 2001) and sunflower (Ghobadi *et al*. 2013) in abiotic stress conditions. In the present study, some defense systems of sunflower genotypes were studied under drought stress conditions. The information presented here could assist sunflower breeders to choose parents of crosses for breeding of resistance to drought stress.

Materials and Methods

Plant material and experimental methodology

Six oilseed sunflower lines were selected from 125 recombinant inbred lines based on their different responses to drought stress (Poormohammad Kiani *et al*. 2009). Prominent features of the studied lines are summarized in Table 1. The experiment was conducted as the randomized complete block design with three

[1]Ascorbate peroxidase (APX)
[2] Catalase (CAT)
[3]Superoxide dismutase (SOD)
[4]Peroxidase (POX)
[5]Monodehydroascorbate reductase (MDHAR)
[6]Dehydroascorbate reductase (DHAR)
[7]Glutathione reductase (GR)

replications. The seeds were planted in the plastic pots with a diameter of 12 and height of 14 cm which filled with soil and peat moss (3:1). The plants grew in a greenhouse with 12 h light and maximum and minimum temperature of 28 and 12˚C, respectively. Amount of water applied was identical for all treatments from the beginning of planting time until the complete establishment of sunflower plants (eight-leaf (V8) stage). When plants grew into the 8-leaf stage, the control pots were maintained at field capacity and other pots were irrigated at 60 and 40% of field capacity until the end of growth period. It should be noted that for the drought stress experiment, first the plants were irrigated at 60% of field capacity during 5 days and leaves were sampled on each pot. Then, the pots were irrigated at 40% of field capacity until the end of growth period. Finally, different physio-biochemical parameters and antioxidant enzymes were measured at two levels of drought stress.

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

Measurement of physio-biochemical characteristics

Relative water content (RWC)

A piece of fresh leaf was removed and fresh weight was measured. The samples were placed in distilled water in a closed container at room temperature for 24 h and then turgid weight was measured. To measure dry weight, the samples were placed in an oven at temperature of 72˚C for 24 h and then weighted. Finally, RWC was calculated by the following formula (Levitt 1980):

$$RWC= (FW-DW/TW-DW) \times 100$$

In this formula, FW, TW and DW refer to fresh, turgid and dry weight of the leaves, respectively.

Chlorophyll a, b and carotenoid contents

Chlorophyll a, b and carotenoid contents were measured using the method described by Lichtenthaler and Wellburn (1985). An amount of 0.1 g of fresh leaf was homogenized in 5 mL of 100% acetone in a mortar. After homogenizing, the extract was centrifuged for 10 min at 2500 rpm. Then absorbance of the supernatant was recorded at 662, 645 and 470 nm by UV/Vis spectrophotometer (WPA S2100, UK) and the

Chlorophyll a, b and carotenoid contents were calculated using the following formula and expressed in µg/g FW:

$$Chla = 11.75\ A662 - 2.350\ A645$$

$$Chlb = 18.61\ A645 - 3.960\ A662$$

$$C_{X+C} = 1000\ A470 - 2.270\ Chla - 81.4\ Chlb\ /227$$

Proline content

Proline content was measured using the method described by Bates *et al*. (1973). An amount of 0.04 g of dried leaf tissue was homogenized in 15 mL of Sulpho salicylic acid (3%) and kept for 72 h in a refrigerator at 4˚C to release the proline. After 72 h, the samples were centrifuged at 3000 rpm for 20 min. Then 2 mL of glacial acetic acid and 2 mL of reagent ninhydrin (containing 20 mL phosphoric acid 6 M, 30 mL glacial acetic acid and 1.25 g ninhydrin) were added to 2 mL of the supernatant. The samples were placed in a water bath at 100˚C for 1 h. The samples were rapidly cooled using ice and then 4 mL of toluene was added to each sample and stirred. After the formation of two phases, absorption of the supernatant was recorded at 520 nm. To determine the amount of proline, standard curve was prepared using known proline concentrations.

