



Stage specific upregulation of antioxidant defence system in leaves for regulating drought tolerance in chickpea

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Abstract: Leaf is one of the early sensors for the drought stress and is important to study drought tolerance mechanism. Activities of antioxidative enzymes and status of malondialdehyde (MDA), hydrogen peroxide (H₂O₂), proline and total phenols were studied in leaves of drought tolerant (PDG 3 and PDG 4) and susceptible (PBG 1, GPF 2, PBG 5, L 550 and BG1053) chickpea cultivars under irrigated and rainfed conditions at different development stages. In general, with the age of plant, the activities of superoxide dismutase (SOD) and catalase (CAT) increased but the activities of glutathione reductase (GR), ascorbate peroxidase (APX) and peroxidase (POX) decreased in leaves. With some exceptions, in general, higher status of APX and POX in leaves at vegetative stage I (30 days after sowing) and II (60 days after sowing); GR at vegetative stage II and pre-flowering stage and SOD and CAT at seed filling stages in tolerant cultivars under drought stress reflected stage specific upregulation of antioxidant defence system in them. The relatively lower activities of APX and POX in old leaves during seed filling stage make them more prone to enhanced oxidative injury than the young leaves. Lower content of hydrogen peroxide and malondialdehyde in leaves of tolerant cultivars during seed filling reflects the impact of antioxidant defence system operative at that time. The higher accumulation of proline and total phenol in leaves of tolerant cultivars might be playing important role in drought stress tolerance. These results indicated the importance of upregulation of different antioxidant enzymes at variable stages of leaf development.

Keywords: Antioxidative enzymes, Chickpea, Drought stress, Leaves

INTRODUCTION

Drought stress caused by soil and atmospheric water deficiency is one of the most significant environmental factors affecting plant growth and productivity worldwide (Salekjalali *et al.*, 2012; Mirzaee *et al.*, 2013). It disrupts major components of the photosynthesis process. Drought stress decreases intracellular CO₂ concentration due to stomatal closure, and this causes suppression of the carboxylation reaction catalyzed by Rubisco, thus increasing photorespiration. A decrease in the photosynthetic fixation of CO₂ reduces the consumption of ATP and NADPH during the Calvin cycle and this causes over-reduction in the photosynthetic electron transport chain due to insufficient electron acceptor NADP⁺. When ferredoxin is over reduced during photosynthetic electron transfer, electrons that have a high-energy state are transferred from the PS I to molecular oxygen forming the superoxide anion radical O₂^{•-} via the Mehler reaction (Hsu and Kao, 2003; Labudda and Azam, 2014), which triggers chain reactions that generate more harmful oxygen radicals like OH•. As a result of drought stress, these reactive oxygen species (ROS) cause damage to cell structures, proteins, lipids, carbohydrates, and nucleic acids, leading

to cell death. However, in spite of their damaging effects, under steady state conditions, ROS play a major physiological role in intracellular signalling and regulation, as secondary messengers (Gill and Tuteja, 2010).

To protect themselves against these toxic ROS, plants have evolved efficient systems which include ROS-scavenging antioxidative enzymes including superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR), and non-enzymatic antioxidants including ascorbic acid, glutathione, carotenoids, and tocopherols (Ashraf, 2009; Singh *et al.*, 2012; Verma *et al.*, 2014). Among these, SOD converts O₂^{•-} to H₂O₂ which is further scavenged by POX in extracellular space and cytosol; and mainly by CAT in peroxisomes. H₂O₂ is also decomposed by APX, one of the Asada-Halliwell enzymes, in different cell compartments. Hence, reacting directly or indirectly with ROS, enzymatic and non enzymatic antioxidants contribute in maintaining the integrity of cell structures and the proper functions of various metabolic pathways (Chaparzadeh *et al.*, 2004). The degree to which the activities of antioxidant enzymes and the amount of antioxidants are elevated under drought stress shows a

remarkable variation among plant species (Zhang and Kirkham, 1996; Turkan *et al.*, 2005) and even between two cultivars of the same species (Bartoli *et al.*, 1999; Perez-Lopez *et al.*, 2009). In recent years, many researchers have established that the induction of the cellular antioxidant machinery is important for tolerance to drought stress (Miller *et al.*, 2010; Chugh *et al.*, 2011; Singh *et al.*, 2012).

Drought stress is a major concern affecting the agriculture production. Generally pulses are very susceptible to drought stress. Chickpea is the second largest cultivated grain legume in the world, grown in about 11.5 million hectares (Varshney *et al.*, 2013) 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). As there is reduction in the translocation of carbohydrates during drought stress, this leads to a change in source-sink relationship (Patel and Hemantaranjan, 2012). Over past several years, there has been a great interest in research investigating the antioxidant defence system in plant leaves, as leaf is the principle plant part which acts as one of the early sensor for the drought stress. It has been demonstrated that the antioxidative response is correlated with the tolerance of individual crop cultivars to drought (Kolarovic *et al.*, 2009).

Most of the studies reported in the literature either focussed on seedlings or if in the field then on one particular stage (Patel and Hemantaranjan, 2012; Kaur *et al.*, 2012; Devi *et al.*, 2012; Battana and Ghanta, 2014). The present study was conducted to study the antioxidant defence response at different developmental stages i.e. vegetative stages, pre-flowering and seed filling stages of the crop to see if there is any differential development specific response of tolerant genotypes to drought. Furthermore, the level of some compounds (proline, phenolics, malondialdehyde and hydrogen peroxide) were considered in an effort to identify the mechanism (s) by which drought stress decreases plant growth.

