R E S E A R C H   A R T I C L E

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Seed priming with ascorbic acid or calcium chloride mitigates the adverse effects of drought stress in sunflower (*Helianthus annuus* L.) seedlings

ABSTRACT:

Drought stress is one of the most important factors limiting the survival and growth of plants in different habitats of Egypt. This study was carried out to investigate the effect of drought stress on growth and some metabolic activities of sunflower seedlings under treatments with ascorbic acid (AsA) or $CaCl_2$. Drought stress showed a marked reduction in shoot length, leaf area, shoot fresh and dry weight, photosynthetic pigments, soluble sugar, and amylase activity. On the other hand, root length, soluble proteins, protease activity, proline content, soluble phenolics and flavonoid contents, phenylalanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO) activities**,** H_2O_2 and Malondialdehyde (MDA) contents, and total antioxidant capacity were induced compared with normal plant. The application of AsA or $CaCl_2$ mitigated the drought stress throughout the increase of growth criteria, antioxidant enzymes and photosynthetic pigments and decreased of H_2O_2, Malondialdehyde (MDA), soluble phenolics and flavonoid contents.

KEY WORDS:

Antioxidant enzymes, Ascorbic acid, $CaCl_2$, Drought, Photosynthetic pigments, Sunflower.

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INTRODUCTION:

Sunflower (*Helianthus annuus* L.) is one of the most important oil crops worldwide due to its higher levels of unsaturated fatty acids, as well as its efficacious in keeping the cholesterol levels down (Razi and Asad, 1998). Drought stress is one the most common plant threats that occurred due to either high rate of transpiration or limited water supply to the roots, the conditions that usually coincide with arid and semi-arid climates. These environmental conditions, as well as the plant genetic constitution severely affect the plant growth (Güler *et al.*, 2012). It was found that water shortage markedly reduced shoot height and dry matter of sunflower under controlled water conditions (Ahmad *et al.*, 2009). One of the most commonly used criteria to assess plant water status is the relative water content (RWC). Lower levels of RWC and leaf water potential noticeably reduced the photosynthetic rate of sunflower plants (Tezara *et al.*, 2002).

Concerning the photosynthetic status, water unavailability stimulated a pronounced decline in photosynthesis, which is reliant on photosynthesizing tissue and photosynthetic pigments (Raza *et al.*, 2006). Photo system II, as well as the proteins of chloroplast stroma severely affected under water shortage (Inzé and Van Montagu, 1995). Moreover, implementation of drought stress was found to retard chlorophyll pigments in sunflower plants as compared to well-watered plants (Manivannan *et al.*, 2008). Furthermore, limited water availability delayed photosynthetic capacity due to the imbalance between light capture and its utilization, so an oxidative stress elevated (Foyer and Noctor, 2005).

Water accessibility plays a significant role in plant life. It is important in all plant biological events (Bewley and black, 1994). Plant stress tolerance could be mitigated through the accumulation of osmolytes like proline, sugars and amino acids that caused osmotic regulation (Azooz, 2009). Proline one of the most important amino acids that rapidly accumulated in plants under water

deficit due to its potentiality to maintain cell turgor, so proline accumulation can be used evaluate of drought tolerance in plant varieties (Gunes *et al.*, 2008). Soluble sugars was found to play a pivotal role in osmotic adjustment of cells during germination (Bolarin *et al.* 1995). Additionally, sugars are involved in the expression of some genes during germination of seeds (Yu *et al.*, 1996). Besides soluble sugars, proteins are commonly involved to cope with prevailing environmental changes including water deficit. Most of these proteins are water soluble so can be implicated in plant stress tolerance through the synthesis of a diversity of transcription factors and stress proteins (Wahid *et al.*, 2007). It was found that water deficit markedly reduced the activity of α- and β-amylase in *Medicago sativa* germinating seeds (Zeid and Shedeed, 2006). In addition to amylases, proteases have a necessary role in the proteins degradation and nutrient mobilization under environmental stresses (Grudkowska and Zagdanska, 2004). Zagdariska and Wisniewski (1996) reported that the total proteolytic activity was increased drastically in response to water shortage in wheat plants.

The antioxidant arsenal in the plant cell includes both enzymatic and non-enzymatic constituents which showed marked improvement under stressful conditions including drought (Apel and Hirt, 2004). Peroxidases, polyphenol oxidases and phenylalanine ammonia lyases considered as examples for the enzymatic components of the plant antioxidant system (Ahmad *et al.*, 2010). Saleh and Madany (2015) found that, peroxidase activity enhanced when wheat plants were grown under salt stress. Moreover, the activity of phenylalanine ammonia lyase (PAL), a key enzyme in the phenylpropanoid pathway, showed a sharp improvement in the leaves of plants under drought stress (Gholizadeh, 2011). Among the non-enzymatic components are phenolics that play multiple roles in plants. They act as structural components of cell walls, involved in growth and developmental processes, as well as in the mechanisms of defense against both biotic and abiotic stresses (Cheynier *et al.*, 2013). Additionally, malondialdehyde (MDA), a lipid peroxidation marker, is accumulated in plants under salt stress (Shalata and Neumann, 2001).

Ascorbic acid, essential vitamin in plants, has the potentiality to scavenge the reactive oxygen species (ROS) which has deleterious effect on plant growth (Shalata and Neumann, 2001). It was shown that ascorbic acid can improve plant tolerance against abiotic stresses (Al-Hakimi and Hamada, 2001). Meanwhile, the exogenous application of ascorbic acid significantly

improved their plant growth (Yazdanpanah *et al.*, 2011). Therefore, besides being important coenzymes, exogenous ascorbic acid was found to play other independent roles in the biochemical processes of plants such as repairing the injurious effects of stressful conditions (Oertli, 1987).

Calcium plays a key role in several plant growth and physiological behaviors and involved in plant resistance against stresses including water scarcity (Mozafari *et al.*, 2008). It also considered as an essential nutrient that improve the plant productivity and increase its biomass production (Srivastava *et al.*, 2013). Moreover, calcium involved in plant cell elongation and division, structure and permeability of cell membranes, nitrogen metabolism and carbohydrate translocation (White, 2000).

The objective of this study was to determine the potentiality of ascorbic acid and calcium chloride to alleviate the drought stress implemented on sunflower plants through investigating its growth and some metabolic activities.

MATERIAL AND METHODS:

Plant material and pot experiment:

Pure strain of sunflower seeds obtained from Agriculture Research Center, Giza, Egypt. Seeds were surface sterilized with 0.1% mercuric chloride for 5 min, then thoroughly washed with distilled water. The sterilized seeds were soaked in ascorbic acid or calcium chloride (0.5 mM or 5 mM, respectively) then sown in plastic pots filled with a mixture of clay-sand soil (2:1 w/w). The pots were kept at 80% soil field capacity (SFC) for ten days until the drought treatments were started. After that, the pots were divided into two groups: (1) normal irrigation that serves as control (80% SFC); (2) mild drought (40% SFC). Drought treatment was initiated by withholding water, the pots were then weighed daily and once they had reached the required SFC they were maintained at that level by the addition of appropriate volumes of nutrient solution. At the end of the experiment (30 days), Plant samples were collected for determination of growth parameters. Some fresh seedlings were stored at - 20˚C after grounding under liquid nitrogen for biochemical analyses.

