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Enhancing antioxidant–yield relationship of pea plant under drought at different growth stages by exogenously applied glycine betaine and proline

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Abstract Pod filling stage considers as a receiver (sink), which reflects plant performance during previous growth stages. In order to study, the influence of drought imposed at different growth stages, and the impact of foliar applied glycine betaine (GB) and proline on the status of osmolytes and antioxidant defense system of pea plant during pod filling stage, a field experiment was conducted in 2012/2013 and 2013/2014 on clay loam soil. Four different irrigation regimes were applied to provide drought at different growth stages: (1) vegetative stage, (2) flowering stage, as short-term drought stress, (3) throughout the stages of vegetative + flowering growth (long-term drought stress), and (4) control (without stress). Foliar applications of GB and proline at 4 mM for each, in addition to distilled water as control, were conducted. Generally, drought applications reduced the growth and yield of pea plant. Long-term drought was more effective to reduce growth and yield than drought at flowering stage. GB increased the yield and its soluble protein concentration more than proline. Proline recorded the maximum increase in non-enzymatic antioxidant defense system under drought. Application of GB or proline enhanced the activity of SOD, APX and catalase in leaves under drought, while in seeds they increased SOD activity under long-term drought stress. APX activity in seeds under drought decreased by GB application. The maximum positive effect was for GB under unstressed condition and drought at vegetative stage, by maximizing APX activity, in addition to enhancing the production and translocation of assimilates from source to sink.

Introduction

Plants in their natural habitat or cultivated crops are exposed to several environmental stresses, that affecting plant growth and productivity. Drought is the most widespread devastating environmental stress, which decreases crop productivity more than any other environmental stress (Farooq et al., 2012), for instance, continuous or frequent drought effect on up to 45% of the world agricultural lands (Ashraf and Foolad, 2007). Drought severely affects plant growth and development with consequence reductions in the rate of cell division and elongation, leaf area, root and stem growth, interrupted

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stomatal conductance, and water use efficiency, which makes photosynthesis very sensitive to drought (Farooq et al., 2009). The detrimental effects of drought on plant growth and development depend on the severity of stress and the crop growth stage. Nutrients require water for uptake and translocation. As water supply decreases, nutrient uptake does (Farooq et al., 2012).

Pea plant as other most legume crops are more sensitive to water stress during flowering and pod filling stage than during vegetative stage. Long-term drought causes destructive effects in pea plants (Karataş et al., 2014). A severe water deficit leads to a fall in the content of the proteins as well as modifying their composition (Lecoeur and Guilioni, 2010). Antioxidant system in leaves of pea plant (seven weeks old) exposed to long-term salt stress (four weeks) was studied by Ozturk et al. (2012), and found that protein content was significantly decreased, while proline was accumulated with increasing in salinity level. Activity of peroxidase and superoxide dismutase (SOD) increased under salt stress, while catalase (CAT) and ascorbate peroxidase (APX) activities generally decreased in salt stressed seedlings. They suggested that increase in the activities of peroxidase and SOD/ascorbate–glutathione (AsA–GSH) cycle, improved the resistance of pea plant to oxidative stress, which enhanced salt tolerance. Moderate water stress in pea marks the beginning of the modification of the physiological status of plant tissues. Stomatal conductance falls with an increase in ABA content, reduces the size of all developing vegetative organs on the plant at the time of its occurrence, and reduces the final number of reproductive branches (Lecoeur and Guilioni, 2010). At increased maturity, the greater decrease in sucrose concentration in peas was in nonstressed than drought-stressed peas (Sorensen et al., 2003).

Reactive oxygen species (ROS) are produced as a normal by-product during plant cellular metabolism with controlled amounts, and effect on the expression of a number of genes (Gill and Tuteja, 2010; Sharma et al., 2012). Exposing to abiotic stresses including drought elevates the oxidative stress with overproduction of ROS, which are highly toxic and trigger impairment to carbohydrates, proteins, lipids, and DNA, leads to deteriorate normal plant metabolism through oxidative damage, and ultimately causes cell death. Superoxide radicals ($O_2^{\cdot-}$), single oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), alkoxy radicals ($RO^{\cdot}$), and hydroxyl radicals ($OH^{\cdot}$) are among the major ROS generated in plants under abiotic stresses (Gill and Tuteja, 2010). The major sites for the production of $O_2^{\cdot-}$ were photosystem I and II in chloroplasts, and complex I, ubiquinone and complex III of electron transport chain in mitochondria (Gill and Tuteja, 2010). Therefore, weakened activity of anabolism and catabolism essential enzymes leads to hamper the photosynthetic and respiratory activities (Farooq et al., 2012). Plants possess very efficient antioxidant defense machinery, which consists of enzymatic and non-enzymatic antioxidants. The enzymatic antioxidants consist of SOD, CAT, and AsA–GSH cycle enzymes; APX, glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), and glutathione s-transferase (GST) (Karataş et al., 2014). The non-enzymatic antioxidants such as ascorbic acid (AsA), glutathione (GSH) phenolic compounds, carotenoids, alkaloids, non-protein amino acids and α-tocopherols (Sharma et al., 2012). Both of enzymatic and non-enzymatic antioxidants work in concert to operate the cascades of uncontrolled oxidation and shield most affected plant cells components by scavenging of ROS (Gill and Tuteja, 2010).

Under environmental stresses such as drought, plants accumulate many of low molecular weight water-soluble compounds, which are known as compatible solutes, osmolytes or osmoprotectants, which decrease the cell water potential without decreasing actual water contents. The most common compatible solutes are betaines (glycine betaine, as the original betaine), soluble sugars (sucrose, trehalose, mannitol, and sorbitol), polyamines, proline and amino acids (Giri, 2011). These compatible solutes not only maintain the turgor pressure within cells, but also protect the enzymes and macromolecules from oxidation by ROS (Farooq et al., 2012).

Exogenous application of GB or proline can play an important role in enhancing plant stress tolerance. This role can be in the form of either osmoprotection or cryoprotection (Ashraf and Foolad, 2007; Giri, 2011). Proline protects cell membranes from oxidative stress by enhancing activities of various antioxidants and facilitated growth (Ashraf and Foolad, 2007). Exogenous application of proline and GB has an important role in upregulating the homeostasis in lentil under stress condition. Proline exhibited better protection than GB under drought stress, suggesting that both proline and GB provided a protective role in drought induced oxidative stress by reducing H_2O_2 levels and by increasing the antioxidant defense system (Molla et al., 2014). Importantly, exogenous application of proline and GB in stressed plants further enhanced the endogenous proline content (Hasanuzzaman et al., 2014). Exogenous amino acids have been shown to promote potassium and calcium uptake. Therefore, proline and other amino acids may contribute to osmoregulation not only per se, but also by regulating the contents of inorganic solutes, which in turn may contribute to osmotolerance (Rai, 2002).

Although, the influence of water deficit on yield of pea plants has been studied previously in different viewpoints (Martin and Jamieson, 1996; Sousa-Majer et al., 2004; Duzdemir et al., 2009) without reviewing the effect of drought on antioxidant system during pod filling stage, screening of antioxidant system during pod filling stage under drought stress was the main target of the present study. Therefore, the objectives of this study revealed the following:

- The effect of drought imposed at different growth stages on yield components, plant growth and assimilates compartmentation between source and sink.
- Assessing the status of antioxidant defense system during pod filling stage under stressed and unstressed conditions.
- The effect of GB and proline application on ameliorating the adverse effects of short- and long-term drought.

Materials and methods

The present study was conducted during the two growing seasons of 2012/2013 and 2013/2014 under open field conditions in the clay loam soil, at the experimental farm, Faculty of Agriculture, Ain Shams University, Qalyubia governorate, Egypt, in order to investigate the influence of foliar application of glycine betaine and proline under drought at different growth stages (at pre–pod filling stage) and their interaction on pea total antioxidant capacity status and assimilates

content during pod-filling stage, and its relationship with alleviating the deleterious effects of short-term and long-term drought stress, which reflected on yield.