Lipid peroxidation and accumulation of MDA

Lipid peroxidation was measured as an amount of TBARS[1] that determined by TBA reaction described by Heath and Packer (1968). 0.2 g of fresh leaf was homogenized in 5 mL of TCA 1% (w/v) and centrifuged at 8000×g for 10 min. To 1 mL of the supernatant, 4 mL TCA 20% containing TBA 0.5% (w/v) was added. The mixture was heated at 95˚C for 30 min and then quickly cooled on ice. Then the extract was centrifuged at 8000×g for 5 min. The amount of TBARS was determined from the difference between absorbance at 532 and 600 nm using extinction coefficient of 155 $mM^{-1}cm^{-1}$ by the following formula:

$$MDA\ (\mu mol/gFW) = (A532\text{-}A600/155) \times 1000$$

Activity of antioxidant enzymes

For extraction of enzymes, 0.5 g of fresh leaf was homogenized in a chilled mortar. Then 3 mL of ice-cold extraction buffer containing Tris-HCl 0.05 M, pH 7.5, $MgCl_2$ 3 mM and EDTA 1 mM was added on powder. The extraction buffer included 2 mM ascorbate that is served for determining APX activity. The homogenate was centrifuged at 15000 g for 15 min at 4˚C and the supernatant was served as an enzyme extract. Guaiacol peroxidase (GPX) activity was also determined according to the method of Chang and Kao (1998). The reaction contained 2.5 mL potassium phosphate buffer 50 mM, 1 mL H_2O_2 1% (w/v), 1 mL guaiacol 1% and 0.3 mL extraction buffer. GPX activity was measured at 420 nm. Extinction coefficient of 26.6 ($mM^{-1}cm^{1}$) in a minute was used to calculate its activity. In addition, APX activity was measured using the modified method originally described by Asada (1992). The reaction contained 2.5 mL potassium phosphate buffer (50 mM, pH 7.0) which included ascorbate 0.5 mM and EDTA 0.1 mM. Then, 0.2 mL H_2O_2 1% (w/v) and 0.1 mL extraction buffer were added. After that, APX activity was measured at 240 nm. Extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ was used to calculate its activity.

[1]Thiobarbituric acid reactive substances (TBARS)

Moreover, CAT activity was determined by measuring H_2O_2 consumption (Maehly and Chance 1959) in a reaction containing 3 mL phosphate buffer (50 mM, pH 7.0), 0.2 mL H_2O_2 1% (w/v) and 50 µl extraction buffer. CAT activity was measured at 240 nm. Extinction coefficient of 0.036 mM^{-1} cm^{-1} was used to calculate its activity. Finally, GR was defined as an amount of the enzyme which decreases A340 (1u per min).

Statistical analysis

The data were analyzed by GLM procedure of SAS 9.2 software. Comparison of mean treatments was made with Tukey test.

Results and Discussion

Relative water content (RWC)

Results pertaining to relative water content revealed significant reduction in RWC of studied lines as compared to the control plants under two drought stresses except for the line LR55 at 60% drought stress condition. Maximum and minimum reduction of RWC was observed in the lines C100 and RHA266, respectively, under two drought stresses (Figure 1a, b). Drought stress resulted in the loss of water in plants as well as reduction of RWC. Therefore, RWC is widely used as one of the most reliable indices for characterization of both sensitivity and tolerance of plants to water stress (Rampino *et al*. 2006). It has been reported that resistant varieties have the highest RWC (Bastide *et al*. 1993; Antolin *et al*. 1993). Rauf and Sadaqat (2008) reported that all the studied sunflower genotypes retained lower RWC under drought regime in comparison to the irrigated condition. Also, Ghobadi *et al*. (2013) observed that RWC decreased in sunflower by drought severity. Significant variability and inhibitory effect has been reported in sunflower for this trait under drought stress condition (Baldini *et al*. 1997). In this study, the more resistant lines had the better osmotic adjustment to prevent their water potential under both drought and normal conditions. The highest RWC was related to the lines LR55 and RHA266 at 60% and the lines C104 and again RHA266 at 40% drought stress.

