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Antioxidant Defence System in Chickpea (*Cicer arietinum* L.): Influence by Drought Stress Implemented at Pre- and Post-anthesis Stage

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ABSTRACT

Significant differences in the antioxidant system of the leaves of four chickpea (*Cicer arietinum* L.) genotypes differing in tolerance to drought were observed in a pot experiment. Drought stress was imposed at pre- and post- anthesis stage (50 and 65 days after sowing). We studied the effect of drought stress on hydrogen peroxide (H_2O_2) content, lipid peroxidation and antioxidant enzyme activities in four chickpea genotypes viz., Tyson, ICC 4958, JG 315 and DCP 92-3. Drought treatment significantly increased hydrogen peroxide accumulation and lipid peroxidation, which indicated the extent of oxidative injury posed by stress conditions. Enzyme activities of Superoxide Dismutase (SOD), Peroxidase (POX), Catalase (CAT) and Ascorbate Peroxidase (APX) increased in chickpea leaves as a consequence of drought conditions and all the enzyme activities were significantly higher in drought stressed genotypes i.e., Tyson and ICC 4958 than in JG 315 and DCP 92-3. Relationship between Lipid Peroxidation (LPO), Hydrogen peroxide (H_2O_2), Superoxide Dismutase (SOD), Catalase (CAT), Peroxidase (POX) and Ascorbate Peroxidase (APX) was determined in order to find out whether these parameters can be used as selection criteria for drought tolerance in this crop. Early drought stress (pre-anthesis drought) was found to be more damaging than the late drought stress (post-anthesis drought).

Key words: Antioxidants, chickpea, drought, lipid peroxidation

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third most important grain legume crop in the world and first in the Mediterranean basin and South Asia that frequently experiences water stress during pod set and seed filling stage (terminal drought) in India and the Mediterranean basin, leading to a substantial yield loss (Turner *et al.*, 2001). Drought stress during seed filling has been reported to be highly detrimental to yield in chickpea (Davies *et al.*, 1999). In the current scenario of water limitation, there is little scope to increase the irrigated area of this crop. Moreover, the slow and steady migrations of this crop towards lower latitudes in India (Gowda *et al.*, 2009).

Plants respond to drought stress and acclimatize through various physiological and biochemical changes (Farooq *et al.*, 2009a). Drought induces oxidative stress in plants, in which reactive oxygen species (ROS), such as superoxide radical ($O^{2-\bullet}$), hydroxy radical ($^\bullet OH$), hydrogen peroxide (H_2O_2) and alkoxy radical ($RO^\bullet$) are produced (Munne-Bosch and Penuelas, 2003;

Esfandiari *et al.*, 2008). Hydrogen peroxide (H_2O_2) is the first stable compound among ROS produced in the plant cell under normal conditions and as a result of stress. It is suggested to be involved in ROS-mediated signaling because of its ability to penetrate the plasma membrane as an uncharged molecule. Therefore, it can be transported to the site of action (Foyer *et al.*, 1997).

Oxidative damage in the plant tissue is alleviated by a concerted action of both enzymatic and non-enzymatic antioxidant metabolism (Hasegawa *et al.*, 2000). These mechanisms include β-carotenes, Ascorbic Acid (AA), α-tocopherol (α-Toc), reduced glutathione (GSH) and enzymes including Superoxide Dismutase (SOD), Peroxidase (POX), Ascorbate Peroxidase (APX), Catalase (CAT), Polyphenol Oxidase (PPO) and Glutathione Reductase (GR) (Prochazkova *et al.*, 2001).