Experimental design, agricultural practices and treatments

Seeds of pea (*Pisum sativum* L. cv. Master-B) were obtained from the Horticulture Research Institute, Agricultural Research Center, Giza, Egypt, and sown on 10th of October during 2012/2013 and 2013/2014 seasons. Treatments were arranged in a split plot design with three replicates. Main factor included drought application. Four different irrigation regimes provided drought at different growth stages:

(1) Control (unstressed treatment) with 4 days irrigation interval, where water level maintained throughout the experiment near field capacity.
(2) Short-term drought stress, where an irrigation interval for about 20 days (until the water level at field capacity decreased to 30%), which applied at the following:
    (a) During the vegetative (veg.) stage.
    (b) During the flowering (flow.) stage (from beginning to the end of flowering).
(3) Long-term drought stress, where soil moisture content was 30% of field capacity during both vegetative and flowering stages (veg + flow).

Foliar applications of glycine betaine, proline and water as control were assigned in sub-plots. Glycine betaine (GB) at 4 mM and proline at 4 mM in addition to distilled water as a control were separately sprayed to foliar system five times with 10-day intervals started at the stage 4 of the leaf development (four leaves with stipules unfolded) of the BBCH scale which used to identify the phenological development stages of a plant (Lancashire et al., 1991). Tween 20 at 0.1% was used as a wetting agent. The experimental plot area was 9.5 m^2 included five rows, each row was 2.7 × 0.7 m. The plant distance was 10 cm apart on one side of the ridge. Agricultural management, fertilization, disease and pest control programs were performed as recommended by the Egyptian Ministry of Agriculture and Land reclamation.

Vegetative growth characteristics

Samples of 10 plants were taken at random from each experimental plot at 90 days after sowing (DAS) to determine the number of leaves per plant and average leaf fresh weight.

Pod yield and its components

Random samples of 20 plants from each plot were labeled. The green ripe marketable pods on the labeled pea plants which had fully formed peas were started to be harvested at 75 days after sowing with 7 days interval to determine pods number/plant, average green pod weight/plant and total green pods yield as ton per feddan.

Biochemical analyses

Leaf and seed samples were collected at 85 days after sowing to determine total soluble sugars, starch, free amino acids, proline, non-enzymatic total antioxidant capacity and total soluble protein concentration, and the enzymatic activity of superoxide dismutase, ascorbate peroxidase and catalase.

Total soluble sugars (SS) and free amino acids were extracted from 1 g leaf and seed tissues separately by 80% hot ethanol as described by Irigoyen et al. (1992) and Katoch (2011) respectively. The homogenate was centrifuged at 10,000 rpm for ten minutes, and then the supernatant was collected. The pellet was re-extracted twice with 3 ml of 80% ethanol, then vortexed and centrifuged. The supernatants were combined and stored at – 20 °C until the determination step of SS and free amino acids concentration. Starch was determined in the residue.

The total soluble sugars and starch concentrations were estimated by anthrone method as described by Sadasivam and Manickam (2010). The extracts in the ethanol-soluble fractions were used for SS estimation, while the extracts from residues by 52% perchloric acid were used for starch determination through treating with the anthrone reagent and read at 625 nm using a spectrophotometer (Mapada UV 1200).

Free amino acids were determined according to the method described by Swamy (2008). The pink color developed was measured using a spectrophotometer (Mapada UV 1200) at 570 nm. The concentration of total free amino acids was calculated from the standard curve. The proline concentration was estimated by the method of Bates et al., (1973). Soluble protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

Non-enzymatic antioxidant capacity (NEAC)

The non-enzymatic total antioxidant capacity in extracts of pea leaves and seeds was estimated by the method of Prieto et al. (1999). Leaves and seeds extracts were obtained by grinding 1 g of organ (leaves or seeds) with 5 ml of pure methanol. Then, extracts were kept at 4 °C for 24 h, filtered and stored at 4 °C until analysis. An aliquot of 0.3 ml sample extract was mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated at 95 °C for 90 min. After cooling the mixture at room temperature, the solution absorbance was measured at 695 nm against a blank. The antioxidant capacity was expressed as equivalents of ascorbic acid (μg g^{-1} f. w.).

Antioxidant enzymes assays

Enzyme extract for superoxide dismutase (SOD) and catalase (CAT) was prepared by first freezing the weighed amount of samples (1 g) in liquid nitrogen to prevent proteolytic activity followed by grinding with 5 ml of cold extraction buffer (0.1 M phosphate buffer, pH 7.8, containing 0.5 mM EDTA, and 2% (w/v) polyvinylpyrrolidone (PVP)). Brie was passed through 4 layers of cheesecloth, filtrate was centrifuged for 20 min at 10,000 g and the supernatant was used as enzyme extract. All steps in the preparation of the enzyme extract were carried out at 4 °C. For measuring ascorbate peroxidase (APX) activity, the tissue was separately ground in homogenizing medium containing 2.0 mM AsA in addition to the other previous ingredients. All assays were done at 25 °C.

SOD (EC: 1.15.1.1) activity was determined by nitro-blue tetrazolium (NBT) photochemical assay following Dhindsa et al. (1981). Three milliliters of the reaction mixture contained 13.33 mm methionine, 75 µm nitroblue tetrazolium chloride, 0.1 mm EDTA, 50 mm phosphate buffer (pH 7.8), 50 mm sodium carbonate, 0.05 ml enzyme extract and 0.95 ml of water. The reaction was started by adding 2 µm riboflavin and placing the tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal color, served as control. Switching off the light and placing the tubes in the dark stopped the reaction. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm (Mapada UV 1200), and one unit of enzyme activity was taken as that amount of enzyme which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme.

APX (EC: 1.11.1.11) activity was assayed following methods adopted by Nakano and Asada (1981). The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, and 1.5 mM H_2O_2 and 0.1 ml enzyme extract. The reaction was started with the addition of H_2O_2. Absorbance change was measured at 290 nm every 30 s for 5 min ($\varepsilon = 2.8$ mM cm^{-1}) using Mapada UV 1200 spectrophotometer. APX activity was expressed as nmol AsA oxidized min^{-1} mg^{-1} protein.

CAT (EC: 1.11.1.6) activity was determined following the reaction of the extract in the presence of 50 mM potassium phosphate buffer (pH 7.0) containing 12.5 mm H_2O_2 and 50 µl enzyme extract and water was made up to 3.0 ml. The reaction took place at 25 °C, by adding H_2O_2 with absorbance monitored at 240 nm for 60 s (Aebi, 1984). CAT specific activity (nmol H_2O_2 degraded min^{-1} mg^{-1} protein) was calculated using the molar absorptivity of 43.6 mM^{-1} cm^{-1} for H_2O_2 at 240 nm.

Statistical analysis

Data of the two seasons were arranged and statistically analyzed using CoStat software (version 6.4, CoHort Software, USA). Duncan's multiple range test was used to compare between means, according to the method described by Gomez and Gomez (1984).

Results and discussion

Vegetative growth characteristics

The most critical vegetative parameters affected by water stress are number of leaves/plant and leaf fresh weight, which are presented in Table 1. Drought stress application at all tested growth stages significantly decreased the number of leaves/plant comparing with control in both seasons. The most effective drought application in decreasing the number of leaves/plant was found under long-term drought, followed by drought at flowering stage. These results are in agreement with the results of Lopez et al. (1997) who mentioned that water stress reduced leaf number per plant of pigeon pea by 15–35% under drought at vegetative stage and by 20–45% under drought at flowering stage. At the vegetative stage, water deficit initially reduced the rate of leaf expansion, followed by an interruption of new leaf production Lopez et al. (1997). Eventually, leaf area increment dropped to zero. However, no leaves were lost. Stressed plants persisted in a stunted state until rewatering (Warrag and Hall, 1984). So, drought at vegetative stage ceased leaves growth along stress period, while after rewatering plants resumed the developmental state and increased the leaves number per plant which still in the second order after unstressed plants (plant growth did not cease for a period of time). Besides the cessation of expansion of the leaves and inhibition of new leaves production, flowering or pod-filling stage in addition to drought stress triggered the senescence and abscission of mature basal leaves and reduced the average leaf size because later produced leaves are smaller (Akyeampong, 1986; Lopez et al., 1997). This observation explains why leaves number per plant decreased in drought at flowering stage and long-term drought comparing with drought at vegetative stage.