Determination of Relative Water Content (RWC):

The relative water content (RWC) of leaves was determined according to the method described by Kavas *et al.* (2013). Using the equation: RWC (%) = (FW – DW)/(TW – DW) × 100. The fresh weight (FW) of the leaves was determined and recorded. Each leaf was placed in a petri dish filled with distilled water for 24 h at 4°C and then weighed to determine the turgid weight (TW).

The dry weight (DW) of the leaves was determined after oven drying for 48 h at 70°C.

Determination of photosynthetic pigments:

Assessments of chlorophyll content were performed during the experimental period. Total chlorophyll, chlorophyll a and b, as well as carotenoids from fully expanded fresh leaves were measured spectrophotometrically using 100% acetone, and their concentrations were calculated as mg g^{-1} FW (Sestak et al., 1971).

Determination of soluble sugars and proteins:

Frozen samples (0.5 g) were ground and extracted in 2 ml of 2.5 N HCl for 30 min, followed by centrifugation at 10,000 × g at 4°C for 10 min. Total soluble sugar content was estimated colorimetrically using anthrone reagent and glucose as standard (Roe, 1955).

The total protein content in the leaves was estimated by adopting the methodology of (Lowry et al., 1951). The protein was extracted with NaOH (0.1 M) and the Folin phenol reagent was added to develop the blue colour which was read at 600 nm.

Protease and amylase Assays:

Fresh powdered tissues were homogenized in 20 mM phosphate buffer, pH 7.6 for estimating protease activity. The reaction was initiated by adding 0.5 ml of the crude extract to 2 ml of the substrate solution (20 mM phosphate buffer, pH 7.0, containing 10 mg/ ml BSA) and incubated at 40°C for one hour. The resulted soluble peptides were recorded using Folin-Lowry method adopted by Hartree (1972). For amylase extraction, fresh powdered tissues were homogenized in 100 mM acetate buffer, pH 6.0. The amylolytic activity was determined by mixing 0.5 ml of the crude extract with 0.5 ml of 0.5% soluble starch prepared in 0.1M of acetate buffer, pH 6.0, containing 5 mM CaCl₂. The resulting reducing sugars were estimated by the Nelson's method (Clark and Switzer, 1964).

Estimation of proline content:

Free proline content was determined according to Bates et al. (1973). A known fresh weight of powdered tissue was homogenized in 3% aqueous sulfosalicylic acid. The reaction was initiated by adding acid ninhydrin reagent and glacial acetic acid to the extract in boiling water bath. After cooling, 4 ml toluene was added and mixed well for 20 sec. The absorbance of chromophore-containing toluene layer was recorded at 520 nm against toluene.

Phenolics and flavonoids content:

Total soluble phenolic compounds were extracted with 70% ethanol (Sauvesty et al., 1992). The Folin-Ciocalteu phenol method was used for phenolic estimation (Carter, 1993). Total flavonoids were extracted and

estimated using the method adopted by Pessarakli (2005).

Activities of antioxidant enzymes:

Polyphenol oxidase (PPO, EC 1.14.18.1) was extracted as described by Kar and Mishra (1976) with slight modification. According to the method proposed by Nguyen et al. (2003), the assay mixture contained the crude enzyme extract and the substrate solution (0.05 M phosphate buffer, pH 6.0, containing 0.05 M catechol). The mixture was incubated at 30°C for 30 min and then the absorbance measured at 420 nm then expressed as nmol guaiacol mg protein^{-1}min^{-1}.

Extraction of peroxidase (POX, EC 1.11.1.7) was carried out according to the method outlined by Kar and Mishra (1976). Based on the method of Wakamatsu and Takahama (1993), the reaction mixture contained the crude enzyme extract and assay mixture (50 mM phosphate buffer, pH 7.2; 0.1 mM EDTA; 5 mM guaiacol; 0.3 mM hydrogen peroxide) and the absorbance was measured at 470 nm then expressed as nmol guaiacol mg protein^{-1}min^{-1}.

Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) activity was assayed using the method outlined by Chandra et al. (2007). The activity was started by mixing enzyme extract and the substrate solution (6 mM of L-phenylalanine in 0.5 mM Tris-HCl buffer, pH 8.0) for two hrs at 37°C. The absorbance was measured at 290 nm and determined as the rate of conversion of L-phenylalanine to t-cinnamic acid.

Estimation of hydrogen peroxide (H₂O₂) content:

H₂O₂ was extracted by homogenizing fresh powdered tissues in 0.1% tri-chloroacetic acid (Alexieva et al., 2001). The homogenate was centrifuged and 0.5 ml of the supernatant was added to 0.5 ml of phosphate buffer (10 mM, pH 7.0) and 0.2 ml of potassium iodide (5 M). Absorbance was followed for 1 min at 390 nm. The blank consisted of a reaction mixture without potassium iodide, and its absorbance was subtracted from the mixture with H₂O₂ extract.

Malondialdehide (MDA) and Total antioxidant capacity:

MDA content was determined using the method of Fu and Huang (2001). Fresh powdered sample was homogenized in 4 ml trichloroacetic acid (0.1%; w/v) in an ice bath and the supernatant was used for lipid peroxidation analysis. MDA content was then estimated using thiobarbituric acid (0.5% in 20% TCA) spectrophotometrically at 532 nm and corrected for nonspecific turbidity at 600 nm.

For extraction of non-enzymatic antioxidants, a known weight of liquid nitrogen-powdered tissues was homogenized

with pre-chilled 80% ethanol. The total antioxidant capacity was determined by de-colorization of the $ABTS^{•+}$, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), radical (Re *et al.*, 1999). Ten ml of the extract was mixed with 1 ml of the diluted $ABTS^{•+}$ solution (A_{734nm} = 0.700 ± 0.020) and O.D. was taken at 734 nm. The TAC was calculated from Trolox standards curve and expressed as µmol Trolox g^{-1} fresh weight.

Statistical Analysis:

Experiments were carried out following a randomized complete block design. Data normality and the homogeneity of variances were checked using the Kolmogorov– Smirnov test and Levene´s test, respectively. All the data were subjected to one-way analysis of variance (ANOVA). Duncan's Multiple Range Test (p B 0.05) was carried out as the post hoc test for mean separations. Data were transformed by log (x + 1) before statistical analysis where needed. All statistical tests were performed using the computer program PASW statistics 18.0 (SPSS Inc., Chicago, IL, USA).