MATERIALS AND METHODS

Plant material and experimental conditions:

Tolerant *desi* (PDG 3 and PDG 4), susceptible *desi* (PBG 1, GPF 2 and PBG 5) and susceptible *kabuli* (L 550 and BG 1053) chickpea cultivars were raised in the field of Punjab Agricultural University (30°54'N, 75°48'E, elevation 247 m above sea level), Ludhiana, following recommended agronomic practices. The crop was sown in the last week of November, 2008-2009 in sandy loam fields with pH of about 7.8 to 8.0 in randomized block design with three replicates of each cultivar under rainfed and irrigated conditions. Each plot had five rows of each cultivar with row length of 4 m and row spacing of 30 cm and there were 20-30 plants in each row. In irrigated area, 3 irrigations

were given after sowing i.e. on 21th December, 28th January and 5th March and plants grown in irrigated area received 369.4 mm water. However, under rainfed conditions all the three irrigations were withheld from sowing to maturity and the plants received water only available through rainfall. The average rainfall during the crop season was 44.4 mm and rainfed crops in total received 144.4 mm water (Table 1). It is cleared from the rainfall data that during the crop season rainfall was scanty resulted in development of drought stress. The plants were uprooted by deep burrowing from the field at the interval of 30 days and were collected at 30, 60, 90 and 120 days after sowing (DAS) and brought buried under ice to the laboratory. These stages have been named as vegetative stage I (VS I), vegetative stage II (VS II), pre-flowering stage (PF) and seed filling stage (SF).

Extraction and assay of antioxidative enzymes: All extractions were conducted in three replicates at 4°C. SOD, POX and GR were extracted by homogenizing the leaf samples (1st to 3rd apical leaf) in 0.1 M phosphate buffer (pH7.5) containing 1% polyvinylpyrrolidone (PVP), 1 mM EDTA and 10 mM β-mercaptoethanol. CAT and APX were extracted with 0.05 M phosphate buffer (pH 7.5) containing 1% PVP (Kaur *et al.*, 2009). Both the homogenates were centrifuged at 10,000 g for 20 min and the supernatant was used for assaying above mentioned enzymes. All enzymes were assayed at 30°C. Components of enzyme assay system, except the enzyme were preincubated at 30°C for 20 min before starting the reaction.

Activity of APX was assayed by taking 1 ml of 50 mM sodium phosphate buffer (pH 7.0), 0.8 ml of 0.5 mM ascorbic acid, 0.2 ml of enzyme extract and 1 ml of H₂O₂ solution in total volume of 3 ml (Nakano and Asada, 1987). Absorbance was recorded at 290 nm at an interval of 30 sec upto 3 min. Extinction coefficient of monodehydroascorbic acid was 2.8 mM⁻¹cm⁻¹. APX activity was expressed as μmoles of mono dehydroascorbic acid formed min⁻¹ mg⁻¹ protein FW.

Activity of CAT was determined by taking 1.8 ml of 50 mM sodium phosphate buffer (pH 7.5) to which 0.2 ml of enzyme extract was added. The reaction was initiated by adding 1 ml H₂O₂ and utilization of H₂O₂ was recorded at an interval of 30 sec for 3 min by measuring the decrease in absorbance at 240 nm (Chance and Machly, 1955). Extinction coefficient for H₂O₂ was 0.0394 mM⁻¹cm⁻¹. CAT activity was expressed as μmoles of H₂O₂ decomposed min⁻¹ mg⁻¹ protein FW.

Assay system of SOD contained 1.4 ml of 100 mM Tris HCl buffer (pH 8.2), 0.5 ml of 6 mM EDTA, 1 ml of 6 mM pyrogallol solution and 0.1 ml of enzyme extract (Marklund and Marklund, 1974). Change in absorbance was recorded at 420 nm after an interval of 30 sec upto 3 min. A unit of enzyme activity was expressed as the amount of enzyme causing 50% inhibition of auto-oxidation of pyrogallol observed in

blank.

Assay of GR contained 0.2 ml of 200 mM potassium phosphate buffer (pH 7.5), 0.1 ml $MgCl_2$ (1.5 mM), 0.1 ml EDTA (0.2 mM), 0.2 ml NADPH (0.025 mM), 0.2 ml enzyme extract, followed by 0.2 ml of oxidized glutathione (0.25 mM) in a quartz cuvette (Esterbauer and Grill, 1978). Decrease in absorbance at 340 nm after an interval of 30 sec upto 3 min was recorded. The molar extinction coefficient for NADPH was $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. GR activity was expressed as η moles of $NADP^+$ formed $\text{min}^{-1} \text{ mg}^{-1}$ protein FW.

Assay system of POX contained 3 ml of 0.05 M guaiacol in 100 mM phosphate buffer (pH 6.5), 0.1 ml of enzyme extract and 0.1 ml of 0.8 M H_2O_2 (Shannon *et al.*, 1966). The reaction mixture without H_2O_2 was taken as a blank. The reaction was initiated by adding H_2O_2 and rate of change in absorbance was recorded at 470 nm for 3 min at an interval of 30 sec. POX activity was expressed as change in absorbance $\text{min}^{-1} \text{ mg}^{-1}$ protein FW. Protein content of all enzyme extracts was determined by the method of Lowry *et al.* (1951).

Extraction and estimation of H_2O_2 and Malondialdehyde: H_2O_2 was extracted from 500 mg of leaf tissue with 3 ml of ice cold 10 mM potassium phosphate buffer (pH 7.5) and was estimated according to the procedure of Noreen and Ashraf (2009). Malondialdehyde was extracted from 1.0 g of leaf tissue with 4 ml of 5% (w/v) trichloroacetic acid and the homogenate was centrifuged at 12,000 g for 15 min at room temperature. The supernatant was used for estimation of malondialdehyde (MDA) (Heath and Packer, 1968).

Extraction and estimation of proline and total phenols: Approximately, 100 mg of sample was homogenized with 4 ml of 3% aqueous sulfosalicylic acid and the homogenate was filtered through Whatman filter paper and filtrate was used for proline estimation by the procedure of Bates *et al.* (1973). Total phenols was refluxed with 80% methanol and then volume was made with 80% methanol to 10 ml and total phenols were then estimated (Swain and Hills, 1959).