Plants experience drought stress either when the water supply to roots is interrupted or when transpiration rate becomes very high. These two conditions often coincide under arid and semiarid climates. Drought stress tolerance has been seen in almost all plant species but its extent varies from species to species (Lin *et al.*, 2006). To date, antioxidant responses of chickpea under abiotic stress conditions such as chilling (Nayar *et al.*, 2005) salt (Eyidogan and Oz, 2007) and also drought (Moinuddin and Imas, 2007) have been reported. Drought stress altered lipid peroxidation, increasing peroxidase and superoxide dismutase activity (Saleh *et al.*, 2007; Tatar and Gevrek, 2008). However, no data are available related to comparative antioxidant study under drought at two critical phases of development i.e., pre- and post- anthesis stages in chickpea. Therefore, the aims of this study were: (1) to understand how leaves of plants differing in drought tolerance respond to oxidative damage, (2) to characterize the genotypic drought tolerance variability in chickpea, (3) to identified the most drought-tolerant genotypes, (4) to determine the selection criteria of drought stress and (5) to identify the most sensitive stage of drought stress.

MATERIALS AND METHODS

Site description: The experiment was carried out during Rabi season 2009-10 in rain-protected wire- house at the Horticulture Research Farm, Institute of Agricultural Sciences, Banaras Hindu University Varanasi, India. The experimental area lies between latitudes 25.18°N, longitude 83.03°E and 123.93 m altitude. The average of climatic conditions calculated during the entire growth period is as follows: maximum/minimum temperature, Relative Humidity (RH) was 28.0°C /13.6°C, 71.3/36.5%, respectively and an average sunshine hour was 6.9.

Plant materials and treatment pattern: The seeds of chickpea (*C. arietinum* L.) genotypes (Tyson, ICC 4958, JG 315 and DCP 92-3) were obtained from Indian Institute of Pulse Research (IIPR-ICAR), Kanpur, India through Material Transfer Agreement (MTA). The healthy seeds of uniform size were selected and surface sterilized with 0.2% $HgCl_2$ solution followed by repeated washing with Double Distilled Water (DDW). Thereafter 10 seeds of each genotype were subsequently sown (10 per pot), pot size "30×30 cm" filled with farm soil having 12-14% moisture at the time of sowing and were thinned to six uniform plants per pot at the first true leaf stage. The experimental soil was sandy loam containing organic carbon 0.31%, available nitrogen 228.00 kg ha^{-1}, available phosphorus 17.00 kg ha^{-1}, available potassium 180.00 kg ha^{-1} and pH 7.3. All the pots were applied with measured dose of fertilizer for chickpea at 20, 40, 20 kg ha^{-1} of N, P_2O_5 and K_2O, respectively.

Drought stress applications: Each genotype was grouped in three sets viz., irrigated, early drought stress and late drought stress. Water stress treatment was instigated at 50 DAS. Normal
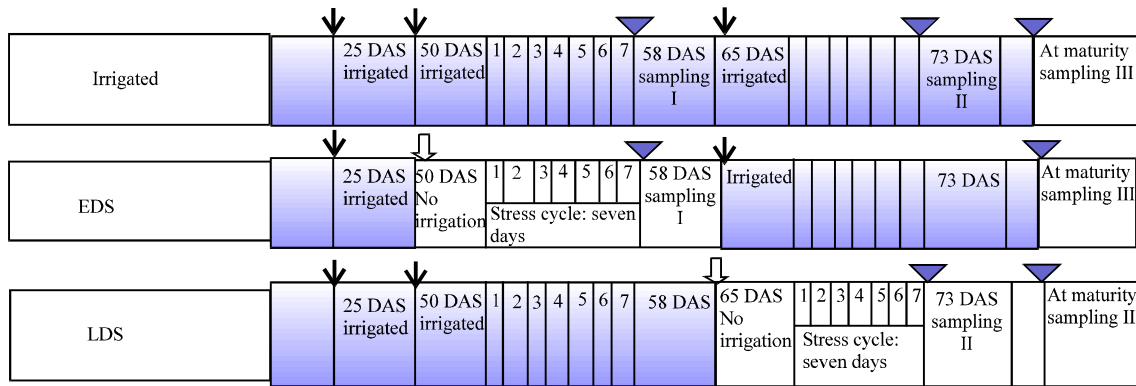
| Irrigated | | 25 DAS irrigated | 50 DAS irrigated | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 58 DAS sampling I | 65 DAS irrigated | | | | | | 73 DAS sampling II | | At maturity sampling III |

| EDS | | 25 DAS irrigated | 50 DAS No irrigation | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 58 DAS sampling I | Irrigated | | | | | | 73 DAS | | At maturity sampling III |
| | | | Stress cycle: seven days | | | | | | | | | | | | | | | | | |

| LDS | | 25 DAS irrigated | 50 DAS irrigated | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 58 DAS | 65 DAS No irrigation | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 73 DAS sampling II | At maturity sampling III |
| | | | | | | | | | | | | | | Stress cycle: seven days | | | | | | | |