RESULTS:

Drought stress showed a non-significant reduction in shoot length, as well as fresh and dry weight of sunflower seedlings, while leaf area showed a marked reduction compared with control plants (Fig. 1 A-D). In contrast, root length was significantly increased under the same level of drought stress as compared with normal plants (Fig. 1E). Ascorbic acid and $CaCl_2$ treatments generally induced a significant increase in the values of shoot and root length, shoot fresh and dry weight and leaf area compared with the reference control plants. The relative water content of the drought plants significantly decreased within 30 days-old sunflower seedlings (Fig. 1F) as compared with the control plants. The application of ascorbic acid under drought stress induced a significant increase in the values of relative water content with about 25% compared with control plants. On the other hand, $CaCl_2$ treatment under the drought stress led to a non-significant increase in the values of relative water content with about 10% compared with reference control.
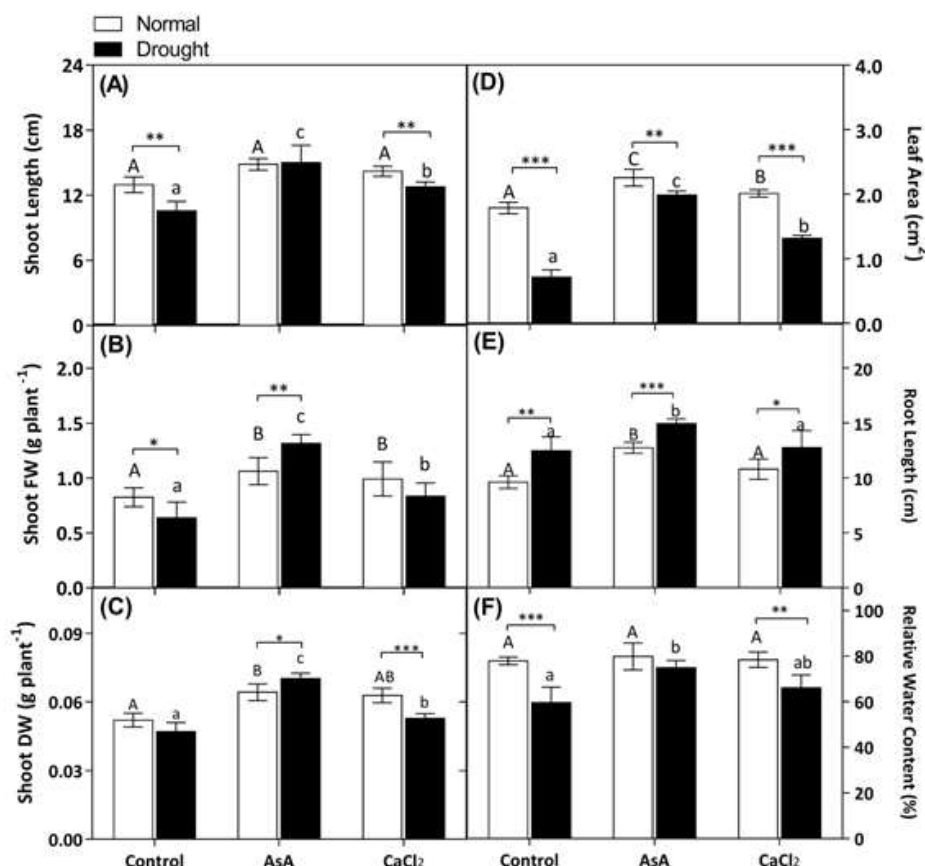

Fig. 1. Effect of AsA and $CaCl_2$ treatments on (A) *shoot length*; (B) *shoot fresh weight*; (C) *shoot dry eight*; (D) *leaf area*; (E) *root length* and (F) *relative water content* of 30-day-old sunflower seedlings under both normal and drought conditions. Each value is the mean of 5 independent replicates and vertical bars represent the standard error. The same letters indicate no significant difference (P < 0.05) as analysed by Duncan's test (upper and lower case letters are used for normal and drought stressed sets, respectively). Differences between normal and drought stressed seedlings in each set were analysed using Student's *t*-test: *= *P* < 0.05; **= *P* < 0.01; *** = *P* < 0.001

The contents of chlorophyll a, chlorophyll b, chlorophyll a + b, carotenoids and total pigments showed a significant decreased under drought level compared with normal plants (Fig. 2). The treatments of sunflower plants with ascorbic acid and CaCl$_2$ did not only alleviate the inhibitory effect of drought on photosynthetic pigment contents, but also induced a significant stimulatory effect on the biosynthesis of pigment fractions and the largest enhancement was observed in chlorophyll b that reach about 121% when treated with ascorbic acid under drought stress as compared with control plant.
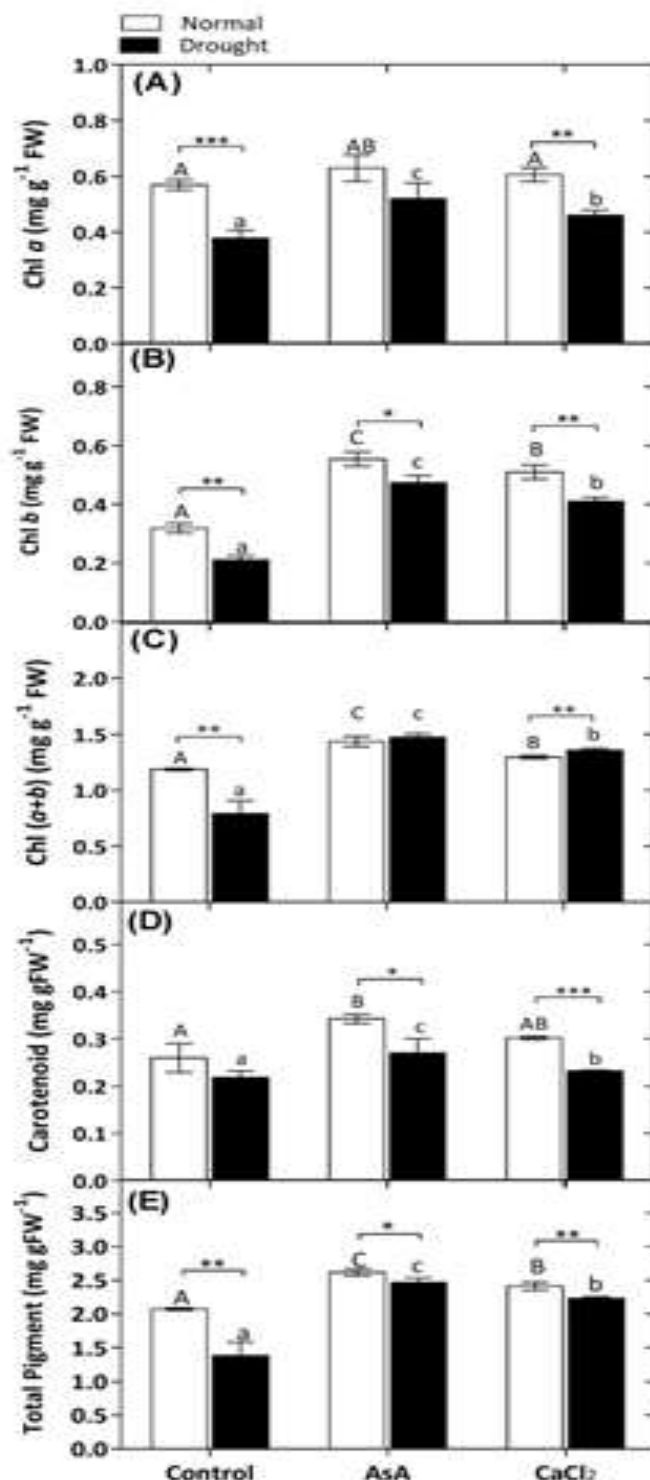