Foliar application of GB and proline respectively, enhanced the growth and increased the number of leaves per plant comparing with control under all drought application cases. Glycine betaine and proline decreased the adverse effects of

Table 1 Effect of glycine betaine (GB) and proline as foliar application on number of leaves per plant and average leaf fresh weight of pea plants at harvest under drought stress at different growth stages during the two seasons (2012/2013 and 2013/2014).

Foliar treatments (mM)	Cont.	GB	Proline	Mean	Cont.	GB	Proline	Mean
	1st Season				2nd Season			
	No. of leaves/plant							
Cont.	15.6 bc	18.6 a	17.9 a	17.3 A	16.6 c	20.4 a	19.1 b	18.7 A
Drought at veg.	14.1 d	15.7 bc	15.4 bc	15.0 B	15.3 e	16.2 cd	16.0 d	15.8 B
Drought at flow.	14.1 d	16.2 b	15.2 c	15.1 B	14.0 f	16.0 d	14.8 e	14.9 C
Drought at veg + flow	11.1 f	13.1 e	12.6 e	12.3 C	12.1 g	14.0 f	13.5 f	13.2 D
Mean	13.7 C	15.9 A	15.3 B	–	14.5 C	16.6 A	15.9 B	–
	Average leaf f.w. (g)							
Cont.	2.21 ab	2.13 b	2.21 ab	2.18 A	2.20 b	2.01 ce	2.12 bc	2.11 B
Drought at veg.	2.04 b	2.01 b	2.09 b	2.05 B	1.81 f	2.01 ce	1.90 ef	1.91 C
Drought at flow.	2.06 b	2.06 b	2.22 ab	2.12 AB	2.09 bd	2.51 a	2.19 b	2.27 A
Drought at veg + flow	2.20 ab	2.12 b	2.38 a	2.23 A	1.96 de	2.10 bc	2.21 b	2.09 B
Mean	2.13 AB	2.08 B	2.23 A	–	2.02 B	2.16 A	2.11 A	–

Means followed by different letters are significantly different at $P \leqslant 0.5$ level; Duncan's multiple range test. Capital letters for mean of overall treatment or drought time, whereas lowercase letters for interaction.

Table 2 Effect of glycine betaine (GB) and proline as foliar application on pods parameters and green pod yield of pea plants at harvest under drought stress at different growth stages during the two seasons (2012/2013 and 2013/2014).

Foliar treatments (mM)	Cont.	GB	Proline	Mean	Cont.	GB	Proline	Mean
	1st Season				2nd Season			
				Pods no./plant				
Cont.	7.13 ab	7.97 a	7.93 a	7.68 A	6.97 ab	7.80 a	7.77 a	7.51 A
Drought at veg.	5.93 bc	7.00 ab	6.00 bc	6.31 AB	6.07 ac	6.83 ab	5.80 bc	6.23 AB
Drought at flow.	5.00 cd	5.90 bc	5.83 bc	5.58 BC	4.83 cd	5.73 bc	5.67 bc	5.41 BC
Drought at veg + flow	3.97 d	4.93 cd	4.97 cd	4.62 C	3.80 d	4.77 cd	4.80 cd	4.46 C
Mean	5.51 B	6.45 A	6.18 AB	–	5.42 A	6.28 A	6.01 A	–
				Average pod f.w. (g)				
Cont.	7.52 a	7.03 a	6.79 a	7.11 A	8.04 a	7.52 a	7.18 a	7.58 A
Drought at veg.	7.45 a	7.21 a	7.58 a	7.41 A	7.85 a	7.76 a	8.47 a	8.03 A
Drought at flow.	8.40 a	7.84 a	7.87 a	8.04 A	8.95 a	8.74 a	8.68 a	8.79 A
Drought at veg + flow	8.83 a	7.63 a	8.18 a	8.21 A	9.71 a	8.21 a	8.89 a	8.94 A
Mean	8.05 A	7.43 A	7.60 A	–	8.64 A	8.06 A	8.31 A	–
				Green pods yield (ton/fed)				
Cont.	3.17 b	3.36 a	3.12 b	3.22 A	3.30 b	3.51 a	3.23 bc	3.35 A
Drought at veg.	2.64 d	2.96 c	2.73 d	2.77 B	2.85 e	3.10 cd	2.93 de	2.96 B
Drought at flow.	2.49 e	2.72 d	2.63 d	2.62 C	2.56 f	2.95 de	2.83 e	2.78 C
Drought at veg + flow	2.00 h	2.26 g	2.37 f	2.21 D	2.10 h	2.34 g	2.49 fg	2.31 D
Mean	2.58 C	2.83 A	2.71 B	–	2.70 C	2.97 A	2.87 B	–

Means followed by different letters are significantly different at $P \leqslant 0.5$ level; Duncan's multiple range test. Capital letters for mean of overall treatment or drought time, whereas lowercase letters for interaction.

drought stress; supporting that GB and proline are actively involved in the regulation of plant growth. Earlier studies have demonstrated that the exogenous application of GB and proline mitigated decrease in plant growth caused by drought is through increasing antioxidant system, relieving oxidative damage, improving the synthesis of compatible solutes, and accelerating proline accumulation, which reflected on enhancing photosynthesis (Ashraf and Foolad, 2007; Mattioli et al., 2009; Szabados and Savouré, 2010; Anjum et al., 2011). Increasing the efficiency of photosynthesis protection system by exogenously-applied GB and proline could be reflected on the total assimilates in plant, which serve as raw material for boosting growth and increase the leaves number per plant comparing with control.

The impact of drought stress on average leaf weight was less than its effect on the number of leaves/plant. Although individual drought application decreased significantly leaf fresh weight in the second season, the first season was insignificant (Table 1). Drought at vegetative stage and vegetative + flowering stages recorded the highest decrease in leaf weight in the second season. A reduction in the specific leaf area (leaf area/leaf dry weight) is generally observed for grain legumes under water stress (Lopez et al., 1997; Ohashi et al., 1999; Erice et al., 2010), which in general refer to the reduction in leaf area; thereby, leaf weight was less affected by drought than leaf area. Proline and GB treatments increased leaf weight under drought at flowering stage and long-term drought (veg + flow) comparing with drought under vegetative stage, conceivably indicating thicker leaves which assists in leaf water conservation under long-term drought (Lopez et al., 1997). This hypothesis was supported by a parallel increase in total soluble sugars in plants (leaves + seeds) and starch concentration in leaves of pea plants (Table 3), which possibly had a role in increasing leaf dry weight and leaf water content. Under unstressed conditions, neither GB nor proline had a positive effect on leaf fresh weight (Table 1), which could refer to that GB and proline enhanced the pea growth through increasing the leaves number per plant (Table 1), which in turn reflected on the pods number per plant (Table 2).