RESULTS

Superoxide dismutase (SOD): It was observed that SOD increased in PBG 1 under rainfed conditions. In other susceptible *desi* cultivar GPF 2 increase in SOD was observed only at seed filling stage and in PBG 5 reduction in SOD was recorded throughout plant development under rainfed conditions (Fig. 1). On the other hand, under rainfed conditions *kabuli* cultivars showed higher activity at vegetative stage II and pre-flowering stage and reduction at seed filling stage. However, drought did not result in increase in SOD activity in leaves of tolerant (PDG 3 and PDG 4) and GPF 2 cultivars till pre-flowering stage, while a significant increase in SOD activity was observed at seed filling stage when compared to irrigated conditions.

In PBG 1 and *kabuli* (L 550 and BG 1053) cultivars, activity increased at vegetative stage II and pre-flowering stage and thereafter *kabuli* cultivars showed significant decline in activity at seed filling stage (Fig. 1).

PBG 5, GPF 2 and BG 1053 susceptible cultivars were severely affected due to drought stress and decline in mean SOD activity as well as specific activity was observed. When compared with irrigated conditions, 1.5 fold increases in average SOD specific activity was found in PBG 1, and comparable activity was observed in L 550 under rainfed environment. In case of drought stress, tolerant genotypes showed much higher increase in SOD specific activity over the activity seen in leaves of irrigated control plants at all growth stages and throughout the plant development period.

Catalase (CAT): An increase in catalase activity and specific activity was observed in the leaf tissue with the age of plant. In PBG 1 and tolerant (PDG 3 and PDG 4) cultivars, significant increase in CAT activity under rainfed conditions at all the stages as compared to irrigated conditions was observed (Fig. 2). However, in PBG 5 significant increase in CAT activity was observed only upto vegetative stage II and then comparable or lower activity was recorded. Tolerant cultivars PDG 3 and PDG 4, as compared to their activity at pre-flowering stage, had significantly higher activity of about 3 fold and 1.7 fold, respectively, under rainfed condition at seed filling stage. On the other hand, *kabuli* cultivars (L 550 and BG 1053) showed significant 2.2 fold increase in CAT activity at seed filling stage under irrigated conditions (Fig. 2).

The average catalase activity and specific activity under drought stress were significantly higher in drought tolerant PDG 3 and PDG 4 *desi* chickpea cultivars as compared to susceptible cultivars (*Desi*-PBG 1, GPF 2 and PBG 5 and *Kabuli*- L 550 and BG 1053) in comparison to activities observed under irrigated conditions. An average increase in CAT specific activity was found to be 103.7% and 76.2% in tolerant PDG 3 and PDG 4 *desi* chickpea cultivars under rainfed conditions. Out of five susceptible cultivars, in PBG 1 and L 550 have increased average

Table 1. Rainfall data and irrigation schedule during chickpea development.

Treatment	2008-2009	Rainfall (mm)	Total water (mm)
Rainfall	2 Jan 2009	8.6	44.4
	3 Jan 2009	9.8	
	7 Feb 2009	2.0	
	8 Feb 2009	23.0	
	4 Mar 2009	1.0	
Irrigation	21 Dec 2008	75	225
	28 Jan 2009	75	
	5 Mar 2009	75	

* Pre-irrigation of 100 mm was given to all the cultivars irrespective of the treatment.

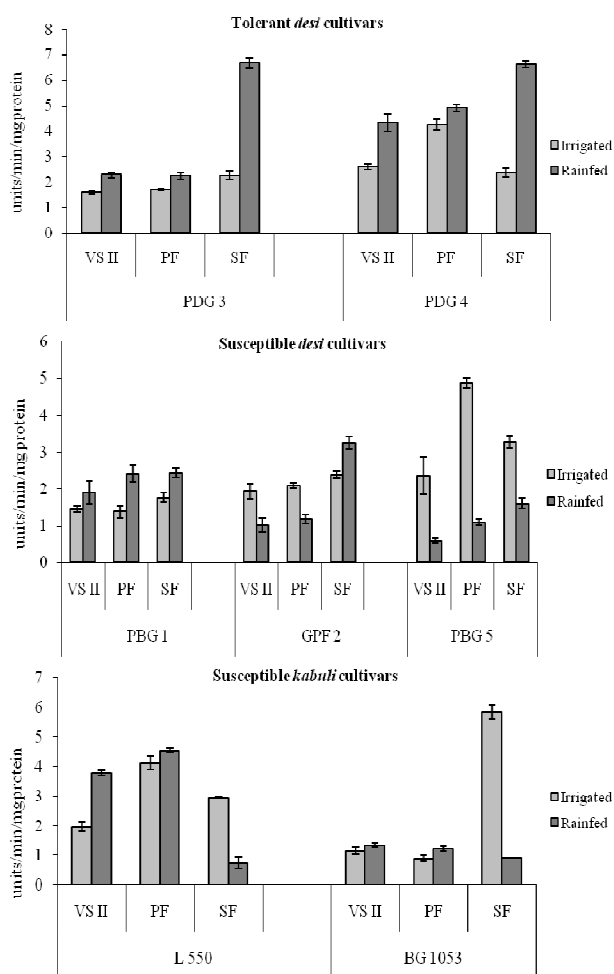


Fig. 1. Superoxide dismutase activity in leaves of *desi* and *kabuli* chickpea under irrigated and rainfed conditions. Vertical bars shows S.D. from mean of three replicates. 'a' represent significance at 1% as compared to control (Student *t*-test). VS II- vegetative stage II, PF- pre-flowering stage, SF- seed filling stage.

catalase activities under rainfed conditions, however, percentage increase in specific activity was relatively less as compared with tolerant cultivars (PDG 3 and PDG 4). Among *kabuli* susceptible cultivars, L 550 leaves showed greater CAT activities as a result of rainfed conditions. Due to drought stress, the percentage increase in average CAT activity in *desi* susceptible cultivars (PBG 1, GPF 2 and PBG 5) was higher compared to *kabuli* susceptible cultivars (L 550 and BG 1053), whereas, it was observed that the *kabuli* cultivars had more average CAT activity under irrigated conditions and comparable under rainfed condition than the *desi* cultivars.