Fig. 1: Schematic representation of experimental layout, ↓(Solid arrow) indicate the irrigated position with respect to DAS, ⇩ (empty arrow) indicate the water withholding position with respect to DAS, ▽ (Solid triangle) indicated the sampling time (I and II for physiological and biochemical parameters and III-for yield and yield attributing parameters), DAS indicate days after sowing (EDS: Early drought stress or pre-anthesis stage, LDS = Late drought stress or post-anthesis stage)

plants were given three irrigations (at 25, 50 and 65 DAS) from the date of sowing to maturity. Early drought stressed or pre-anthesis drought stressed plants (EDS) received two irrigations (25 and 65 DAS) whereas post-anthesis drought stressed plants (LDS) received two irrigations at 25 and 50 DAS. Stress cycle given seven days in both the conditions (Fig. 1).

Methodology

Hydrogen peroxide estimation: Hydrogen peroxide was estimated with titanium reagent (Terenashi *et al.*, 1974). 1 g of titanium dioxide and 10 g potassium sulphate was digested in 150 mL concentrated sulphuric acid over a hot plate for 2 h. The digested mixture was diluted to 500-600 mL and stirred with a magnetic stirrer cum heater at 70-80°C till a clear transparent solution was obtained. This solution was diluted to 1.5 L and stored in a dark brown bottle and used as titanium reagent. Sample preparation and H_2SO_4 estimation was done as described by Mukherjee and Choudhuri (1983). Leaf material (0.5 g) was homogenized in 10 mL of cold acetone (90%, v/v). The homogenate was filtered through Whatman No. 10 filter paper. To the filtrate, 4 mL of titanium reagent was added followed by 5 mL of concentrated ammonium solution to precipitate the peroxide-titanium complex. The reaction mixture was centrifuged in a refrigerated centrifuge for 5 min at 10000x g, the supernatant was discarded and precipitate was dissolved in 10 mL of 2 M H_2SO_4. It was re-centrifuged to remove the undissolved material and the absorbance was recorded at 415 nm against blank. Concentration of H_2O_2 was determined using a standard curve plotted with known concentration of H_2O_2. All spectro-photometric measurements were carried out by spectrophotometer (Shimadzu UV-VIS, Japan).

Lipid peroxidation: The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content, a product of lipid peroxidation (Hodges *et al.*, 1999). Leaf sample (0.5 g) was homogenized in 10 mL of 0.1% TCA. The homogenate was centrifuged at 15000x g for 5 min. To 1.0 mL aliquot of the supernatant, 4.0 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA was added. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10000x g for 10 min, the absorbance of supernatant was recorded at 532 nm. The

value for nonspecific absorption at 600 nm was subtracted. The MDA equivalent was calculated as follows:

$$\text{MDA [nmol / (mL FW)]} = \frac{A_{532} - A_{600}}{155000} \times 10^6$$

Antioxidant enzyme assays: Superoxide dismutase (EC 1.15.1.1) assay was performed as per the protocol of Dhindsa *et al.* (1981). Leaf sample (100 mg) was homogenized in 5 mL extraction buffer (0.1 M phosphate buffer of pH 7.5 containing 0.5 mM EDTA). Enzyme extract was centrifuged at 10,000 x g for 10 min at 4°C. After centrifugation, supernatant was collected and used as enzyme source. Reaction mixture (3.0 mL) consisted of 0.1 mL 1.5 M sodium carbonate, 0.2 mL 200 mM methionine, 0.1 mL 2.25 mM NBT, 0.1 mL 3 mM EDTA, 1.5 mL 100 mM potassium phosphate buffer, 0.7 mL distilled water and 0.2 mL enzyme extract. The reaction was started by adding 0.1 mL riboflavin (60 µM) and placing the tubes below a light source of two 15 W florescent lamps for 15 min. Illuminated and non-illuminated reactions without enzyme were used for calibration. Absorbance was recorded at 560 nm in spectrophotometer. One unit of SOD activity was defined as 50% inhibition of the basic rate of the reaction.