Pod yield and its components

The general tendency of the pods number per plant as overall mean was to decrease with increasing duration of drought, especially in drought at veg + flow and drought at flowering stage (Table 2). These results concur with other studies (Akyeampong, 1986; Sorensen et al., 2003; Mafakheri et al., 2010) which have shown that the decrease in seed yield of legumes grown under drought conditions is largely due to the reduction in the number of pods per plant. Control treatment (unstressed) recorded the maximum pod number per plant followed by drought at vegetative stage. This decrease in the pods number per plant could refer to after re-watering, stressed plants at the vegetative stage resumed growth by increasing leaves number per plant (Table 1), which was not at the expense of pod development. In contrast, at the flowering stage, re-watering did not alleviate the detrimental effect of drought stress. Re-watering plants at the flowering stage resumed reproductive activity, but the majority of the new pods failed to reach maturity due to insufficient resources, as a result of decreasing leaf number (Table 1) and leaf area (Akyeampong, 1986), which in turn reduced pods number per plant. In this connection, Nuñez Barrios et al. (2005) mentioned that, in most legume plants, the number of flowers and pods decreased due to a limitation in vegetative growth.

Exogenously supplied GB and proline increased the pods number per plant either in well watered plants or under all treatments of drought. The highest values in pods number

Table 3 Effect of glycine betaine (GB) and proline as foliar application on total soluble sugars and starch concentration in pea leaves and seeds under drought stress at different growth stages during the two seasons (2012/2013 and 2013/2014).

Season	Foliar treatments (mM)	Cont.	GB	Proline	Mean	Cont.	GB	Proline	Mean
		Leaves				Seeds			
		Total soluble sugars (mg g^{-1} f.w.)							
1st Season	Cont.	18.3 b	16.4 c	15.7 c	16.8 A	46.3 e	48.0 e	58.7 c	51.0 B
	Drought at veg.	18.5 b	21.2 a	8.1 d	15.6 B	47.1 e	59.4 c	56.7 cd	54.4 AB
	Drought at flow.	21.7 a	16.8 c	7.3 d	15.3 C	51.8 de	67.2 b	60.6 c	59.9 A
	Drought at veg + flow	15.8 c	15.5 c	18.5 b	16.6 A	37.0 f	60.6 c	76.0 a	57.8 A
	Mean	18.6 A	17.5 B	12.4 C	–	45.6 C	58.8 B	63.0 A	–
2nd Season	Cont.	20.8 b	18.6 c	18.3 c	19.2 A	51.6 d	51.7 d	62.7 c	55.3 C
	Drought at veg.	21.7 b	24.8 a	12.4 d	19.7 A	53.0 d	62.2 c	60.4 c	58.5 B
	Drought at flow.	24.3 a	20.2 bc	11.7 d	18.7 A	55.5 d	70.6 b	63.7 c	63.3 A
	Drought at veg + flow	18.0 c	18.5 c	21.8 b	19.4 A	40.7 e	63.4 c	83.1 a	62.4 A
	Mean	21.2 A	20.5 A	16.0 B	–	50.2 C	62.0 B	67.5 A	–
1st Season		*Starch (mg g^{-1} f.w.)*							
	Cont.	4.1 de	4.8 c	3.3 f	4.1 C	15.1 h	29.8 c	21.9 fg	22.3 B
	Drought at veg.	4.4 d	4.1 de	4.9 c	4.5 B	27.4 d	22.4 ef	33.6 b	27.8 A
	Drought at flow.	5.6 a	3.9 e	2.9 g	4.1 C	22.4 ef	23.8 e	21.9 fg	22.7 B
	Drought at veg + flow	5.4 ab	5.2 b	4.8 c	5.1 A	42.8 a	15.8 h	20.7 g	26.4 A
	Mean	4.9 A	4.5 B	4.0 C	–	27.0 A	22.9 C	24.5 B	–
2nd Season	Cont.	4.9 de	5.5 c	4.1 f	4.8 C	17.9 e	33.5 b	25.3 c	25.6 B
	Drought at veg.	5.4 cd	4.9 de	5.6 c	5.3 B	28.4 c	25.8 c	35.4 b	29.8 A
	Drought at flow.	6.6 a	4.8 e	3.9 f	5.1 BC	25.8 c	26.6 c	25.6 c	26.0 B
	Drought at veg + flow	6.4 ab	5.9 bc	5.7 c	6.0 A	43.1 a	19.2 e	22.5 d	28.3 A
	Mean	5.8 A	5.3 B	4.8 C	–	28.8 A	26.3 B	27.2 B	–

Means followed by different letters are significantly different at $P \leqslant 0.5$ level; Duncan's multiple range test. Capital letters for mean of overall treatment or drought time, whereas lowercase letters for interaction.

per plant under different drought levels were recorded by GB (Table 2). These results highly correlated with increase in leaves number per plant by exogenous application of GB and proline respectively (Table 1). This increase in leaves number led to an increase in the podding nodes per plant, which reflect on augmenting the pods number per plant. GB and proline not only affected the leaves number per plant, but also enhanced antioxidant capacity. Non-enzymatic total antioxidant capacity (Table 5) increased by exogenous application of GB and proline under drought. Activity of superoxide dismutase (Fig. 1) under drought was enhanced by exogenous application of GB and proline, which in turn reflect on decreasing oxidative damage and enhancing net photosynthesis. This increase in photosynthate, mostly did not remain in leaf cells to share the protective role with GB and proline against stress. Most of photosynthate was translocated from leaves to seeds under stress as affected by exogenous application of GB and proline (Table 3), which re-shaped the source-sink relationship, and increased pods number and weight. In this regard, Rezaei et al. (2012) reported that the seed yield of soybean was significantly increased under salt stress due to foliar application of GB, which was associated with increase in pods number. If the drought stress imposed at the vegetative stage, foliar application of GB was more effective and enhancing the hundred achene weight of sunflower (Iqbal et al., 2005).

Despite fresh weight of the pods was insignificant under all drought treatments, the pod fresh weight unpredictably increased with concomitant increase in drought level (drought at veg + flow). The maximum values as mean in pod fresh weight were in control and proline respectively (Table 2). This increase in the pod fresh weight under drought, especially at flowering stage and veg + flow stages might be due to not only decreasing the pods number per plant, but also ceasing most of the new flowering and vegetative growth under stress. Restricting the new flowering growth could influence the source to sink relationship among leaves and pods. In this regard, Tanaka and Fujita (1979) mentioned that, developing of flowers and pods of pea plant adjacent to smaller leaves tended to abscise. In addition, most of the photosynthates of a labeled leaf recuperated in the flowers and pods of the same leaf. As a result, under drought at flowering stage and veg + flow stages, the vegetative growth may have a surplus of photosynthate to fill the pods in treatments with low pods number per plant, even with less favorable conditions for growth (Martin and Jamieson, 1996; Nuñez Barrios et al., 2005).

Green pods yield (ton/feddan) significantly decreased with concomitant increase in the duration of the exposure to drought (drought at vegetative stage, flowering stage, and veg + flow stages). The yield components (pods number per plant and average pod weight respectively) notably affected by water deficit stress (Table 2). In this respect, Martin and Jamieson (1996) mentioned that pea is an indeterminate plant, where all growth stages (vegetative, flowering, and pod filling) be able to take place at the same time. These developments compete on assimilates, and the response of yield components will differ according to the relative strengths of the sources and sinks for assimilates. These results are in accordance with the

Table 4 Effect of glycine betaine (GB) and proline as foliar application on free amino acids and proline concentration in pea leaves and seeds under drought stress at different growth stages during the two seasons (2012/2013 and 2013/2014).