Glutathione reductase (GR): Under rainfed conditions, activity and specific activity of GR increased in leaves with the plant development, peaked at vegetative stage II and thereafter declined towards plant maturity in all *desi* chickpea cultivars, however, in *kabuli* cultivars GR showed peak activity at pre-flowering stage (Fig. 3). Increase in average specific activity of GR under

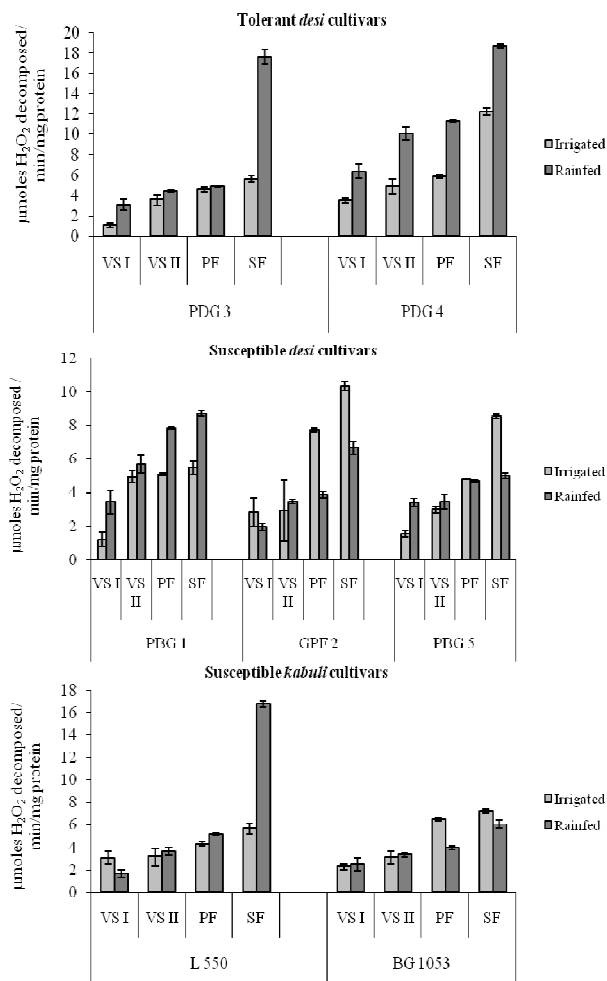


Fig. 2. Catalase activity in leaves of *desi* and *kabuli* chickpea under irrigated and rainfed conditions. Vertical bars shows S.D. from mean of three replicates. 'a' represent significance at 1% as compared to control (Student *t*-test). VS I- vegetative stage I, VS II- vegetative stage II, PF- pre-flowering stage, SF- seed filling stage.

stressed conditions over irrigated samples was observed in tolerant cultivars PDG 3 and PDG 4. This increase was 188% and 117%, respectively, which was significantly more than the susceptible cultivars which in general, with exception to L 550, showed either decline or were comparable in specific activity over the control crop.

Peroxidase (POX): Under both irrigated and rainfed conditions, the chickpea cultivars (tolerant and susceptible) showed significant variation in peroxidase activity and significant variation was seen at all the growth stages and even within the cultivars. Maximum activity of POX was found at vegetative stage II in all the seven cultivars under both stress and normal control conditions and thereafter, the activity declined till maturity (Fig. 4). However, at vegetative stage II and seed filling stage upregulation in POX activity in tolerant cultivars was noticed to be more under rainfed conditions compared to irrigated conditions and the induction in activity was more in tolerant than in

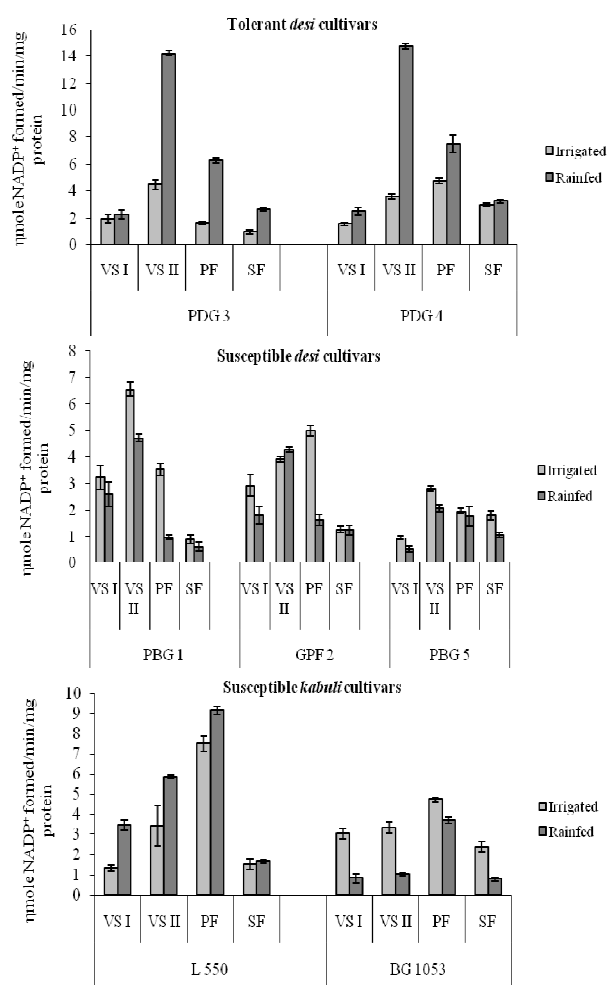


Fig. 3. Glutathione reductase activity in leaves of *desi* and *kabuli* chickpea under irrigated and rainfed conditions. Vertical bars shows S.D. from mean of three replicates. 'a' represent significance at 1% as compared to control (Student *t*-test). VS I- vegetative stage I, VS II- vegetative stage II, PF- pre-flowering stage, SF- seed filling stage.

susceptible at these stages.