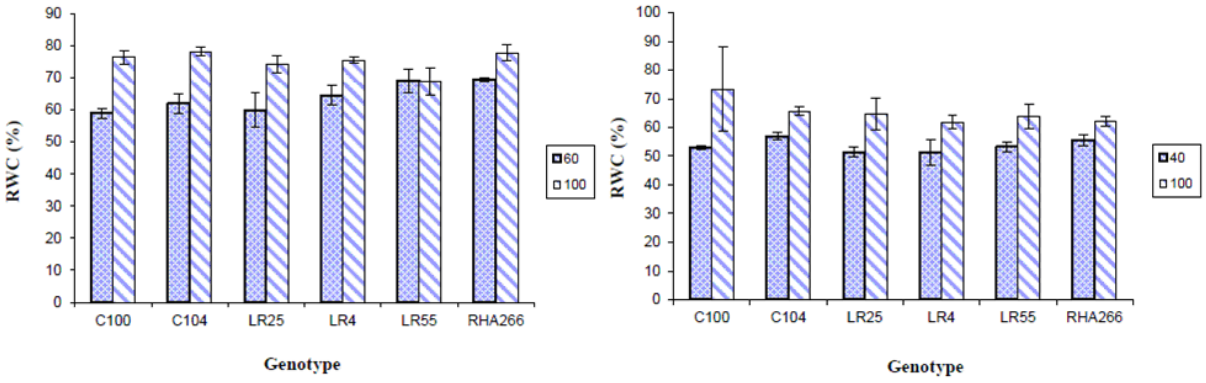

Figure 1. Relative water content (RWC) of the studied sunflower lines at 60% (a) and 40% (b) drought stress as compared to the normal conditions (100%)

Chlorophyll a, b and carotenoid contents

Chlorophyll a and b contents were increased under both drought stresses as compared to the control except for the line LR55 at 60% drought stress. The highest increase in chlorophyll was observed in the line LR25 at 60% and the line RHA266 at 40% drought stress (Figure 2a, b). Chlorophyll is one of the major chloroplast components for photosynthesis and its content has a positive relationship with photosynthetic rate (Anjum *et al*. 2011). The chlorophyll content is considered as one of the most important indicators of vegetative stage and its degradation is normally considered as a measure of drought susceptibility (Beard 1973; Kim *et al*. 1989; Manivannan *et al*. 2007). In this study, the chlorophyll content decreased with decreasing the irrigation water. The decrease correlates with RWC in the leaves (Munne Bosch and Alegre 2000). Drought stress caused a large decline in the chlorophyll a, chlorophyll b and total chlorophyll content in different sunflower varieties (Manivannan *et al*. 2007). Jabari *et al*. (2009) reported that drought resistant wheat varieties have higher chlorophyll content under drought stress. Ghobadi *et al*. (2013) stated that the chlorophyll a and b content in sunflower decrease with drought severity. Some authors found an opposite trend of chlorophyll increase by deficit irrigation. Khayatnezhad (2011) and Alaei (2011) showed that drought stress increase leaf chlorophyll content in maize and wheat. In other studies, chlorophyll content was decreased (Jung 2004; Nayyar and Gupta 2006) and increased (Schurr *et al*. 2000; Jiang and Huang 2001; Barraclough and Kate 2001) in plants under drought stress conditions. According to our results, the chlorophyll content increased under drought stress. It seems that increase in chlorophyll content under stress condition, is probably due to the decrease in leaf area which causes accumulation of chlorophyll. The highest chlorophyll content was observed in line LR4 at 60% and line RHA266 at 40% drought stress.

Carotenoid content increased in most of the studied lines as compared to the control under drought stress except for the lines C104, LR25 and LR4 at 60% drought stress. The highest increase in carotenoid content was observed in the line C100 at 60% and the line RHA266 at 40% drought stress (Figure 3a, b). The line C100 had the highest carotenoid content as compared to the others under both drought stress conditions. According to the results of Ghobadi *et al*. (2013), carotenoid content increases in sunflower with drought severity.