Fig. 2. Effect of AsA and CaCl$_2$ treatments on (A)*soluble proteins;* (B) *soluble sugars;* (C) *protease activity* and (D) *amylase activity* of 30-day-old sunflower shoots under both normal and drought conditions. Each value is the mean of 5 independent replicates and vertical bars represent the standard error. The same letters indicate no significant difference (P < 0.05) as analysed by Duncan's test (upper and lower case letters are used for normal and drought stressed sets, respectively). Differences between normal and drought stressed seedlings in each set were analysed using Student's *t*-test: *= $P < 0.05$; **= $P < 0.01$; *** = $P < 0.001$

The interactive effects of drought, ascorbic acid and $CaCl_2$ on soluble proteins and soluble sugar of sunflower seedlings were investigated (Fig. 3A & B). The data clearly indicate that the soluble proteins were significantly increased, where the soluble sugar significantly decreased when the plants subjected to drought stress compared with those of normal plants. The application of ascorbic acid and $CaCl_2$ under drought stress induced a stimulatory effect on the accumulation of soluble proteins as compared with reference control.

Additionally, both proteolytic and amylolytic activities were affected in sunflower seedlings under implementation of drought stress (Fig. 3C & D). Their values were a highly increased in case protease activity and significantly decreased in case amylase activity in drought treated plants as compared with plants grown without drought. The application of ascorbic acid and $CaCl_2$ led to a significant increase in both protease and amylase activity and the maximum values observed in plants subjected to drought stress with ascorbic acid as compared with reference control.
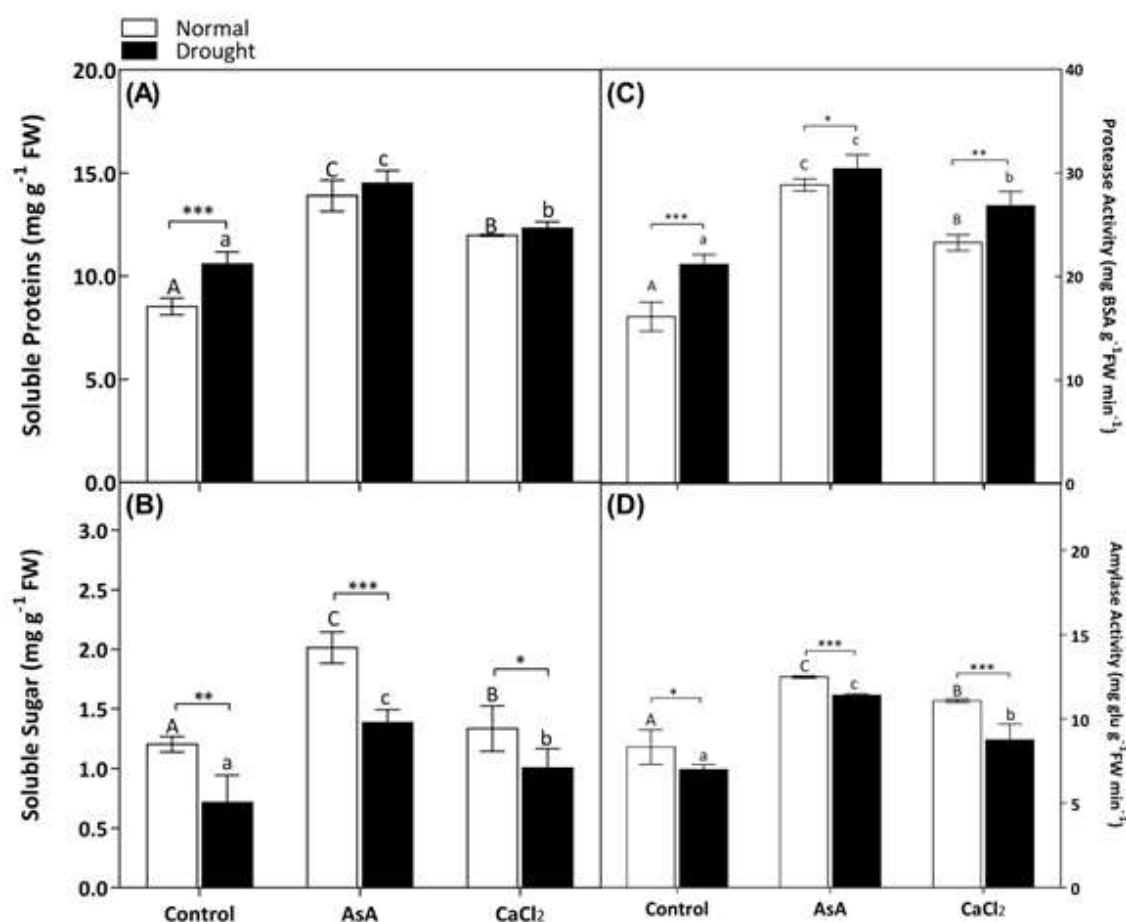

Fig. 3. Effect of AsA and $CaCl_2$ treatments on (A) *soluble proteins;* (B) *soluble sugars;* (C) *protease activity* and (D) *amylase activity* of 30-day-old sunflower shoots under both normal and drought conditions. Each value is the mean of 5 independent replicates and vertical bars represent the standard error. The same letters indicate no significant difference ($P < 0.05$) as analysed by Duncan's test (upper and lower case letters are used for normal and drought stressed sets, respectively). Differences between normal and drought stressed seedlings in each set were analysed using Student's *t*-test: *= $P < 0.05$; **= $P < 0.01$; *** = $P < 0.001$.

The changes in proline content of sunflower seedlings in response to treatment with drought and/or AsA and $CaCl_2$ were recorded (Fig. 4A). The data clearly showed that, drought treatment caused highly significant increases in proline content as compared with untreated control plants. Plants resulted from treatments with AsA and $CaCl_2$ either alone or in combination with drought level showed significant increases in

proline content as compared with reference control. Where, proline content increased by about 114% in seedlings treated with AsA and by about 60% in seedlings treated with AsA under drought stress. Thus, AsA is more effective than $CaCl_2$.

Moreover, our results clearly demonstrated that, both flavonoid and soluble phenolics under drought treatment highly increased compared with normal plant (Fig.