Peroxidase (EC 1.11.1.7) activity was estimated as per the protocol of Kar and Mishra (1976) with slight modifications. The enzyme was extracted by homogenizing 100 mg leaf tissue in 5.0 mL 0.1 M phosphate buffer (pH 6.4). The crude extract was centrifuged at 10,000 x g for 20 min at 4°C. Supernatant was collected. Reaction mixture was prepared by adding 4.5 mL 0.1 M phosphate buffer (pH 6.4), 0.2 mL pyrogallol (50 µM) and 0.1 mL 50 µM H_2O_2 and 0.2 mL supernatant. The mixture was incubated at 25°C for 5 min. Then 0.5 mL of 5.0% H_2SO_4 was added to terminate the reaction. Absorbance was recorded at 420 nm. One unit of enzyme activity is defined as that amount of enzyme, which forms 1 mol of purpurogallin formed per minute under the assay conditions.

Catalase (EC 1.11.1.6) activity was assayed as per the protocol of Aebi (1983) with suitable modifications.100 mg leaf tissue was homogenized in 5 mL of 0.1 M phosphate buffer (pH 6.4) in a chilled pestle and mortar. The extract was centrifuged at 10,000 x g for 20 min at 4°C. Supernatant was collected and used in the enzyme assay. Reaction mixture consisted of 2.6 mL 0.1 M phosphate buffer (pH 6.4), 0.2 mL enzyme extract and 0.1 mL, 10 mM H_2O_2 with final volume 3.0 mL. The reaction mixture was mixed rapidly at room temperature. Changes in absorbance at 230 nm at an interval of 15 s were noticed for 2 min on an UV visible spectrophotometer. One unit of catalase activity was defined as the amount of enzyme catalyzing the decomposition of 1 nmol H_2O_2 per minute (extinction coefficient 36 mM cm^{-1}).

Ascorbate peroxidase (EC 1.11.1.11) was assayed as per the protocol of Nakano and Asada (1980). Enzyme extract for APX was prepared by grinding 100 mg sample with 5.0 mL of extraction buffer (0.1 M phosphate buffer pH 7.5 containing 0.5 mM EDTA and 1 mM ascorbic acid). The homogenate was centrifuged at 10,000x g for 10 min at 4°C. After centrifugation, supernatant was collected and was used as enzyme source. The 3 mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2, 0.2 mL enzyme extract. Reaction was started by adding H_2O_2. Decrease in absorbance for a period of 30 sec was measured at 290 nm in an UV-visible spectrophotometer. One unit was defined as micromoles of ascorbate oxidized per minute (extinction coefficient 2.8 mM cm^{-1}).

Statistical analysis: All experimental data recorded were average mean values for at least three independent assays with three replicates each. The data were subjected to ANOVA for completely