Season	Foliar treatments (mM)	Cont.	GB	Proline	Mean	Cont.	GB	Proline	Mean
		Leaves				Seeds			
		Free amino acids (mg g^{-1} f.w.)							
1st Season	Cont.	3.9 d	6.6 b	5.8 c	5.4 B	13.3 c	13 cd	10.4 d	12.2 D
	Drought at veg.	5.8 c	7.6 a	3.8 d	5.7 A	13.4 c	15.1 c	12.3 cd	13.6 C
	Drought at flow.	4.0 d	5.6 c	2.7 e	4.1 C	18.8 b	14.3 c	20.4 ab	17.8 B
	Drought at veg + flow	2.1 f	2.7 e	1.1 g	2.0 D	20.2 ab	14.9 c	22.6 a	19.3 A
	Mean	3.9 B	5.6 A	3.3 C	–	16.4 A	14.3 B	16.4 A	–
2nd Season	Cont.	4.4 de	6.7 b	6.1 bc	5.7 A	14.9 gh	13.8 hi	12.8 i	13.8 D
	Drought at veg.	5.3 cd	7.8 a	5.1 cd	6.0 A	15.8 fg	17.3 e	14.3 h	15.8 C
	Drought at flow.	4.3 de	5.9 bc	3.4 ef	4.5 B	22.5 c	16.7 ef	20.0 d	19.7 B
	Drought at veg + flow	2.7 fg	3.5 ef	1.9 g	2.7 C	28.1 b	16.6 ef	30.2 a	24.9 A
	Mean	4.2 B	6.0 A	4.1 B	–	20.3 A	16.1 C	19.3 B	–
1st Season		*Proline (μmole g^{-1} f.w.)*							
	Cont.	3.7 c	1.7 f	2.2 e	2.5 C	0.9 e	2.3 bc	1.8 cd	1.7 B
	Drought at veg.	4.6 b	2.2 e	3.0 d	3.3 B	0.9 e	2 cd	1.9 cd	1.6 B
	Drought at flow.	5.4 a	3.8 c	2.4 e	3.9 A	1.4 de	4.1 a	1.9 cd	2.4 A
	Drought at veg + flow	5.8 a	2.0 ef	2.1 ef	3.3 B	1.7 cd	3.6 a	2.6 b	2.6 A
	Mean	4.9 A	2.4 B	2.4 B	–	1.2 C	3.0 A	2.0 B	–
2nd Season	Cont.	5.3 b	3.1 e	3.5 de	4.0 C	1.0 f	2.5 c	2.1 ce	1.9 C
	Drought at veg.	5.9 b	3.8 cd	4.4 c	4.7 B	1.0 f	2.3 cd	2.1 cd	1.8 C
	Drought at flow.	6.7 a	5.4 b	3.9 cd	5.3 A	1.6 e	4.1 a	2.1 ce	2.6 B
	Drought at veg + flow	6.9 a	3.6 de	3.5 de	4.7 B	2.0 de	3.9 a	3.0 b	3.0 A
	Mean	6.2 A	4.0 B	3.8 B	–	1.4 C	3.2 A	2.3 B	–

Means followed by different letters are significantly different at $P \leqslant 0.5$ level; Duncan's multiple range test. Capital letters for mean of overall treatment or drought time, whereas lowercase letters for interaction.

finding of Martin and Jamieson (1996), where the yield reduction was correlated with low numbers of podding nodes per stem and pods per node and a slower increase in pod weight.

The maximum significant increase in yield of green pods was recorded with the exogenous application of GB comparing with control plants under all drought application times. This could be due to the fact that GB application significantly increases the pods number per plant and insignificantly decreases the pod weight, which reflects on increasing the yield of green pods. Although exogenous application of GB and proline increased yield of green pods under drought treatment comparing with its control, their yield amount did not reach the amount of unstressed plants. The yield of GB treatment under drought at vegetative stage was in the second order after yield of unstressed plants (Table 2).

Biochemical analyses

Total soluble sugars and starch concentration

Drought as individual treatment at flowering stage recorded the highest value for SS in leaves and seeds, whereas the lowest SS value was in long-term drought (vegetative + flowering stages) for both analyzed organs (Table 3). Increasing in SS concentration in leaves under drought at flowering stage could refer to more than one reason. Firstly, the fact that leaf area for plants exhibited to drought at flowering was higher than leaf area of plants exhibited to drought at vegetative stage, since plants before flowering grew under normal conditions (unstressed), so leaves reach maximum area before drought application at flowering. Therefore, the bigger leaves could produce more photosynthate, which reflect on SS concentration in leaves. Secondly, plants have some adaptation mechanisms against drought, which pronouncedly activated at flowering stage. These mechanisms could alter plant metabolism to preserve more SS in leaves to maintain high relative water content, which reflected on leaf area and leaf photosynthetic activity. This hypothesis was supported by decreasing the starch concentration in seeds for plants under drought at flowering stage than starch concentration at vegetative stage (Table 3). This amount of starch could be hydrolyzed to synthesize more soluble sugars, which serve as osmolytes. The osmolytes have a direct role on osmotic adjustment, which in turn has an important role in maintaining cell turgor, growth, and photosynthesis (Sorensen et al., 2003). Water stress accelerates the maturation process, which affects the chemical composition of green peas. Osman and Abd El-Gawad (2013) showed that, as green pods maturation process increased, total soluble sugars concentration decreased with concomitant increase in starch concentration. In this regard Sorensen et al. (2003) mentioned that, sucrose was the most important soluble sugar in green peas. Water deficit stress imposed during the flowering stage significantly increased the total soluble sugars concentration (Table 3). Sucrose content increased in pods and leaves of many plant species exposed to drought stress, which might affect osmotic adjustment (Wager, 1954; Sorensen et al., 2003).

Glycine betaine and proline revealed significant effects in the overall means of total soluble sugars (Table 3). These significant effects were less than control for leaves and more than control for seeds. The level of total soluble sugars decreased in

Table 5 Effect of glycine betaine (GB) and proline as foliar application on non-enzymatic total antioxidant capacity and total soluble proteins concentration in pea leaves and seeds under drought stress at different growth stages during the two seasons (2012/2013 and 2013/2014).

Season	Foliar treatments (mM)	Cont.	GB	Proline	Mean	Cont.	GB	Proline	Mean
		Leaves				Seeds			
		Non-enzymatic total antioxidant capacity (μg g^{-1} f.w.)							
1st Season	Cont.	213 bc	214 bc	221 b	216 A	354 a	206 fg	220 ef	260 B
	Drought at veg.	193 d	188 d	247 a	209 B	282 d	205 fg	361 a	283 A
	Drought at flow.	160 e	188 d	135 f	161 C	227 e	280 d	333 b	280 A
	Drought at veg + flow	116 g	206 c	168 e	163 C	192 g	314 c	234 e	247 C
	Mean	171 C	199 A	193 B	–	264 B	251 C	287 A	–
2nd Season	Cont.	253 b	259 b	263 b	259 A	380 ab	255 ef	260 e	298 B
	Drought at veg.	236 cd	237 cd	291 a	255 A	326 c	246 ef	397 a	323 A
	Drought at flow.	196 f	233 d	200 f	210 B	271 de	323 c	373 ab	322 A
	Drought at veg + flow	144 g	249 bc	217 e	203 B	229 f	355 b	288 d	291 B
	Mean	207 B	244 A	243 A	–	302 B	295 B	330 A	–
1st Season		*Total soluble proteins (mg g^{-1} f.w.)*							
	Cont.	17.9 e	22.3 a	18.4 de	19.5 B	16.3 b	19.9 a	15.1 c	17.1 B
	Drought at veg.	21.0 ad	22.0 ab	23.2 a	22.0 A	20.4 a	20.5 a	14.0 d	18.3 A
	Drought at flow.	17.8 e	21.3 ac	18.8 ce	19.3 B	16.2 b	19.9 a	12.5 e	16.2 B
	Drought at veg + flow	12.8 f	19.2 be	12.9 f	14.9 C	13.5 d	20.9 a	10.1 f	14.8 C
	Mean	17.3 B	21.2 A	18.3 B	–	16.6 B	20.3 A	12.9 C	–
2nd Season	Cont.	20.0 ce	25.2 a	21.5 bd	22.3 A	18.6 b	23.6 a	18.0 bc	20.1 A
	Drought at veg.	21.7 bd	23.4 ab	25.0 a	23.4 A	21.9 a	23.4 a	16.3 cd	20.5 A
	Drought at flow.	19.1 de	23.1 ac	21.7 bd	21.3 A	18.0 bc	22.6 a	15.3 d	18.6 AB
	Drought at veg + flow	15.3 f	21.4 bd	17.3 ef	18.0 B	15.4 d	22.5 a	13.3 e	17.0 B
	Mean	19.0 C	23.3 A	21.4 B	–	18.5 B	23.0 A	15.7 C	–