Average increase in specific activity of PDG 3 and PDG 4 was 189% and 161%, respectively. On the contrary, in *desi* chickpea cultivars, decline or comparable in specific activity of POX under water deficit conditions was observed. However, under irrigated condition activities of susceptible cultivars (*desi* and *kabuli*) was found to be more compared to activities in leaves of tolerant cultivars. Rainfed condition resulted in significant reduction in activities in leaf tissue of PBG 5. *Kabuli* susceptible cultivar L 550 compared to *desi* susceptible cultivars and *kabuli* susceptible cultivar BG 1053 had higher POX activities under both normal and stressed conditions.

Ascorbate peroxidase (APX): At vegetative stage I, higher activities of ascorbate peroxidase were observed in all the cultivars under both irrigated and rainfed conditions (Fig. 5). APX activity declined thereafter, with growth and finally at seed filling stage APX activity was negligible. However, an increase in APX

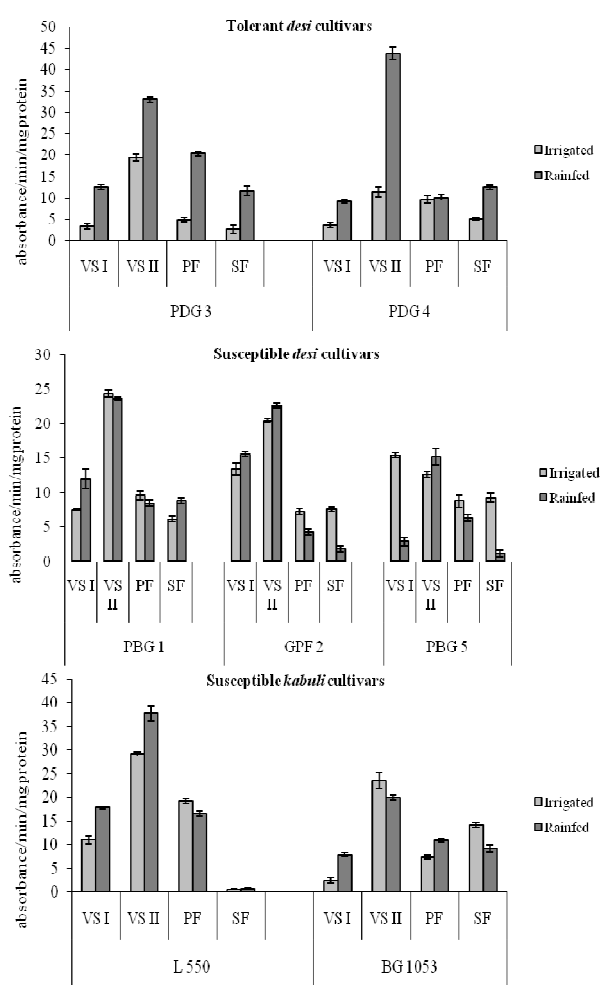


Fig. 4. Peroxidase activity in leaves of *desi* and *kabuli* chickpea under irrigated and rainfed conditions. Vertical bars shows S.D. from mean of three replicates. 'a' represent significance at 1% as compared to control (Student *t*-test). VS I- vegetative stage I, VS II- vegetative stage II, PF- pre-flowering stage, SF- seed filling stage.

activity in PDG 3 and PDG 4 leaves in response to drought stress at all stages of plant growth was observed. Increase in activity as well as specific activity of APX in PDG 3 and PDG 4 at vegetative stage I under rainfed conditions was much more as compared to susceptible cultivars. The drought tolerant genotypes have significant capacity to upregulate APX under drought stress as compared to almost negligible capacity of susceptible cultivars as reflected by mean activity data (Fig. 5).

Hydrogen peroxide (H₂O₂): In general, the content of hydrogen peroxide increased with progress of crop development (Fig. 6). Average H₂O₂ content in leaves of tolerant cultivars PDG 3 and PDG 4 and sensitive cultivars PBG 1 and L 550 was less in stressed leaves. However, major changes in H₂O₂ status occurred between pre-flowering and seed filling stages. At first instance, there appears to be no relationship of H₂O₂ content with drought tolerance. However on close scrutiny, it was observed that H₂O₂ level is less in

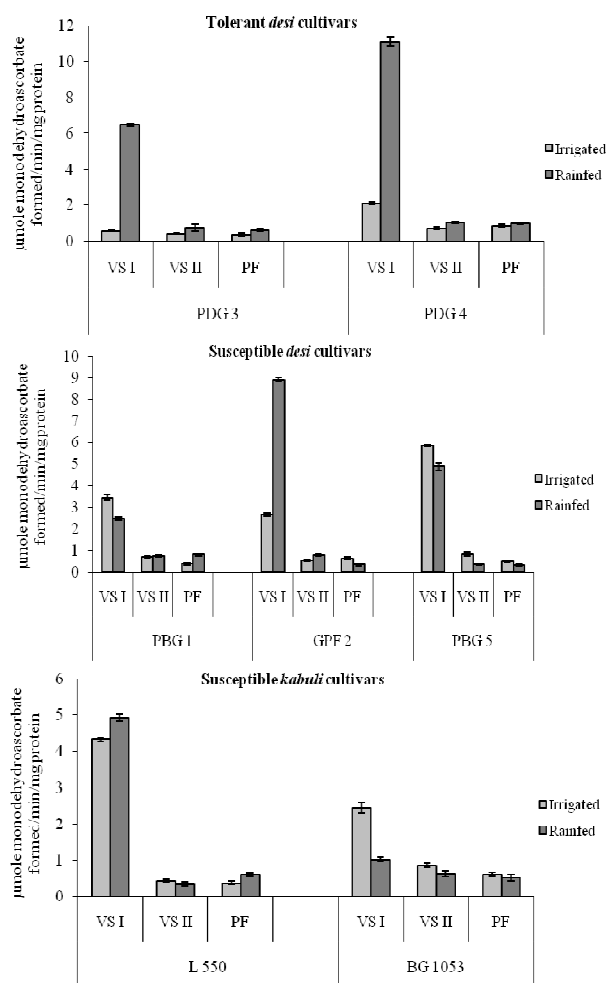


Fig. 5. Ascorbate peroxidase activity in leaves of *desi* and *kabuli* chickpea under irrigated and rainfed conditions. Vertical bars shows S.D. from mean of three replicates. 'a' represent significance at 1% as compared to control (Student *t*-test). VS I- vegetative stage I, VS II- vegetative stage II, PF- pre-flowering stage.

leaves of tolerant cultivars PDG 3 and PDG 4 both at pre-flowering and seed filling stages under rainfed conditions. Such behavior was not observed in sensitive *desi* chickpea cultivars.