randomized design factorial. Differences at p<0.05 were considered statistically significant (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Effect of drought stress on malondialdehyde (MDA) content in four chickpea (*C. arietinum* L.) genotypes are presented in Fig. 2. Lipid peroxidation estimated as MDA content was varied among the genotypes and, it was generally increased under both, early and late drought stress as compared to optimal conditions. Lipid peroxidation was significantly higher in pre- anthesis compare to post-anthesis, as for as genotype is concern, JG 315 (3.23 nmol g^{-1} fresh weight (FW)) showed higher lipid peroxidation over control (1.19 nmol g^{-1} FW) followed by genotype DCP 92-3 (3.11 nmol g^{-1} FW) whereas, minimum was recorded in ICC (1.51 nmol g^{-1} FW) (Fig. 3). Drought induces oxidative stress in plants by generation of reactive oxygen species (ROS) (Farooq *et al.*, 2009b). As increasing ROS level causes oxidative damage to biomolecules such as lipids, proteins and nucleic acids (Mittler, 2002). Reactive oxygen species e.g., peroxides of polyunsaturated fatty acids generate malondialdehyde (MDA) on decomposition and in many cases MDA is the most abundant individual aldehydic lipid breakdown product (Esterbauer and Cheeseman, 1990) and strikingly malondialdehyde (MDA) is a widely used marker of oxidative lipid injury whose concentration varies in response to abiotic stress (Moller *et al.*, 2007; Davey *et al.*, 2005). A decrease in membrane stability reflects the extent of lipid peroxidation caused by ROS. Furthermore, lipid peroxidation is an indicator of the prevalence of free radical reaction in tissues. Present findings support that the prevention of membrane damage may be related to the induction of antioxidant responses which protects the plant from oxidative damage.
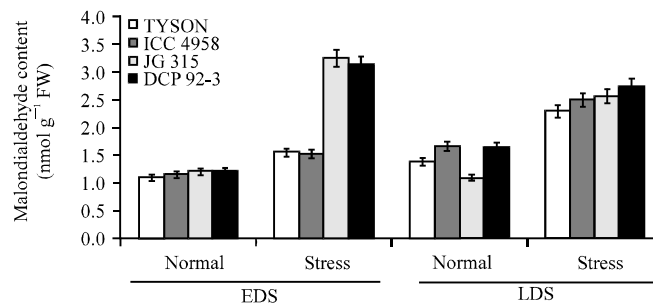

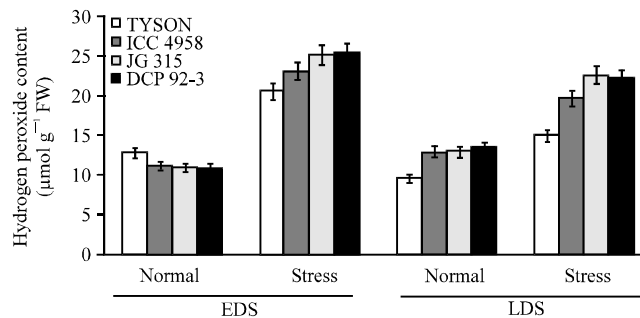Fig. 2: Effect of drought stress on Malondialdehyde (MDA) content in four chickpea (*C. arietinum* L.) genotypes

Fig. 3: Effect of drought stress on H_2O_2 content of four chickpea (*C. arietinum* L.) genotypes

Hydrogen peroxide (H_2O_2) concentrations of the genotypes greatly differed among the genotypes in optimal and both pre- and post-anthesis (early and late) drought stress conditions. The accumulation of H_2O_2 as a result of drought stress was generally increased in the genotypes of chickpea. Hydrogen peroxide was higher in pre- anthesis compare to post-anthesis, as for as genotype is concern, JG 315 (25.10 µmol g^{-1} FW) showed higher H_2O_2 content whereas, minimum was recorded in Tyson (20.60 µmol g^{-1} FW) (Fig. 3). Under drought conditions, plants produce reactive oxygen species, which are harmful to plant growth due to their detrimental effects on the sub-cellular components and metabolism of the plant leading to the oxidative destruction of the cells. It is generally accepted that might be converted to hydrogen peroxide (H_2O_2). Hydrogen peroxide is a toxic compound produced as a result of scavenging of superoxide radical and its higher concentrations are injurious to plants, resulting in lipid peroxidation and membrane injury (Sairam *et al.*, 1998; Baisak *et al.*, 1994; Menconi *et al.*, 1995). In the present work, in parallel to H_2O_2 accumulation, membrane damage (MDA content and membrane permeability) of the chickpea cultivars were generally increased. But there were no significant relationship between the H_2O_2 concentration and drought resistance. In addition to this membrane permeability positively correlated with MDA at both stress conditions and lipid peroxidation in the stress-tolerant genotypes (Tyson and ICC 4958) were found to be lower as compared to stress-susceptible genotypes (JG 315 and DCP 92-3). Higher membrane stability (lower MDA content) has also been reported.