Means followed by different letters are significantly different at $P \leqslant 0.5$ level; Duncan's multiple range test. Capital letters for mean of overall treatment or drought time, whereas lowercase letters for interaction.

leaves of untreated plants from 18.6 mg g^{-1} f.w. to 17.5 mg g^{-1} f.w. for GB and 12.4 mg g^{-1} f.w. for proline in the first season, whereas in the seeds the opposite trend was recorded. The highest values were for proline 63 mg g^{-1} f.w. followed by GB 58.8 mg g^{-1} f.w. The lowest significant overall mean of SS under drought treatments in leaves was in drought at flowering stage (15.3 mg g^{-1} f.w.) in the first season, whereas the highest mean was in control (16.8 mg g^{-1} f.w.). The opposite trend was observed in seed case, where the highest significant overall mean under drought treatments was in drought at flowering stage (59.9 mg g^{-1} f.w.), whereas the lowest mean (51 mg g^{-1} f.w.) was for control (Table 3). The opposite trend between leaves and seeds in SS concentration might be explained by that both GB and proline have a potential to increase the remobilization process of photosynthate. Since the highest SS concentration in seeds was for proline, so the application of proline was more efficient than GB in photosynthate translocation, especially under long-term drought (veg + flow). Under short-term drought (at vegetative or flowering stage), GB is more efficient than proline in photosynthate translocation. These observations suggest that, proline application basically, directed most of the photosynthate to the pods as main sink, which led to increase pod weight (Table 2). Application of GB directed the photosynthate to more than one sink, which increased pod weight (Table 2), vegetative growth (Table 1) and flowers number, which in turn led to an increase in pods number (Table 2). In this connection, Moustakas et al. (2011) found that, exogenously applied proline in *Arabidopsis* under drought, increased proline and total soluble sugars content, and suggest that, signaling pathway of proline interacts with soluble sugars signaling pathway. Several previous studies reported that, proline and GB have direct and indirect effects on many function processes in plant especially under stress. In this regard, Szabados and Savouré (2010) and Ashraf and Foolad (2007) mentioned that, both of proline and GB can protect and stabilize the antioxidant enzymes which reduce ROS. The reduction in ROS reduces its damaging effects on Photosystem II (PSII), which in turn reflect on increasing photosynthesis process and produce more photosynthate under drought. The individual treatments of GB and proline showed the same trend of the overall mean of GB and proline for leaves and seeds.

The interaction between GB or proline treatments and drought levels reveals that SS levels in leaves were higher in GB than the proline treatment under drought at vegetative stage and drought at flowering stage, respectively. For SS in seeds under the same previously mentioned drought conditions, the GB treatments were higher than proline treatments under drought at flowering stage and vegetative stage, respectively. The highest value for SS in seeds was recorded with proline treatment under long-term drought treatment (Table 3).

The highest levels of starch as overall mean in leaves were recorded with untreated plants, whereas the lowest significance values were recorded with proline treatment (Table 3). Same observation was recorded in seeds case, but the lowest significance values were recorded with GB treatment. On the other

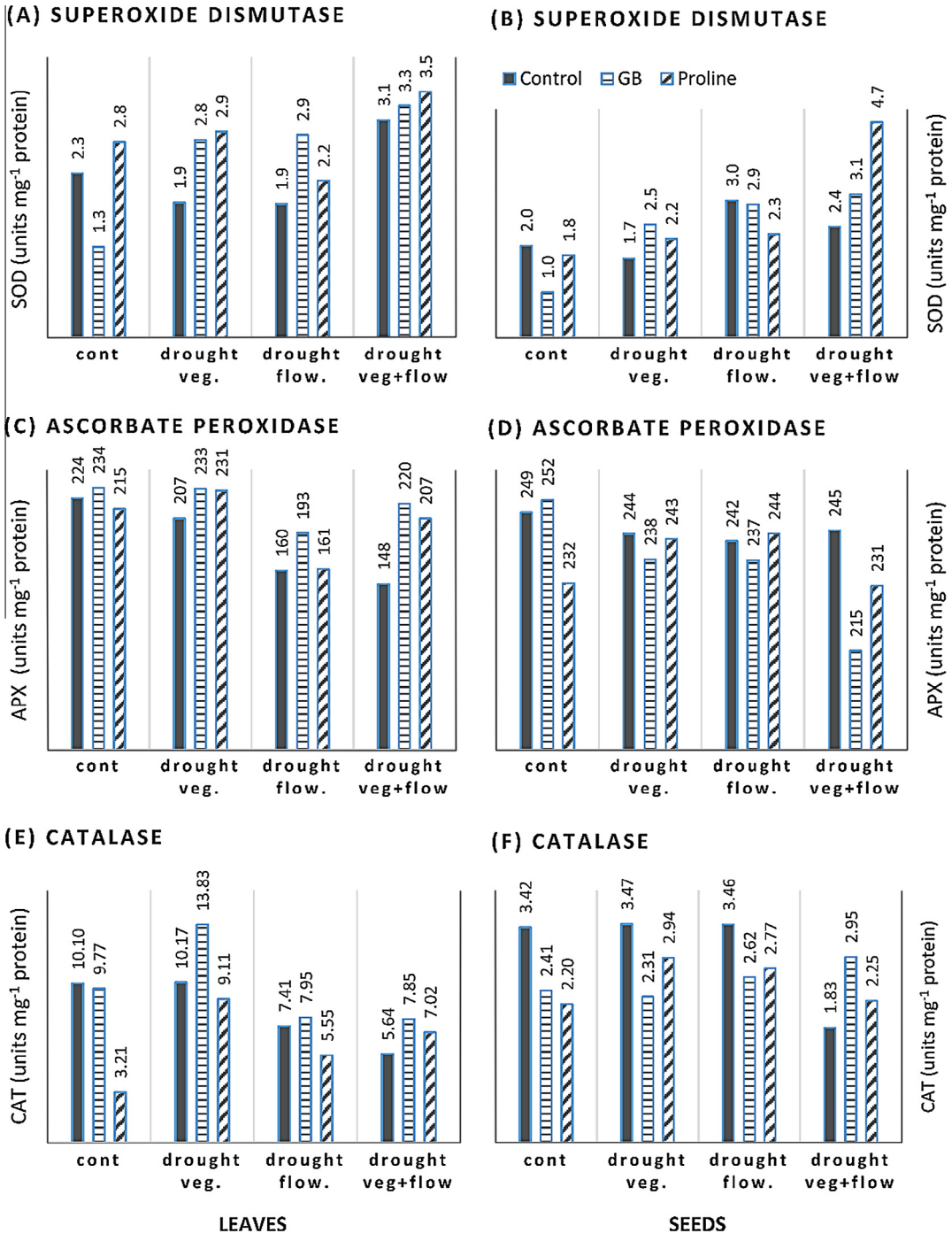

Fig. 1 Effect of foliar application of glycine betaine (GB) and proline on SOD (A and B), APX (C and D) and CAT (E and F) activities in pea leaves (A, C and E) and seeds (B, D and F) under drought stress at different growth stages (main of two seasons).

hand, overall mean of starch in leaves and seeds under drought levels were highly significant with drought at veg + flow stages and at vegetative stage, comparing with unstressed plants (the lowest level). Starch concentration in matured pea seed reaches 50%, the rest being mostly protein and fiber (Wang et al., 1998). As maturation process increased, conversion process of sucrose to starch increased in pea seeds (Osman and Abd El-Gawad, 2013). Concerning the present results, all drought treatments led to increase in starch concentration in untreated plants, so drought has a positive effect on senescence process. Plants under drought stress may alter the direction of metabolism process by accelerating the translocation process of sucrose from leaves to seeds, and also accelerate the conversion process from sucrose to starch in seeds. As seeds have more starch content, they have advanced level of maturity. The matured seeds can survive under stress more than immature seeds (Soeda et al., 2005). In consequence of producing mature seeds under drought, the plant priority is surviving, through ending its life cycle quickly. Both of GB and proline decreased the starch concentration under drought levels, especially under

long-term drought. This observation reveals that GB and proline decreased the deleterious effects of drought on plant growth and in turn directed the plants to grow in conditions near to normal.