Malondialdehyde (MDA): Rainfed induced drought stress may disturb normal ROS balance and promote lipid peroxidation of membranes, either by increased production or decreased scavenging of ROS in the cell. The detrimental effect of drought stress and genotypic variation in drought tolerance could be associated with levels of MDA. Drought stress resulted in increase in MDA content in cultivars (tolerant and susceptible) (Fig. 7). Differences in MDA content were significant at seed filling stage, under irrigated conditions tolerant cultivars PDG 3 and PDG 4 have MDA content between 13-21 $\mu\text{moles g}^{-1}$ FW which is lower than MDA content of 16-42 $\mu\text{moles g}^{-1}$ FW of susceptible cultivars. Under drought stress, MDA content though increased in leaves but at seed filling stage the tolerant cultivars had lower MDA content

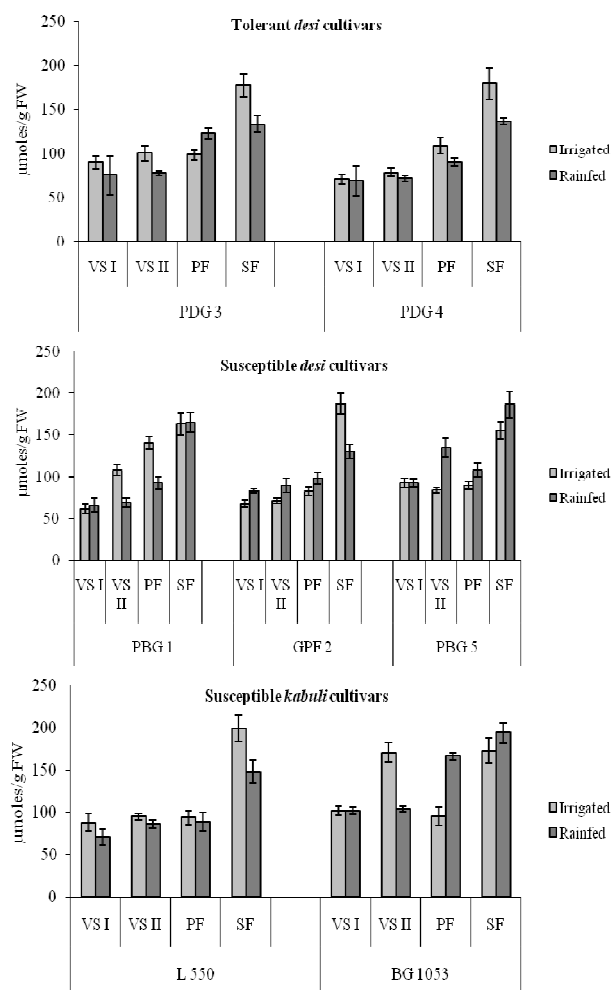


Fig. 6. Hydrogen peroxide content in leaves of *desi* and *kabuli* chickpea under irrigated and rainfed conditions. Vertical bars shows S.D. from mean of three replicates. 'a' represent significance at 1% as compared to control (Student *t*-test). VS I- vegetative stage I, VS II- vegetative stage II, PF- pre-flowering stage, SF- seed filling stage.

than that observed in susceptible cultivars (Fig. 7). Therefore, possibly it can be concluded on the basis of data obtained at seed filling stage the membrane instability, as reflected by lipid peroxidation, was higher in susceptible than in the tolerant chickpea cultivars under both stress and control condition.

Proline (PRO): Cultivar differences in proline content or interaction between cultivars and drought treatment or at different growth stages was observed (Fig. 8). Proline content increased gradually in all cultivars following stress and then decrease was observed at seed filling stage. An increase in proline content in response to drought in chickpea cultivars was seen in all cultivars at all stages compared to irrigated condition except PBG 5 (Fig. 8).

Total phenols: Total phenol accumulation was found to increase with age of plants (vegetative stage I to seed filling stage) in under drought and irrigated conditions (Fig. 9). The data obtained at pre-flowering and seed filling stages showed that tolerant cultivars

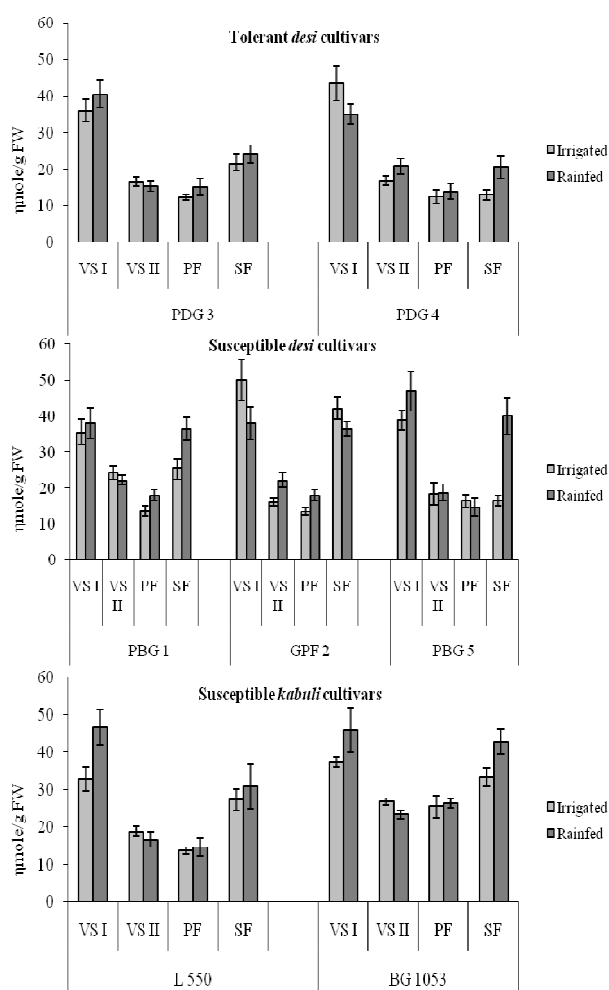


Fig. 7. Malondialdehyde content in leaves of *desi* and *kabuli* chickpea under irrigated and rainfed conditions. Vertical bars shows S.D. from mean of three replicates. 'a' represent significance at 1% as compared to control (Student *t*-test). VS I- vegetative stage I, VS II- vegetative stage II, PF- pre-flowering stage, SF- seed filling stage.