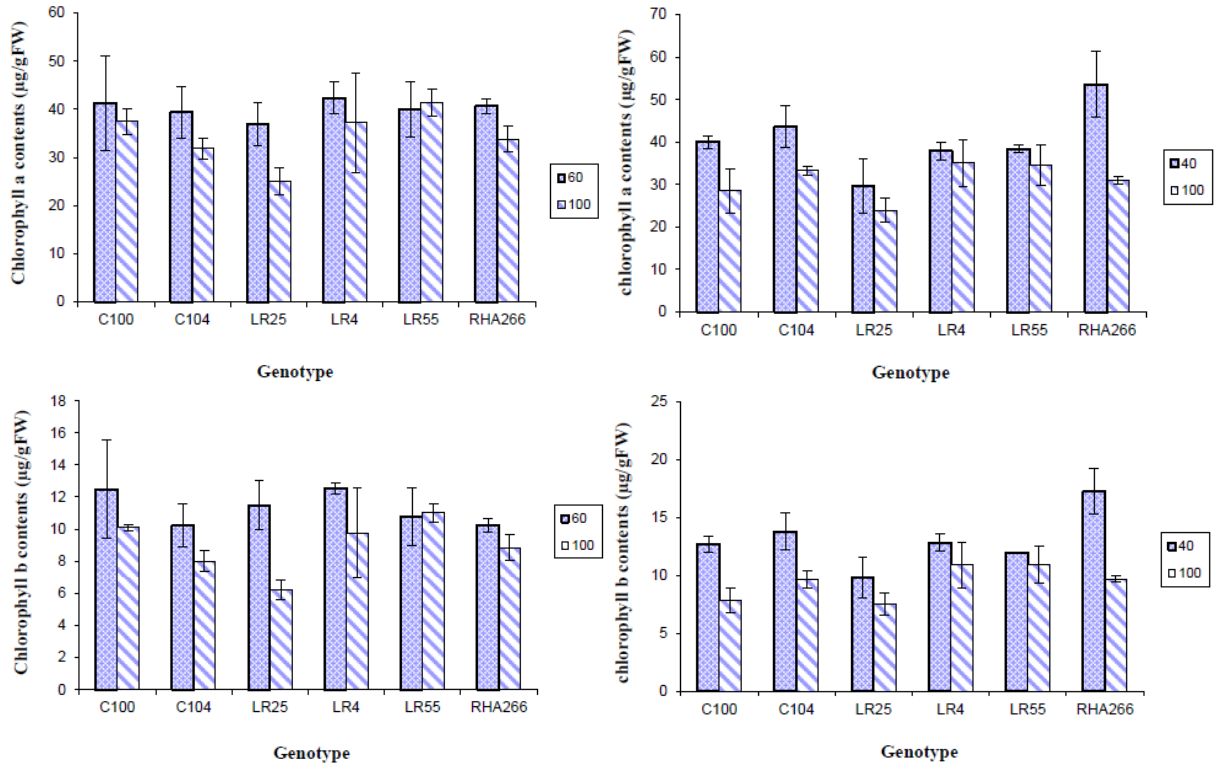

Figure 2. Chlorophyll a and b contents of the studied sunflower lines at 60% (a, c) and 40% (b, d) drought stress as compared to the normal conditions (100%)