Individual treatment of GB increased the level of starch in leaves and seeds comparing with control and proline treatments, whereas proline individual treatment decreases the level of starch in leaves than control. Plants under favorable conditions for growth do not need to accumulate more osmolytes than normal amount. As GB and proline accumulated in many plants subjected to different types of environmental stresses (Ashraf and Foolad, 2007; Szabados and Savouré, 2010). Therefore, additional amounts of GB and proline by exogenous application before exposing to drought could put the pea plant in standby mode to tolerate the drought stress, while under favorable conditions, exogenous application of GB and proline may alter the metabolism of the plant to act as if it was under stress. These hypotheses were supported by data of unstressed plants located in Table 3, both of GB and proline led to an increase in starch concentration in seeds comparing with its control, which was the same observation for pea plants subjected only to drought. In this regard, Giri (2011) mentioned that, GB and proline have the ability to destabilizing DNA, which suggested main role for both of them in the regulation of gene expression by activating replication. Transgenic plants with choline oxidase A (*codA*) have the ability to convert choline into GB in addition to H_2O_2 as by-product. Hydrogen peroxide acts as signal for gene expression under different stresses. In this connection, Park et al. (2006) found that, in tomato plants exogenous application of GB increases H_2O_2 content than control for unstressed treatment, but decreasing its level in stressed plants. These findings support my suggestion that, exogenously applied GB or proline in unstressed plants, could activate some or all of stress genes, which accelerate pod ripening process.

Starch levels in leaves and seeds increased significantly under the individual treatments of drought, especially in drought at flowering stage and drought at veg + flow stages in leaves respectively, and drought at veg + flow stages and drought at vegetative stage in seeds respectively.

Interaction effects between foliar application of proline or GB and drought levels on starch concentration showed that proline application under drought at vegetative stage recorded the highest significant values in the leaves and seeds, while the lowest significant value in seeds was recorded with GB and drought stress through entire season (Table 3). These observations suggest that under drought stress, proline is considered more effective than GB in accelerating the pod ripening process. From another point of view, GB could be more effective than proline in slowing down the pod ripening process under stress.

Free amino acids, free proline and soluble protein concentration

Data presented in Table 4 show the effect of foliar application of glycine betaine and proline on free amino acids and proline concentration in pea leaves and seeds under drought stress at different growth stages. The maximum free amino acids level was recorded with drought at vegetative stage in leaves and the minimum level was observed under long-term drought; the opposite response was recorded in the seeds, where the highest free amino acids level was for long-term drought and the lowest for drought at vegetative stage. These results confirmed by similar results were found in cowpea plants exposed to drought stress during the flowering stage which led to an increase in the free amino acids content with concomitant decrease in protein content in cowpea seeds. The lowest values in free amino acids content were recorded with unstressed plants and plants under drought at vegetative stage (Labanauskas et al., 1981). The present results reveal that the highest level of overall mean for free amino acids concentration in leaves was recorded by GB application, while same application recorded the lowest level of free amino acids in seeds. Interaction between treatments reveals that GB was the best treatment in increasing the levels of free amino acids in leaves under all levels of drought. Increasing free amino acids concentration in leaves by GB application may have positive effects not only in osmoregulation, but also on enhancing plant nutrition. In this connection Rai (2002) mentioned that exogenously applied amino acids promoted the uptake of potassium and calcium, which in turn had a positive effect on osmoregulation. He suggested that contribution of amino acids to osmoregulation under stress might be not only per se, but also by regulating the contents of inorganic solutes. In seeds case, the highest free amino acids levels were recorded with proline and untreated plant under drought at veg + flow stages (Table 4). During pod filling stage, plants directed most of assimilates from its sources to pods as the main sink. Eventually, most of translocated assimilates converted to storage starch and proteins in cotyledons of pea seeds. So, the concentration of free amino acids and proline in leaves (Table 4) did not multiply under drought stress as expected, which could refer to in this phase of growth, and plants prefer to maintain high amounts of free amino acids in seeds than leaves by accelerating the translocation process, especially under drought stress. The free amino acids concentration in seeds under long-term stress is about two-folds of its concentration under unstressed conditions, which suggest the importance of increasing free amino acids concentration in seeds under drought stress, through delaying dehydration and senescence in pea seeds. Thakur and Rai (1985) found that, amino acids application in maize seedlings exposed to osmotic stress delayed plant wilting. Altman et al. (1977) mentioned that polyamines delayed senescence in maize protoplast, and suggested that amino acids were converted to polyamines, which delayed senescence.

Exogenous application of proline might be not only accelerated the translocation process of amino acids from source to sink, but also suppressed the conversion process from amino acids to proteins. Total soluble proteins concentration in seeds (Table 5) corroborates the previous observation, since application of proline decreased the soluble proteins concentration in seeds under all levels of drought. Decreasing free amino acids concentration in seeds under the application of GB (Table 4), while soluble proteins concentration increased under same application (Table 5), leads to the suggestion that, GB is more efficient than proline in ameliorating the adverse effects of drought stress on pea yield (Table 2) by increasing osmoprotectants content (Tables 3–5) and antioxidant enzymes (Fig. 1).

Glycine betaine and proline individual treatments increased the levels of free amino acids in leaves and decreased in seeds comparing with control (Table 4). Drought at veg + flow stages decreased the free amino acids levels in leaves, but the opposite trend was recorded in the seeds.

Free proline concentration recorded the highest levels in leaves for all control treatments comparing with GB or proline application under the same level of stress, whereas the highest level in seeds was observed in GB treatment (Table 4). In this concern, Mafakheri et al. (2010) and Ozturk et al. (2012) stated that, proline content increased in leaves under short- or long-term drought as individual treatment. Proline concentration under drought at flowering stage and drought at veg + flow stages recorded the highest levels in leaves and seeds (Table 4). The unexpected observation was that the exogenous application of proline did not increase the internal level of proline in leaves, but contrarily decreased proline concentration comparing with its control. Not only exogenous proline application decreased the level of internal proline in leaves at pod filling stage against control, but also GB treatment did. These observations may have more than one explanation. The first explanation directed from the reduction of proline accumulation by exogenous GB or proline suggesting that proline accumulation is just a symptom of stress rather than a trigger of tolerance (Ashraf and Foolad, 2007; Hu et al., 2012). Therefore, the strong relationship between proline accumulation and stress tolerance may not be universal. The second explanation directed from rapid breakdown of proline in mitochondria upon rehydration or relief of stress to generate ATP for repairing the stress-induced damages (Ashraf and Foolad, 2007). Osmotic stress activates the biosynthesis process of proline and represses its catabolism, whereas rehydration activates the opposite regulation (Szabados and Savouré, 2010). Proline catabolism is controlled by proline dehydrogenase (PDH) and pyrroline-5-carboxylate dehydrogenase (P5CDH) in mitochondria. The transcription of PDH in *Arabidopsis* was activated as a result of rehydration or exogenous proline in the medium, whereas the dehydration repressed PDH activity to prevent proline degradation throughout abiotic stress (Kiyosue et al., 1996). *Arabidopsis* transcription profile revealed that one-third of plant genes induced by rehydration could also be induced by proline (Oono et al., 2003). Therefore, exogenous application of GB or proline may be led to an increase in leaf water content and activated the transcription process of PDH gene, which rapidly degraded the accumulated proline. Another explanation pointed from the present results, since GB or proline application led to an increase in internal content of proline in seeds, so decreasing in proline concentration in leaves by same applications could refer to its promoting effect on translocation process for proline from source to sink. Also, increasing the level of soluble proteins in leaves under GB and proline application comparing with control (Table 5), suggests that these applications led to an increase in proline-rich proteins which were critical for development and abiotic stress tolerance (Zhan et al., 2012).