Antioxidant defense system plays vital role in plant's tolerance to stressful conditions. It is obvious from the results that drought has induced antioxidant enzymes activities more than the normal plants during the drought period. Results showed that increase in SOD, POX CAT and APX activities in chickpea may be related to the induction of antioxidant responses that protect the plant from oxidative damage. Among antioxidant enzymes, SOD converts the toxic O_2^- to H_2O_2 which must be scavenged to the O_2 and H_2O by the antioxidant enzymes such as CAT, POX and APX.

Superoxide dismutase (SOD) activity increase in all chickpea genotypes under drought condition. The higher SOD activity was noticed at post-anthesis stage than pre-anthesis stage. As for as genotype was concern maximum in ICC 4958 [243.59 units mg^{-1}(protein) min^{-1}] and it was minimum in DCP 92-3 [124.05 units mg^{-1}(protein) min^{-1}] (Fig. 4). Superoxide dismutase constitutes the first line of defence via detoxification of superoxide radicals (Sairam and Saxena, 2000), thereby maintaining membranes of plant tissue.

Catalase (CAT) eliminates H_2O_2 by breaking it down directly to water and oxygen. In our study, total CAT activity was enhanced by drought in the leaves of chickpea. Drought leads to significant
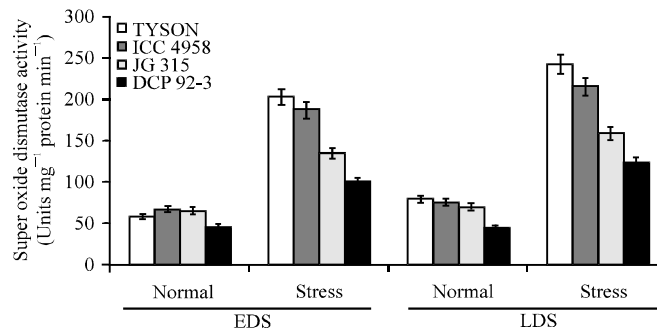

Fig. 4: Effect of drought stress on superoxide dismutase (SOD) activity in four chickpea (*C. arietinum* L.) genotypes
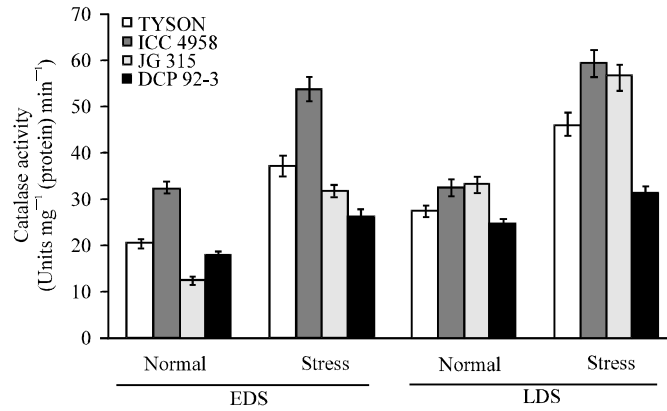
Fig. 5: Effect of drought stress on catalase (CAT) activity in four chickpea (*C. arietinum* L.) genotypes
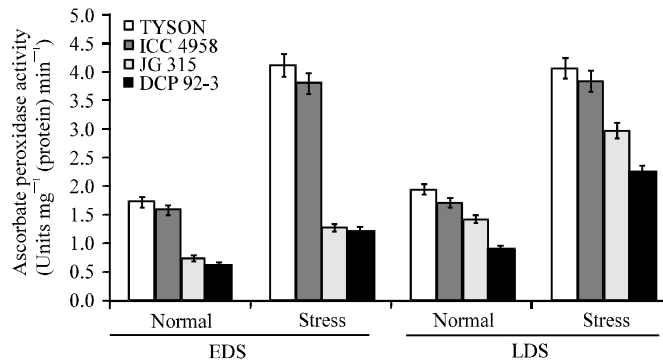