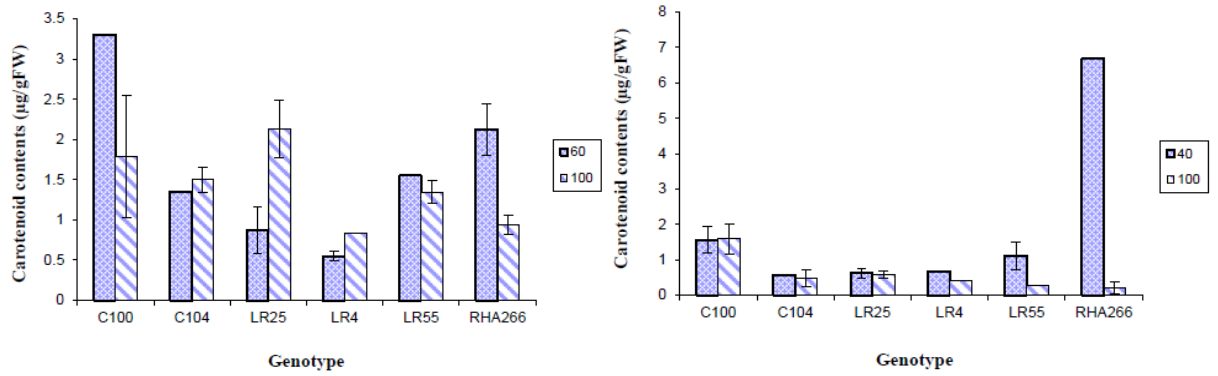

Figure 3. Carotenoid content of the studied sunflower lines at 60% (a) and 40% (b) drought stress as compared to the normal conditions (100%)

Proline content

There was different response of the genotypes to drought stress in terms of proline content. Based on the Figure 4a, proline level was increased in the lines C104, LR25, LR55 and decreased in the lines C100, LR4 and RHA266 at 60% drought stress as compared to the control. But at 40% drought stress, the proline content significantly reduced in all of the studied lines, so the maximum and minimum reduction were observed

in the lines C100 and LR25, respectively (Figure 4b). Accumulation of proline occurs in response to the stress (Hare and Cress 1997; Slama *et al*. 2006). Proline acts as a potent scavenger of ROS and prevents the induction of programmed cell death by ROS (Chen and Dickman 2005). Many plants use organic osmolits such as proline for osmotic regulation and to better tolerate the stress. Proline is a reservoir for carbon, nitrogen and also scavenges free radicals. Moreover, proline stabilizes ultra-cellular structures such as membranes and proteins and removes cellular redox potential that is caused by stress (Chen and Muruta 2000; Errabii *et al*. 2006). In many crops such as sorghum, rice, Indian mustard and tomato, differences in proline content has been reported under stress conditions. Ghobadi *et al*. (2013) reported that the proline content in sunflower increases with drought severity. However, some researchers did not found any increase in proline content under stress condition (Naik and Joshi 1983; Chavan and Karadje 1986). According to our results, the more resistant lines had the highest proline content under both drought and normal conditions. Thus, the highest proline content was seen in lines C104 and LR25 at 60 and 40% drought stress, respectively.

Lipid peroxidation and accumulation of MDA

Regarding data for MDA accumulation that indicates lipid peroxidation, all of the studied lines showed an increase in MDA content as compared to the control under both drought stresses except for the line RHA266 at 60% drought stress. The lowest increase in MDA was observed in the line C100 and LR25 at 60%, and the lines LR25 and LR55 at 40% drought stress

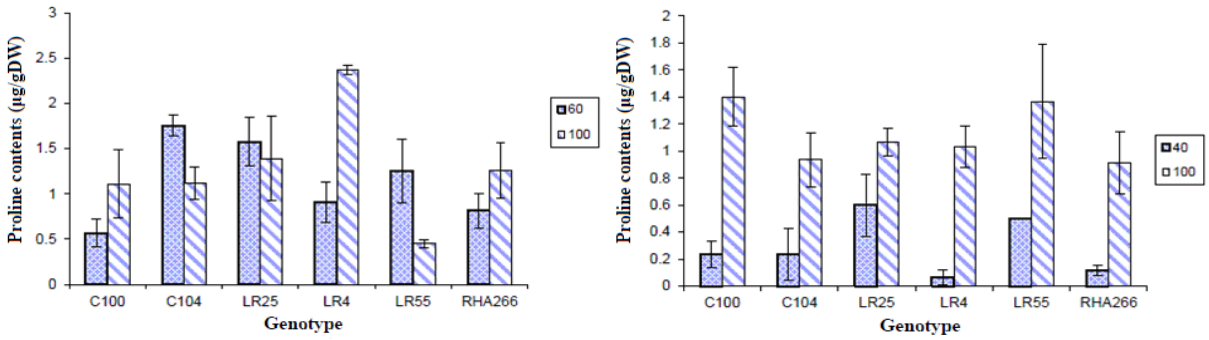

Figure 4. Proline content of the studied sunflower lines at 60% (a) and 40% (b) drought stress as compared to the normal conditions (100%)