Total soluble protein concentrations presented in Table 5 show that GB treatment recorded the highest values under all levels of drought in leaves and seeds comparing with proline treatment and untreated plants, especially under drought at vegetative stage. Drought stress may cause a decrease in protein content in plants. It has been reported that drought stress decreased protein concentration of pea leaves (Karataş et al., 2014). As free amino acids accumulated under drought stress, proteins content decreased (Labanauskas et al., 1981). In the present study, long-term water-shortage treatments decreased soluble protein concentration markedly. The decline in the soluble protein level could be caused by denaturation as ROS production increases. Proline has been shown to function as a molecular chaperone able to protect protein integrity under stress (Szabados and Savouré, 2010) which may maintain proteins content from degradation through oxidation by ROS. Proline treatment in seeds recorded the lowest levels in soluble proteins.

Non-enzymatic total antioxidant capacity (NEAC)

Ascorbate (AsA) and glutathione (GSH) as non-enzymatic antioxidants have vital roles in the development of plant stress tolerance under adverse environmental conditions. Increasing the level of AsA or GSH can effectively reduce ROS production under stress conditions including salt stress and thus prevent oxidative damage (Hasanuzzaman et al., 2014).

Total antioxidant capacity was classified into two groups, enzymatic and non-enzymatic antioxidant capacity. Non-enzymatic total antioxidant capacity is presented in Table 5. The highest levels of NEAC in leaves were recorded by GB and proline treatments comparing with control as overall mean. Drought treatments decreased the levels of NEAC in leaves comparing with unstressed plants. Interactions between treatments show that proline and drought at vegetative and flowering stages recorded the highest values in seeds, followed by GB and drought at veg + flow stages. Individual treatments of GB and proline decreased the levels of NEAC in seeds comparing with untreated plants under same conditions (Table 5).

In addition to antioxidant enzymes system, ascorbate–glutathione cycle considers another essential defense system of plants to protect cells against the detrimental ROS. Exogenously applied proline upregulates the activities of enzymes in the ascorbate–glutathione cycle. The activities of ascorbate peroxidase, monohydro-ascorbate reductase and dihydro-ascorbate reductase, which are the components of ascorbate–glutathione cycle, were significantly enhanced by exogenously applied proline in tobacco cultures under salinity stress (Hayat et al., 2012). Exogenously applied proline and GB significantly enhanced the activities of monohydro-ascorbate reductase and dihydro-ascorbate reductase, which reflect on improving ascorbic acid levels in rice plant (Hasanuzzaman et al., 2014).

Most of the stresses were associated with generation of free radicals (Rai, 2002). Free radical levels were reduced in transgenic tobacco plants engineered for hyper-accumulation of proline by pyrroline-5-carboxylate synthetase (P5CS) overexpression and acceleration of the proline biosynthetic pathway. Numerous studies recognized that proline has an antioxidant feature, which acts as a singlet oxygen quencher (Szabados and Savouré, 2010), suggesting that proline has a direct ROS scavenging feature, which in turn increases the number of antioxidant in the cell by one. Subsequently, according to its amount in the cell, the non-enzymatic total antioxidant capacity increased.

Antioxidant enzymes

Most mesophyte plants imposed to water stress have the ability to induce some or more of physiological changes. Accumulating the compatible solutes, increasing the level of non-enzymatic antioxidants and activating the antioxidant

enzymes were the most adaptive mechanisms under such conditions. The most important antioxidant enzymes involved in scavenging the excess content of ROS formed under stress are superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX).

Decreasing activity of SOD, APX and CAT in leaves was generally observed for untreated plants (control of GB and proline application) subjected to drought at different growth stages. The highly affected enzymes under long-term drought stress were APX and CAT in both evaluated organs (leaves & seeds), whereas SOD activity increased under same drought condition comparing with control (Fig. 1). Such increase in the activity of SOD for untreated plants subjected to long-term drought stress suggests that osmotic stress exhibits an oxidative stress by forming ROS. SOD is the first enzyme working on scavenging the first free radical (superoxide $O_2^{\cdot-}$) formed under stress. The observed increase in SOD activity suggests that the antioxidant defense system would play an important role in the drought tolerance of pea plant. SOD in leaves showed a considerable increase of activity than that recorded in seeds. This tendency could be attributed to the higher SOD biosynthesis in leaf than in seeds tissues.

SOD activity increased markedly under long-term drought stress (Fig. 1). This increase leads to enhanced production of H_2O_2 from superoxide, and the possible H_2O_2 build-up could be attended by increasing the activity of APX and CAT. SOD transforms $O_2^{\cdot-}$ to H_2O_2 by acting as the first line of defense against ROS (Karataş et al., 2014). The present findings showed that SOD activity was increased by the long-term drought stress, whereas CAT and APX in leaves did not follow this increase, which in turn increases the ROS levels leading to oxidative damage to photosynthetic apparatus and reduces the amount of assimilates (Table 3), which in turn reflected on pod yield (Table 2). Focused on APX activity in seeds, results indicate that APX activity changed only slightly, which suggest that APX has an important role in seeds rather than leaves, which could prevent degradation in storage content of the seeds. Under drought stress, increase in APX activity was higher than that of CAT (Fig. 1). APX has an important role in the AsA–GSH cycle. In plants under stress, activity of this enzyme is usually considered as an indicator of the plant tolerance level against the stress condition. The AsA–GSH cycle is known to be responsible mainly for H_2O_2 scavenging in the chloroplast (Asada, 1992). In this respect, a synchronic increase in some components of the antioxidative would be necessary in order to obtain an improvement in stress tolerance (Karataş et al., 2014). In this connection, exogenous application of proline and GB increased the activity of antioxidant enzymes to a significant level comparing with control. Proline has been shown to function as a molecular chaperone able to protect protein integrity and enhance the activities of different enzymes (Szabados and Savouré, 2010). Hoque et al. (2007) reported that the activities of antioxidative enzymes CAT and SOD were significantly improved when proline was applied exogenously in tobacco suspension cultures exposed to salinity stress. Both exogenous proline and GB may improve salt tolerance in tobacco BY-2 suspension-cultured cells by enhancing the activity of enzymes involved in the ASC–GSH cycle. Taken together, the results suggest that antioxidant protection activity of proline against salt stress is stronger than that of GB because of the superior ability of proline to increase the enzyme activity of the antioxidant system (Hoque et al., 2007).

Application of GB and proline under drought at different growth stages increased the activity of SOD and APX in leaves comparing with its control. Proline application decreased the activity of CAT in leaves under drought at different times except for long-term drought, whereas application of proline led to an increase in the activity of CAT comparing with its control of same conditions. Under unstressed conditions, GB decreases the activity of SOD, while proline decreases the activity of CAT in leaves.

Drought at flowering and veg + flow stages increased the activity of SOD in seeds. Levels of activity for APX and CAT in seeds for untreated plants under drought imposed at different growth stages were unaffected, except for CAT under long-term drought (Fig. 1). Application of GB decreased the activity of APX in seeds comparing with control, whereas both of GB and proline application decreased the activity of CAT in seeds under all drought application times, except for long-term drought, which increases the activity of CAT comparing with control (Fig. 1).

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