4B & C). The application of AsA or CaCl₂ alone or in interactions with drought level led to significant increase as compared with untreated plants. Although, AsA mitigate the effect of drought stress more than CaCl₂ in case flavonoid content and soluble phenolics by about 23 and 39%, respectively as compared with control plants.
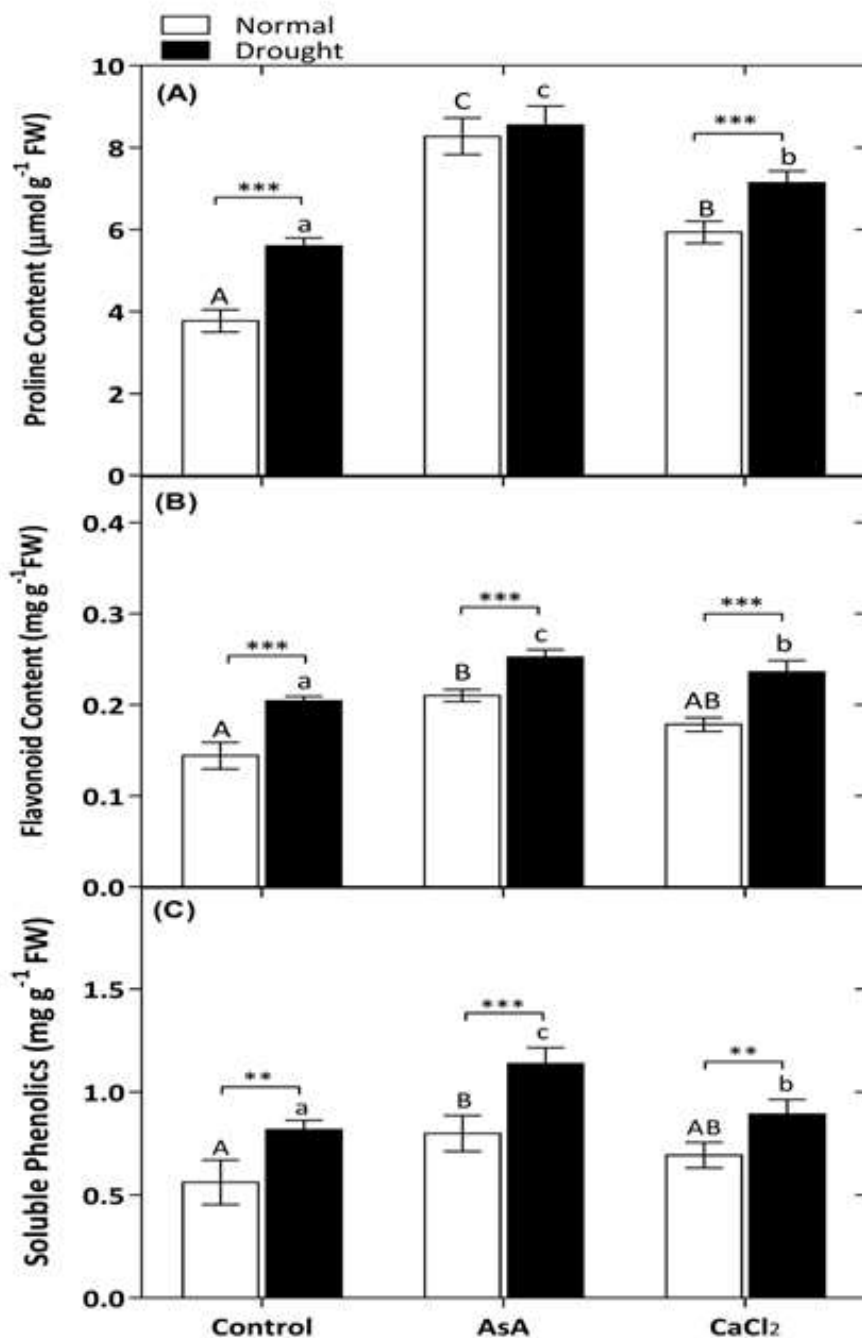

Fig. 4. Effect of AsA and CaCl₂ treatments on (A) *proline content;* (B) *flavonoid content* and (C) *soluble phenolics* of 30-day-old sunflower shoots under both normal and drought conditions. Each value is the mean of 5 independent replicates and vertical bars represent the standard error. The same letters indicate no significant difference (P < 0.05) as analysed by Duncan's test (upper and lower case letters are used for normal and drought stressed sets, respectively). Differences between normal and drought stressed seedlings in each set were analysed using Student's *t*-test: *= P < 0.05; **= P < 0.01; *** = P < 0.001.

The activities of PPO, POX and PAL were traced to stand upon the influence of drought, AsA and CaCl₂ upon them (Fig. 5). Nevertheless, PAL activity showed non-significant increased, drought stress markedly enhanced POX and PPO activity when compared with untreated plants. On the other hand, the treatments with either AsA or CaCl₂, as well as in combination with drought level highly induced the activities of PAL and POX and significantly increase in PPO compared with the well-watered seedlings.
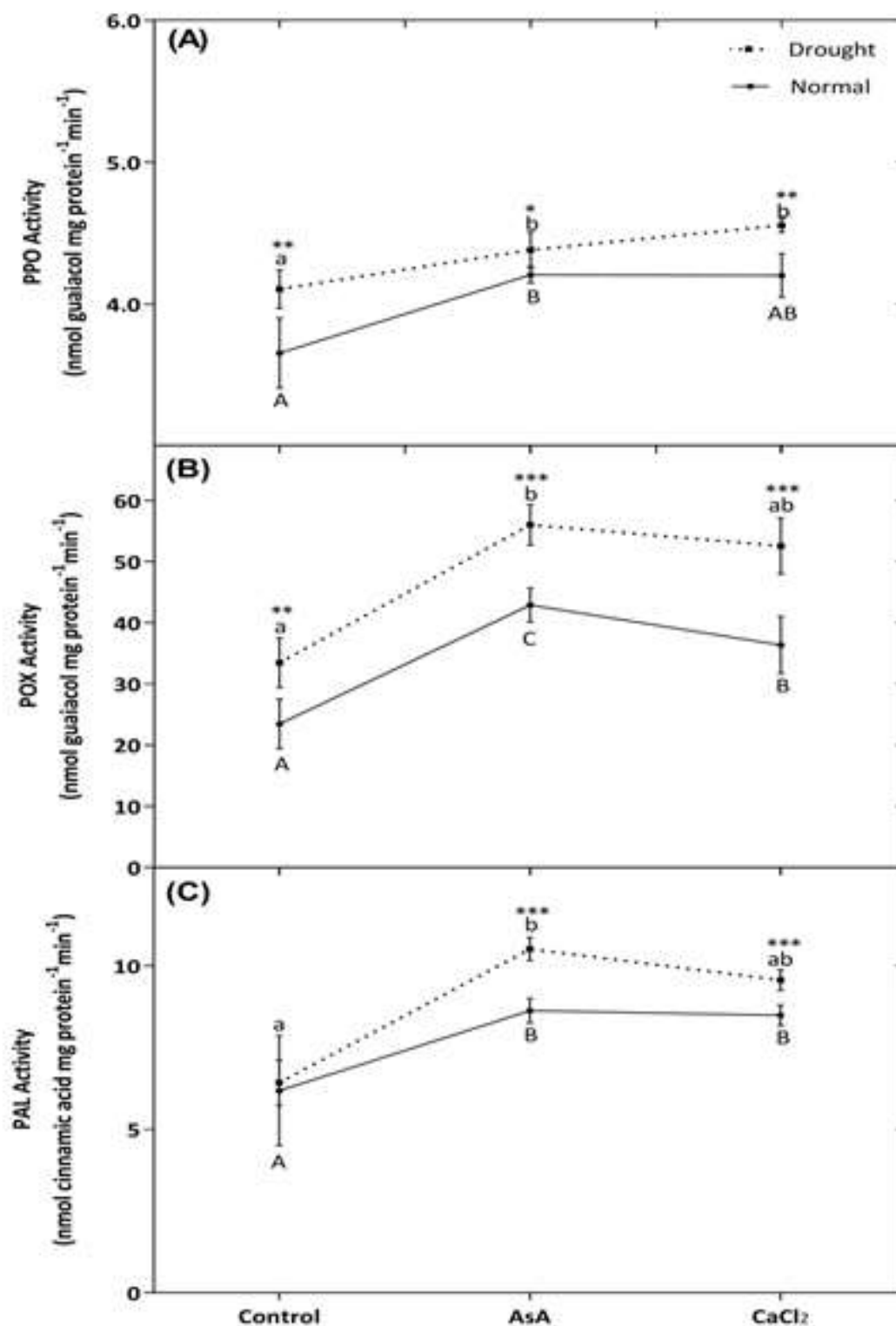
Fig. 5. Effect of AsA and CaCl₂ treatments on the activities of (A) *PPO;* (B) *POX* and (C) *PAL* of 30-day-old sunflower seedlings under both normal and drought conditions. Each value is the mean of 5 independent replicates and vertical bars represent the standard error. The same letters indicate no significant difference (P < 0.05) as analysed by Duncan's test (upper and lower case letters are used for normal and drought stressed sets, respectively). Differences between normal and drought stressed seedlings in each set were analysed using Student's *t*-test: *= *P* < 0.05; **= *P* < 0.01; *** = *P* < 0.001.