(Figure 5a, b). Lipid peroxidation, estimated by the change in MDA content, is used as a reliable marker of oxidative stress (Davar *et al*. 2013). It is reported that the accumulation of MDA under oxidative stress is a by-product of fatty acid peroxidation, synthesized by cellular membrane lipid peroxidation in different plants species during oxidative stress. Our results are in consistent with the finding of Sairam and Srivastava (2001) that showed the MDA increase

in wheat under drought stress. They have reported that when antioxidant defenses decrease or the formation of free radicals increase, oxidative stress is produced that lead to the increase in the lipid peroxidation of unsaturated fatty acids, lipid membrane damage and subsequent withdrawal of various aldehydes such as MDA. In a study on a salt sensitive variety of wheat, MDA accumulation was greater than the resistant variety (Khanna Chopra and Selote 2007). Our results confirmed that high accumulation of ROS has overcome the antioxidant system of plants and increased membrane lipid peroxidation under stress. Considering these results, the lowest values of MDA belonged to the lines LR4 and RHA266 at 60% drought while the line LR55 possessed lowest values at 40% drought stress.

GPX

As shown in Figure 6a, GPX activity increased significantly in all of the studied lines except for C100 whose activity declined at 60% drought stress. Under 40% drought stress, the GPX activity increased in lines C100, LR55 and RHA266 (Figure 6b). GPX can breaks down indole-3-acetic acid and has important role in lignin biosynthesis and the defense against the live stresses consuming H_2O_2 in cytosol, vacuole, cell wall and the extracellular space (Gill and Tuteja 2010). Increasing of GPX activity has been reported in previous experiments. Smirnoff (1993) indicated that increasing of the guaiacol peroxidase, ascorbate peroxidase and catalase activity is an outcome to the ROS and especially H_2O_2 production under drought stress. Increasing of the GPX activity has also been reported by Zhang *et al*. (1995) in maize under drought stress. Based on the results of Ghobadi *et al*. (2013) POX in sunflower was not affected by drought stress. According to our results, the more resistant and susceptible lines had the highest GPX activity under drought stress. So, the highest GPX activity belonged to lines LR25 and LR55 at 60 and 40% of drought stress, respectively.

APX

APX activity significantly increased in the lines C100 and C104 but, declined in other lines at 60% drought stress (Figure 6c). At the state of 40% drought stress, APX activity significantly increased in the lines LR4 and LR55 (Figure 6d). APX is placed in both the cytosol and chloroplast and can be used to effectively destroy hydrogen peroxide, especially in chloroplasts, where there is no catalase (Groden and Beck 1979). APX activity was studied under stress conditions and the results showed that in some studies APX activity increased (Sairam *et al*. 2002; Jung 2004), while in some other its activity decreased (Sharma and Dubey 2005). However, in another study no change in APX activity was observed (Bartoli *et al*. 1999). Considering our results, the highest APX activity belonged to the lines C100 and LR4 at 60 and 40% drought stress, respectively.