Fig. 6: Effect of drought stress on ascorbate peroxidase (APX) activity in four chickpea (*C. arietinum* L.) genotypes

increase in CAT activity. Maximum CAT activity under drought stress noticed in genotype ICC 4958 (59.45 Units mg^{-1} (protein) min^{-1}) while minimum in JG (31.44 Units mg^{-1} (protein) min^{-1}) (Fig. 5). Catalase and peroxidases are the most important enzymes involved in regulation of intracellular level of H_2O_2 (Prasad *et al.*, 1995). APX convert H_2O_2 into H_2O along with the regeneration of NADP+, therefore, the enzymes play important roles in stress conditions (Jimenez *et al.*, 1998). When one considers that CAT acts in peroxisomes, it is logical to suggest that photorespiration in the leaves of chickpea was also affected by drought treatment. Omidi (2010) recently noticed that enzyme activities of Ascorbate Peroxidase (APX) and Catalase (CAT) were highly significant difference between drought and genotypes in respect to CAT and APX. These were increase under drought stress.

APX which is primarily located both in chloroplasts and cytosol acts as a key enzyme of the glutathione-ascorbate pathway. It scavenges peroxides by converting ascorbic acid to dehydroascorbate and it is one of the most important enzymes playing a vital role in eliminating toxic H_2O_2 from plant cell in Asada-Halliwell pathway. In the present study, drought stress increased total APX activity differed prominently in stressed leaves as compared to that of non-stressed leaves. Higher activity of APX in Tyson and ICC 4958 under stressed leaves, generally its activity was 2-fold more over control, suggests a more effective H_2O_2 removal which might be produced by an enhanced activity of SOD in chickpea (Fig. 6).
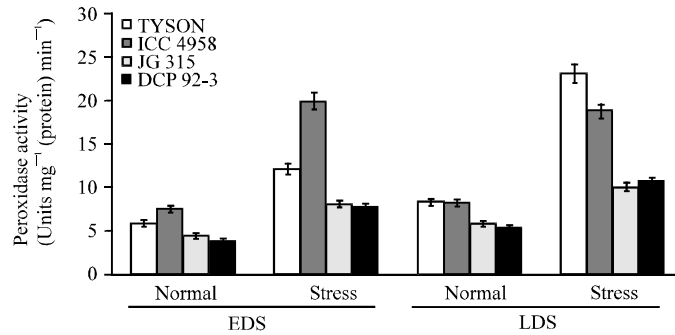
Fig. 7: Effect of drought stress on peroxidase (POX) activity in four chickpea (*C. arietinum* L.) genotypes

This well elucidates that POX should play a more significant role than CAT in detoxifying the produced H_2O_2 since the activity of POX increased, in contrast to that of CAT (Dey *et al.*, 2007). It is well documented that CAT is less efficient than POX in scavenging of H_2O_2 because of its low substrate affinity (Erdal and Dumlupinar, 2010). Highest values of POX activity were observed in Tyson [22.88 unit mg^{-1}(protein) min^{-1}] followed by ICC 4958 [18.66 unit mg^{-1}(protein) min^{-1}] (Fig. 7). In tolerant genotype, POX activity was found to be higher, providing protection against the oxidative stress. Remarkably higher levels of POX in drought-stressed seedlings of chickpea might also be considered as a higher capacity to decompose H_2O_2 more rapidly.

CONCLUSION

Based on analyses of four chickpea genotypes and there were substantial variation in tolerance to drought within chickpea genotypes. A perusal of the results shows that drought tolerance genotypes have higher enzymatic activities such as SOD, POX, CAT, APX and lower level of MDA and H_2O_2 in comparison to drought susceptible genotypes. These parameters showed a considerable variability and heritability under drought stress conditions. Based on the significant and well defined relationships between lipid peroxidation and antioxidant enzyme activities, we concluded that these parameters could be instrumental in predicting the drought tolerance of chickpea genotypes. It is also concluded that pre- anthesis stage was most sensitive whereas, genotype ICC 4958 was most tolerant to drought.

Meagre information is available regarding genotypic variation for drought tolerance in chickpea. Henceforth, this study could help to understand some adaptive mechanisms developed by chickpea genotypes in contributing useful identified traits for chickpea breeding programme.

ACKNOWLEDGMENTS

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