H₂O₂ content markedly increased with drought treatment compared with normal plants (Fig. 6A). Under AsA and CaCl₂ treatment in combination with drought reduced the H₂O₂ content higher than in unstressed plants. While, CaCl₂ alone

increased the H_2O_2 content by 15.39% as compared with well-watered plants. The total antioxidant capacity has been increased in drought treated plants and when treated with AsA and CaCl₂ or in combination with drought led to the increased in the total antioxidant status of sunflower seedlings (Fig. 6B). The maximum value recorded at AsA treatment alone or in combination with drought stress by about 78% and 71%, respectively as

compared with control seedlings. Oxidative damage to tissue lipid was estimated by the content of MDA. The plants subjected to drought showed a trend of increasing content of MDA (Fig. 6C). The AsA and CaCl₂ treatments were a significantly reduced the MDA content in combination with drought stress by 32.11% and 20.62%, respectively as compared with control plants.
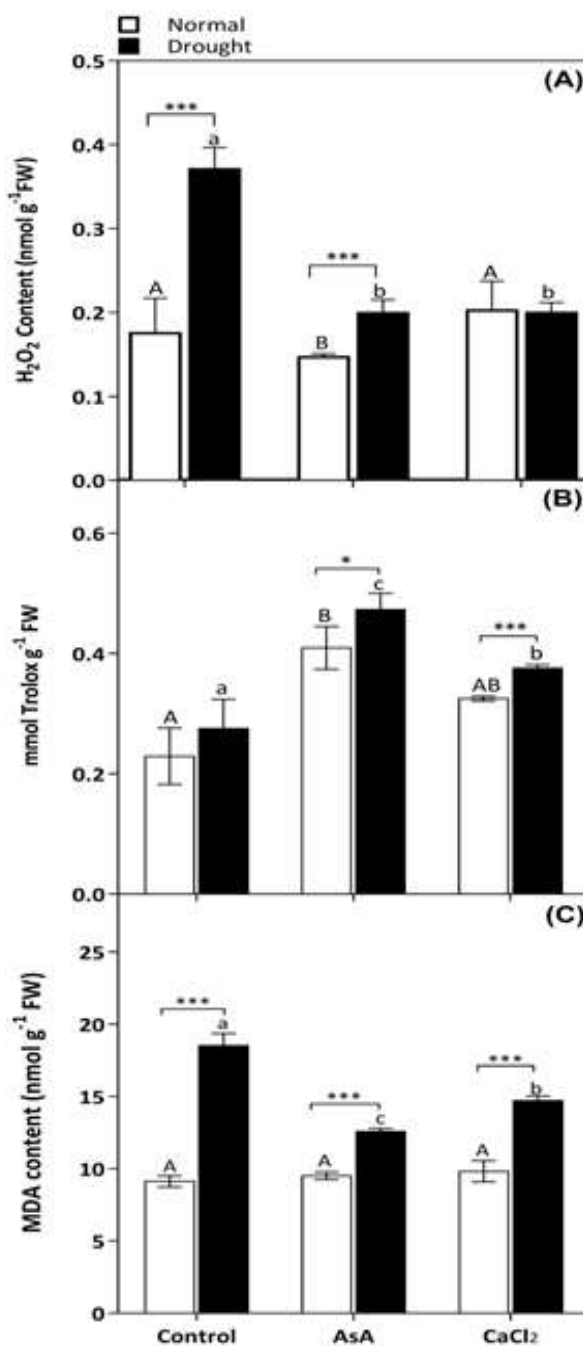

Fig. 6. Effect of AsA and CaCl₂ treatments on (A) *H₂O₂ content;* (B) *total antioxidant capacity* and (C) *MDA content* of 30-day-old sunflower seedlings under both normal and drought conditions. Each value is the mean of 5 independent replicates and vertical bars represent the standard error. The same letters indicate no significant difference (P < 0.05) as analysed by Duncan's test (upper and lower case letters are used for normal and drought stressed sets, respectively). Differences between normal and drought stressed seedlings in each set were analysed using Student's *t*-test: *= $P < 0.05$; **= $P < 0.01$; *** = $P < 0.001$.

DISCUSSION:

In the present results, a reduction in growth parameters under water stress was found. The plant growth depend on cell division, enlargement and differentiation, all of these events are affected by water stress (Kusaka et al. 2005). This might be the reason for the reduced growth of sunflower plants under water deficit. Reduction in leaf area by drought stress is a vital causative agent for reduced crop yield through reduction in photosynthesis (Tezara et al., 2002). Leaf area expansions depend on leaf turgor, temperature and assimilate supply, which are all affected by drought (Reddy et al. 2004). The reduction in plant height may be associated with decreased cell enlargement and cell growth due to the low turgor pressure and also more leaf senescence under drought stress. Mohammadian et al. (2005) reported that the leaf area index, a well as leaf, shoot, and root dry weights decreased under drought stress, as compared to non-stress conditions. Our results revealed that relative water content (RWC) of sunflower leaves decreased under water stress. Water limitation has an effect on plant growth and development. Li et al. (2011) showed that, drought treatment significantly decreased the leaf relative water content in Cotinus coggygria seedlings. Decreasing leaf relative water content is an indication of decrease of swelling pressure in plant cells and causes growth to decrease.