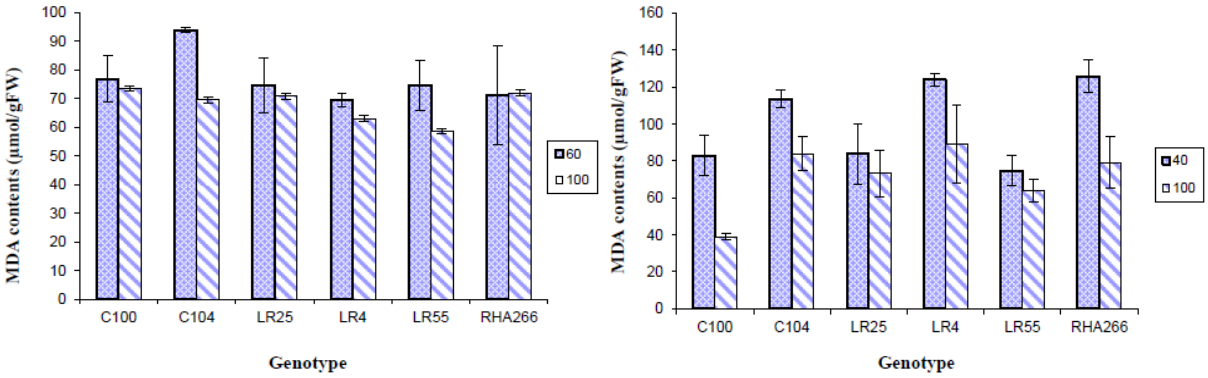

Figure 5. Malondialdehyde (MDA) content of the studied sunflower lines at 60% (a) and 40% (b) drought stress as compared to the normal conditions (100%)

CAT

CAT activity increased in the lines C100 and RHA266 at 60% drought stress, while it declined significantly in the lines LR4 (Figure 6e). Under 40% drought stress, CAT activity increased significantly in the lines C104, LR55 and RHA266 (Figure 6f). CAT is usually located in peroxisomes and hydrolyzes and detoxifies hydrogen peroxide. CAT activity was studied under stress conditions and the results have shown that in some studies CAT activity increased (Wang *et al*. 2001; Sairam *et al*. 2002), while in some other its activity decreased (Jiang and Huang 2001; Sharma and Dubey 2005). However, in another study no change in APX activity was observed (Bartoli *et al*. 1999). Ghobadi *et al*. (2013) reported that the CAT activity increases in sunflower with drought severity. Also, Jung (2004) showed that the CAT and POX activity in the mature leaves of tall grass increased as compared to the control under water stress conditions. But any increase was not observed in the young leaves. In another study, it was observed that the activity of these two enzymes increase during the early stages of water stress. It seems that water stress prevents the detrimental effects of ROS on cell membrane via increasing the CAT activity as much as possible (Jiang and Huang 2001). Considering our results, CAT activity increased in the line RHA266 under both water stress conditions compared to the control. The highest CAT activity observed in the line C100 at 60% and in the lines C104 and RHA266 at 40% drought stress.

GR

GR activity increased in the lines C100 and RHA266 at 60% drought stress and the increase was higher in RHA266 (Figure 6g). Under 40% drought stress, level of the GR activity increased in the lines C100, C104, LR4 and RHA266 while it decreased in the lines LR25 and LR55 (Figure 6h). GR is the last enzyme in glutathione-ascorbate pathway which consumes NADPH as an electron donor to reduce glutathione (Noctor and Foyer 1998). Increasing of GR activity in cells is a way to increase the glutathione level. This increases the cell tolerance toward ROS