PDG 3 and PDG 4 have higher phenolic content as compared to sensitive cultivars under drought conditions. In general, *kabuli* cultivars have higher phenolic content than susceptible chickpea cultivars under stressed conditions (Fig. 9).

DISCUSSION

Experimental findings on antioxidant system indicate that tolerant and sensitive cultivars responded differently under irrigated and rainfed conditions. Abiotic stresses are known to induce and enhance the expression of antioxidant genes encoding SOD, CAT, GR, POX and APX (Jiang and Zhang, 2002; Unyayar and Cekic, 2005; Gharari *et al.*, 2014). Status of SOD activity modulates the relative amounts of superoxide radicals and hydrogen peroxide, the Haber-Weiss reaction substrates and decreases the risk of OH radical formation, which is highly reactive and may cause severe damage to membranes, protein and DNA (Bowler *et al.*, 1992; Azooz *et al.*, 2009). In this work,

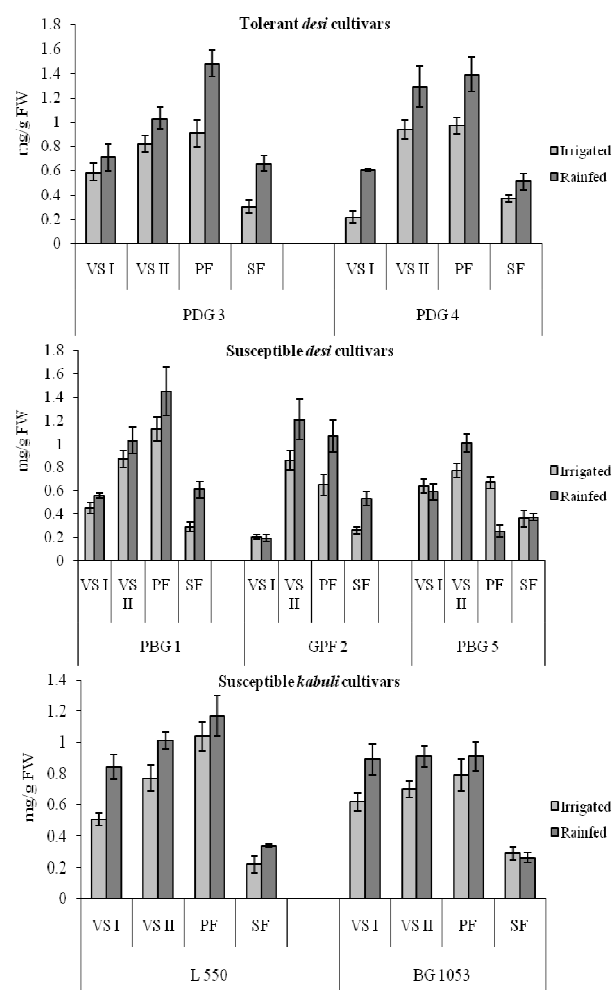


Fig. 8. Proline content in leaves of *desi* and *kabuli* chickpea under irrigated and rainfed conditions. Vertical bars shows S.D. from mean of three replicates. 'a' represent significance at 1% as compared to control (Student *t*-test). VS I- vegetative stage I, VS II- vegetative stage II, PF- pre-flowering stage, SF- seed filling stage.

drought stress increased SOD activity in leaves of tolerant PDG 3 and PDG 4 cultivars followed by susceptible PBG 1 and L 550 cultivars and reduced it in other sensitive ones suggesting that drought tolerant cultivars have a better superoxide radical scavenging ability (Fig. 1). Badawi *et al.* (2004) reported that transgenic tobacco plant over expressing Cu/Zn SOD showed tolerance to salt and water stresses as well as polyethylene glycol induced stress. It has been shown that drought increased SOD activity in drought tolerant maize genotype and decreased in drought sensitive genotype (Moussa and Abdel-Aziz, 2008).

The lower SOD activity at vegetative stages (I and II) and pre-flowering stage under both irrigated and rainfed conditions indicated the lower synthesis of SOD at these stages compared to its activity at seed filling stage in leaves of chickpea plants (Fig. 1). The initial lower SOD activity could improve superoxide scavenging system of cells and favor accumulation of superoxide which mainly contributes in damaging the