Application of ascorbic acid (AsA) or $CaCl_2$ improved all plant growth criteria compared to untreated plants. Regarding the interaction effects, ascorbic acid or $CaCl_2$ significantly increased all growth parameters of sunflower plants and decreased the harmful effect of water stress on plant growth. Ascorbic acid or $CaCl_2$ may act as growth promoters which can play a role in alleviating the contrary effect of stress on metabolic activities appropriate to growth through increasing cell division and/or cell enlargement. These were further substantiated by the significantly higher levels of sugars by vitamin treatment (Hassanein and Bassuony, 2009). Also, The effect of Ca^{+2} on enhancing growth characters is due to some reasons including that Ca^{+2} contributes in the structure of the cell wall and the cell membrane, therefore it keeps the balance and stability of membranes through the contacting of types of protein and lipids on the surface of the membrane (Davis et al., 2003). As well as, affecting the pH in the cell which prevents the solvent exit out of the cytoplasm and works on increasing of the shoot length (Hirschi, 2004).

Vitamins such as ascorbic acid probably reflect the efficiency of water uptake and reduce water loss through increasing the relative water content (RWC) of leaves and reducing the transpiration rate and consequently cause an increase in leaf water potential. Hence, it could be concluded that the beneficial effect of ascorbic acid on growth parameters of sunflower plants has been related to their water uptake and utilization efficiency. It was found that some crop plants improved their growth and yield upon treatment with vitamins using optimal concentrations under saline conditions (Ekmekçi and Karaman, 2012). The treatment with $CaCl_2$ showed a slight increase in RWC compared with control plants. Supplemental Ca^{2+} was found to prevent the inhibition of hydraulic conductance in maize (He and Cramer, 1992).

The reduction of photosynthesis during exposure to stress is a kind of defence mechanism used by plants. In wheat plant, the amount of photosynthesis reduced significantly due to drought stress (Jones and Corlett, 1992). Reduction of chlorophyll surface is mostly due to lack of activity in photosynthetic system. Therefore, the drought stress causes the chlorophyll surface to be reduced and the chloroplast membrane to be destructed and finally it would result in reduction of photosynthesis pigments. The drought stress resulted in reduction of total chlorophyll in Festuca and Kentucky bluegrass (Fu and Huang, 2001). Under water scarcity, plants tend to close their stomata in order to avoid water loss through transpiration. Stomatal closure prevents the passage of CO_2 through the leaves leading to retardation of photosynthetic carbon fixation even in daytime (Sanda et al., 2011).

It is well known that application of ascorbic acid or Ca^{+2} enhances the total chlorophyll through either the encouragement of its accumulation and/or hindrance of its degradation. This improvement could be attributed to efficient role of antioxidants in stabilizing active sites of the enzymes involved in photosynthetic reactions, as well as their role in scavenging the harmful ROS that may destroy the chlorophyll pigments. Moreover, chloroplast is considered as a major source of ROS in plants, but it lacks catalase enzyme that can scavenge ROS. Instead, ascorbic acid can act as a substrate for ascorbate peroxidase (APX) to scavenge ROS produced in the thylakoid membranes (Davey et al., 2000). Additionally, the stimulating effect of antioxidants on chlorophyll content may also be due to ascorbic acid has a major role in photosynthesis, acting in the Mehler peroxidase reaction with ascorbate peroxidase to regulate the redox state of photosynthetic electron carriers and as a co-factor for violaxanthin de-epoxidase, an enzyme involved in xanthophyll cycle-mediated photoprotection (Smirnoff and

Wheeler, 2000). Fu and Huang (2001) observed that foliar application of ascorbic acid ameliorates the adverse effect of water stress due to stomata closure, nutrient uptake, total chlorophyll content, protein synthesis, transpiration, photosynthesis and plant growth. The addition of Ca^{+2} to the drought stressed plants affects most of the physiological processes within the plant and our data showed that the addition of Ca^{+2} worked on increasing the total content of photosynthesis pigments compared to untreated plants. Mozafari *et al.* (2008) found that application of $CaCl_2$ with the concentration 5mM the chlorophyll *a* was increased significantly and it is probably due to the Ca^{+2} work on protecting the chloroplasts wall and helps the activity of photosynthesis enzymes as reported by Siddiqui *et al.* (2012) on faba bean (*Vicia faba*) stressed with the cadmium element, where it was found that there was an increase in the content of chlorophyll *a* and chlorophyll *b* when the plant treated with each of Ca^{+2} and K^+.

Water deficit caused a pronounced change in protein synthesizing apparatus in plant tissue (Genkel *et al.*, 1967). In the present study the results obtained with higher protein content in sunflower plants are in agreement with Chinoy *et al.* (1974) who found that rice plants showed a marked increase in protein content under water stress. Ashraf and Foolad (2007) revealed the marked accumulation of protein in tolerant genotypes under water stress to higher DNA and RNA contents, which enhance synthesis of protein.

The present study showed a noticeable reduction in the carbohydrate content. This may be attributed to the deleterious effect of water stress on the membrane of thylakoids and the amount of photosynthetic pigments that in turn will decrease the carbohydrate content in the leaves stressed plants. On the other hand, water shortage leads to the reduction in turgor pressure and hence the closeness of the stomata then finally decrease the photosynthetic rate (Yazdanpanah *et al.* 2011).

Amylase activity was decreased under water stress as compared with control plants. In this context, Pratap and Sharma (2010) showed that amylase exhibit a pronounced reduction in *Phaseolus mungo* under osmotic stress. Similarly, water shortage severely reduced α and β-amylases, as well as soluble sugars in *Pisum sativum* seedlings which showed a marked increase in proline accumulation under these conditions (Al-Jebory, 2012).

Proline consider one of the most important amino acids in plants that is rapidly accumulated in plants under environmental stresses (Gunes *et al.*, 2008). It is synthesized in plants under water stress from glutamic acid, which acts as osmoprotectant for keeping the water balance in cells and outer environment and protecting cellular structures during dehydration (Lehmann *et al.*, 2010). Furthermore, proline act as enzyme protectant and stabilizes the structure of macromolecules and organelles (Ermak and Kelvin, 2000). Our study indicated that proline showed marked accumulation in response to drought stress. In this context, Saleh and Madany (2015) reported that proline showed marked accumulation under salt stress. Moreover, Reddy *et al.* (2004) reported that the amount of proline under drought stress was highly accumulated, indicating that proline is a key amino acid in osmosis regulation. The effect of ascorbic acid was clear, suggesting an interaction between the proline synthesis and ascorbate function. Also, addition of $CaCl_2$ together with water stress increased the proline content under drought level, mainly due to the breakdown of proline rich protein and fresh synthesis of proline and amino acids (Huang *et al.*, 2000). It could also be due to prevention or feedback inhibition of synthesis of the biosynthetic enzyme caused by sequestering of proline away from its site of synthesis or by relaxed feedback inhibition of regulatory step enzymes (Kishor *et al.*, 2005). Increased proline in the stressed plants may be an adaptation to compensate the energy for growth and survival and thereby help the plant tolerate stress, as reported in spinach leaves (Öztürk and Demir, 2003).