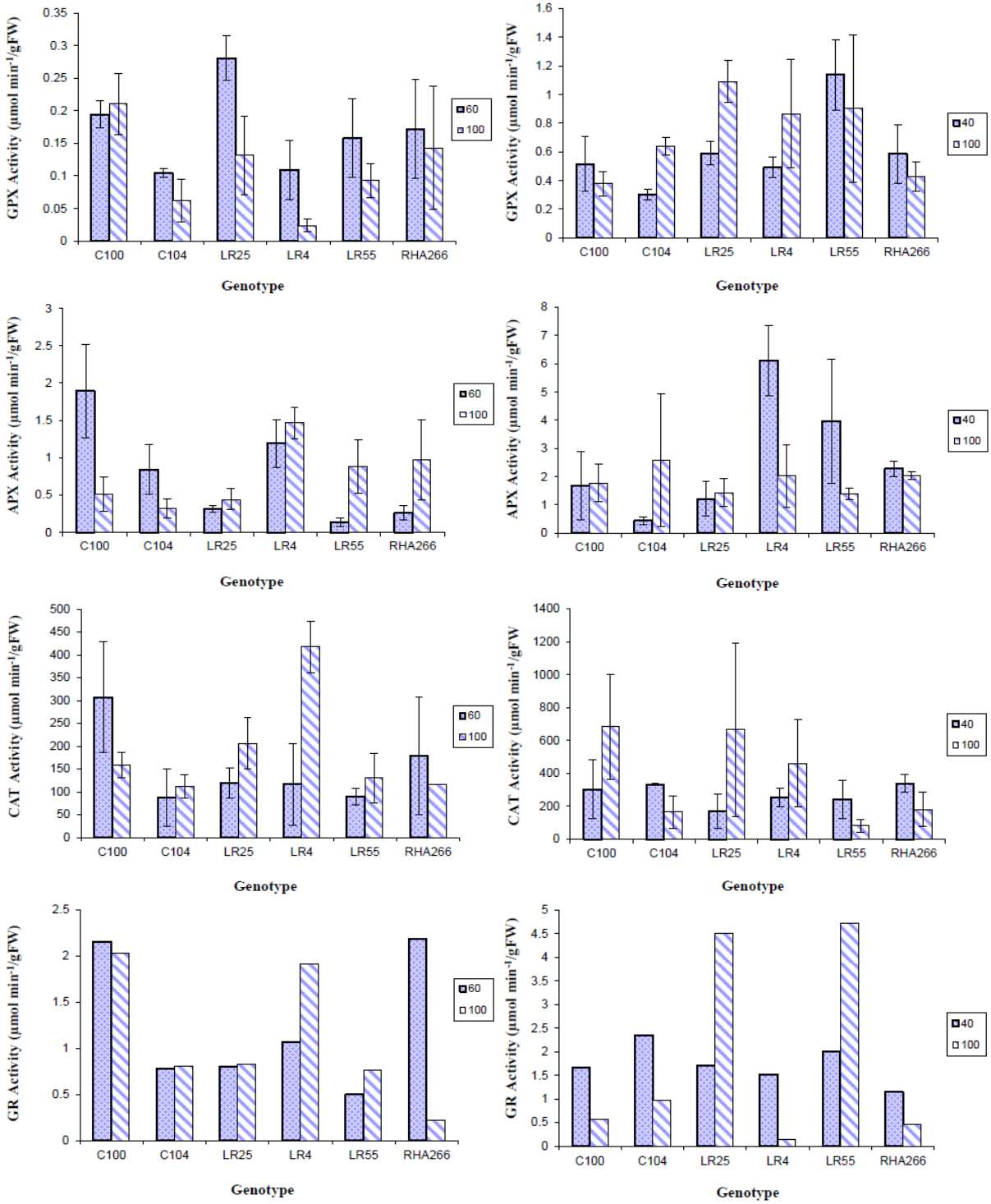

Figure 6. Antioxidant enzymes' activity of the studied sunflower lines at 60% (a, c, e, g) and 40% (b, d, f, h) drought stress as compared to the normal conditions (100%)

(Jiang and Huang 2001). Regarding the increase in cellular GR activity under drought stress and its role in glutathione reduction, GR is probably one of the most important enzymes in plant that the increase in its activity can enhance plant tolerance against oxidative stress (Khanna Chopra and Selote 2007). There are several conflicting reports about the activity of the GR under drought stress. According to the reports, both increasing (Sairam *et al*. 2002; Jung 2004) and decreasing (Jiang and Huang 2001) of the GR activity have been reported under drought stress. Considering our results, the highest GR activity belonged to the lines C100 and RHA266 at 60% and the line C104 at 40% drought stress.

Conclusion

The results showed significant differences for physio-biochemical and enzymatic responses of sunflower lines to the drought stress. Resistant and susceptible lines showed different responses to the stress. Some lines which were recognized as a drought sensitive genotype based on non-enzymatic variables were drought resistant genotype based on enzymatic characters. However, the antioxidant enzymes' activity was much more pronounced in the resistant lines of sunflower compared to the susceptible lines. It seems that each of enzymatic and non-enzymatic variables could clarify some aspects of drought resistance and must be used simultaneously for the screening of sunflower genotypes against drought stress. The lines C104 and RHA266 had good water status and osmotic adjustment and showed better tolerance to the drought stress. The finding of this study can be useful in sunflower breeding programs for producing resistant cultivars to drought stress.

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