The present results showed that both soluble phenolics and flavonoid contents significantly increased under drought stress and interaction with ascorbic acid or $CaCl_2$. This could be important for preventing the lipid peroxidation by hydroxyl radicals of the cell membrane lipids. The lipid peroxidation altered the signal transduction and initiate the metabolic alteration and promotes the accumulation of secondary metabolites is important to protect the cell membrane lipid from the oxidative stress and the reactive oxygen species (Zhu *et al.* 2009). Moreover, Sánchez-Rodriguez *et al.* (2010) found an enhancement of some phenolics and flavonoids under moderate water stress (50% of the field capacity) in the more tolerant cultivars of cherry tomatoes, but a reduction in the more sensitive ones, in partial agreement with the data presented here.

Drought stress induces the activity of antioxidative system that may contribute to drought resistance in sunflower plants. Phenylalanine ammonia lyase (PAL) catalyzes deaminating reaction of the amino acid phenylalanine at the gateway from the primary metabolism into the important secondary phenylpropanoid metabolism in plants (Hahlbrock and Scheel, 1989).

Phenylpropanoid compounds not only fulfil various essential functions during plant development, but also, they act as important protectants against various biotic and abiotic environmental stresses. High activities of antioxidant enzymes also improved drought tolerance olive (Ben Ahmed *et al.*, 2009). It seemed to be that higher activity of POX provided higher protection against oxidative stress under drought stress, as judged from higher increases of proline and total protein content and this results agreement with (Shanjani *et al.*, 2014). The observed positive correlations among activities of POX and PPO in this study suggested that these enzymes might be involved in the elimination of the reactive oxygen species (ROS) within the peroxide/phenols/ascorbate system in drought stress (Sgherri *et al.*, 2004). Ben Ahmed *et al.* (2009) reported that proline accumulation could activate the antioxidant defence mechanisms.

Additionally, ascorbic acid is a co-factor for prolyl-hydroxylase that post-translationally hydroxylates proline residues in cell wall hydroxyl proline rich glycoproteins required for cell division and expansion (Smirnoff and Wheeler, 2000). The depressive effect of water stress on growth parameters may also be attributed to a drop in leaf water content, and a reduction in the assimilation of nitrogen compounds (Reddy *et al.*, 2004), affecting the rate of cell division and enlargement. Drought stress also reduced the uptake of essential elements and photosynthetic capacity, as well as the excessive accumulation of intermediate compounds such as reactive oxygen species (Yazdanpanah *et al.* 2011) which cause oxidative damage to DNA, lipid and proteins and consequently a decrease in plant growth. Finally, water stress leads to increases in abscisic acid which cause an inhibition of the growth (Abdalla, 2011). In addition, a secondary aspect of water stress in plants is the stress-induced production of ROS (Razaji *et al.*, 2014). The enhanced production of ROS during water stress lead to the progressive oxidative damage and ultimately cell death and growth suppression (Ruiz-

Lozano *et al.*, 2012). These results are in agreement with those obtained by others (Azooz 2009; Ekmekçi and Karaman 2012). They indicated that, vitamins (such as ascorbic acid) could accelerate cell division and cell enlargement and induce improvement.

In the present study, activities of antioxidant enzymes (PAL, POX, and PPO) in sunflower plants were increased in response to water stress, as well as after AsA application. Whereas, AsA application may act to protect plants from oxidative injury induced by water stress (Athar *et al.*, 2009). On the other hand, the treatment with exogenous $CaCl_2$ demonstrated lower MDA levels when compared to seedlings treated with drought level. Diminished MDA levels in the presence of $CaCl_2$ were reported in cucumber treated with exogenous $CaCl_2$ (Liang *et al.*, 2009).

The $CaCl_2$ treatment enhanced different H_2O_2 scavenging enzymes, like SOD, APX and CAT and non-enzymatic antioxidants. This enhancement would have helped in scavenging of ROS in *Pennisetum.* H_2O_2 is an endogenous signaling molecule involved in plant responses to abiotic and biotic stresses such as extremes of temperature, light intensity, drought, pathogen, salinity, as well as stimuli such as plant hormones and gravity (Hernández *et al.*, 2001). Accumulation of H_2O_2 will also lead to enhance potential for production of hydroxyl radicals, which leads to lipid peroxidation and membrane deterioration (Axelrod, 1981).

It can be concluded that ascorbic acid and $CaCl_2$ can play an important role in the growth and biochemical activities of sunflower plants grown under water stress conditions, perhaps through maintaining relative water content and other chemical compositions within plant tissues, and because it has the potential to stimulate the production of various metabolites which cause a reduction in transpiration and thus more water become available to plants for better growth and productivity.

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نقع البذور مع حمض الأسكوربيك أو كلوريد الكالسيوم يخفف من الآثار السلبية لإجهاد الجفاف في بادرات عباد الشمس

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يعد إجهاد الجفاف أحد أهم العوامل التي تحد من بقاء ونمو النباتات في البيئات المختلفة في مصر. وقد أجريت هذه الدراسة لدراسة آثار إجهاد الجفاف منفردا أو مع المعالجة بحمض الأسكوربيك وكلوريد الكالسيوم على النمو وبعض الأنشطة الأيضية لنباتات عباد الشمس. أدت معاملة اجهاد الجفاف الى انخفاض طول النبات، مساحة الأوراق، والوزن الطازج والجاف للنبات، ومحتوى الأصباغ، السكر الذائب، ونشاط الأميليز من ناحية أخرى، وجد أن طول الجذر، البروتينات الذائبة، نشاط انزيم البروتييز، محتوى البرولين، الفينوليات الذائبة ومحتويات الفلافونويد، الفينيل ألانين امونياليز، البيروكسيديز، البولي فينول أوكسيديز، ومحتوى الهيدروجين بيرأوكسيد وفوق أكسدة الدهون، ومضادات الأكسدة الكلية زاد بالمقارنة مع النبات الغير معاملة. أدت المعالجة بحمض الأسكوربك وكلوريد الكالسيوم الى تخفيف إجهاد الجفاف من خلال زيادة النمو، والانزيمات المضادة للأكسدة وأصباغ التمثيل الضوئي وانخفاض الهيدروجين وفوق أكسدة الدهون، الفينولات الذائبة ومحتويات الفلافونويد.