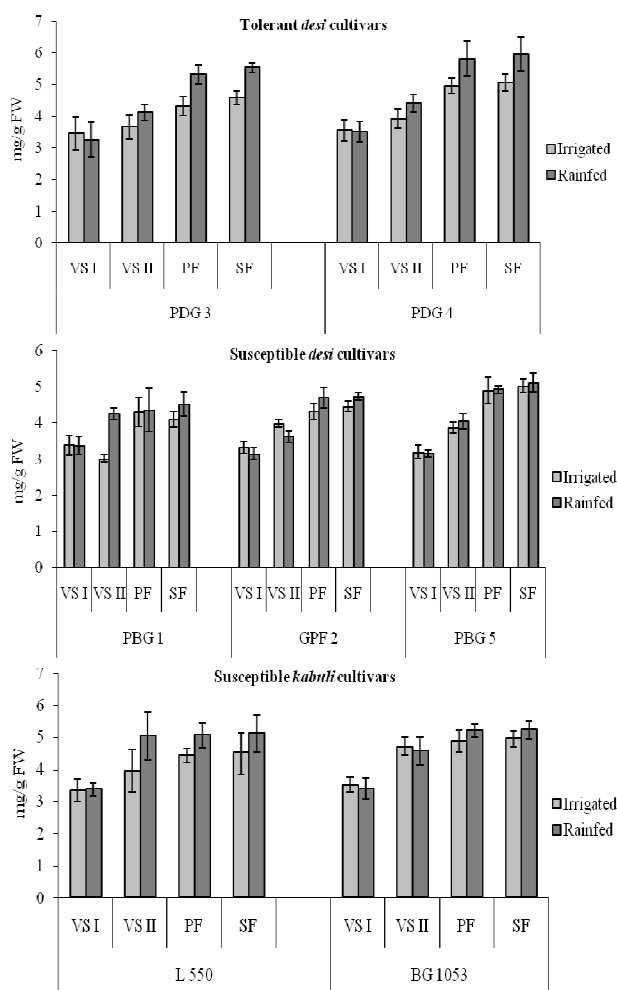


Fig. 9. Total phenols content in leaves of *desi* and *kabuli* chickpea under irrigated and rainfed conditions. Vertical bars shows S.D. from mean of three replicates. 'a' represent significance at 1% as compared to control (Student t-test). VS I- vegetative stage I, VS II- vegetative stage II, PF- pre-flowering stage, SF- seed filling stage.

membrane (Pandey *et al.*, 2010). However, leaves of tolerant cultivars were observed to have comparatively higher activity at vegetative and pre-flowering stages compared to sensitive cultivars and a significant higher induction at the of seed filling can be correlated with better seed development and thereby higher yield. These observations indicate that the leaf machinery in tolerant cultivars is not damaged to the extent as in susceptible cultivars due to oxidative stress and have impact on yield.

Catalase has a significant role in plant defence against drought induced oxidative stress. The main loci of CAT action are known to be the peroxisomes. The activity of this enzyme is sensitive to both drought and heat stresses (Jiang and Hoang, 2001). Decreasing activity of this enzyme in susceptible cultivars may relate to photo inactivation of the enzyme which is a sign for the advent of light stress in the plant that usually cause photo-inhibition of photosystem II, and this condition itself leads to hydrogen peroxide

concentration and damage to cell membrane. Under salt stress, CAT was hypothesized to be the most important among H_2O_2 scavenging enzymes in chickpea (Eyidogan and Oz, 2007). Possibly, increase in catalase activity under rainfed conditions is related to increased level of drought tolerance in drought tolerant cultivars (Fig. 2). The increased activity of CAT at seed filling stage compared to other stages might be due to the enhanced superoxide dismutase activity and the increase in CAT activity might be useful in dismutating hydrogen peroxide that is the key product in reducing senescence under prolonged water stress conditions (Casano *et al.*, 1999).

APX activity is rapidly induced by various stress conditions including drought (Mittler and Zilinskas, 1994), chilling (Prasad *et al.*, 1994) and salt stress (Lopez *et al.*, 1996) and has an important role in stress tolerance. Ascorbate peroxidase plays a vital role in scavenging hydrogen peroxide in water-water and ascorbate-glutathione cycles (Gill and Tuteja, 2010). APX scavenges hydrogen peroxide and uses ascorbate as an electron donor in plants. According to the results of present study, at vegetative stage I APX activity was noticed to be maximum and the activity was relatively lower during other stages under both conditions (Fig. 5). Water stress induced stimulation of APX activity was more pronounced in tolerant cultivars than in sensitive ones. Moreover, the activities of APX in both tolerant cultivars were increased under drought stress by 168% and 256% indicating their possible role in drought tolerance (Fig. 5). Sairam *et al.* (1998) have also observed higher APX activity in tolerant genotypes compared to susceptible ones under water stress. Higher APX activity in drought tolerant cultivars under water deficit conditions might help cultivars in maintaining the required levels of hydrogen peroxide. Glutathione reductase, which catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) is an important endogenous antioxidant (Chalapathi Rao and Reddy, 2008). GR activity was higher in PDG 3 and PDG 4 under control and stress conditions and maximum activity was evident at vegetative stage II and pre-flowering stage (Fig. 3). As a result of rainfed condition, average GR specific activity in leaves of both *kabuli* susceptible cultivars was noticed to be more than *desi* susceptible cultivars. Increase in GR activity has been correlated with increased synthesis of GR protein under stress (Edwards *et al.*, 1993). Edjolo *et al.* (2001) reported greater GR activity in leaves of tolerant poplar genotypes under drought stress than sensitive genotypes. Similar results came from Kellos *et al.* (2008) who reported significantly higher GR activity in leaf tissues of tolerant maize variety than the sensitive variety under ABA induced stress conditions. Due to higher APX activity at vegetative stage I of chickpea cultivars observed under both conditions resulted in accumulation of monodehydroascorbate

and therefore, enhanced GR activity at later vegetative stage II and pre-flowering stage (Fig. 3) may help in maintaining a high ratio of GSH/GSSG which is required for the generation of ascorbate by utilizing monodehydroascorbate and also for activation of several chloroplastic CO₂ fixing enzymes (Asada and Takahashi, 1987). Moreover, regenerated ascorbate might further play a role in non-enzymatic scavenging of superoxide and hydrogen peroxide radicals and protect chickpea leaves from ROS. Thus elevated GR levels may prevent the formation of free radicals in the leaves of tolerant chickpea cultivars under water deficit stress conditions.

Peroxidases are the major scavengers of hydrogen peroxide in chloroplasts and cytosol. They also have important roles in growth, development, lignification and subarization processes (Asada and Takahashi, 1987). So, increasing activity of this enzyme in drought stress perhaps shows the accumulation of hydrogen peroxide in this condition (Csizsar *et al.*, 2005). Therefore, higher POX activity under drought stress in tolerant cultivars and L 550 (susceptible *kabuli* cultivar) could be a sign of an efficient stress response mechanism (Fig. 4). Simova-Stoilova *et al.* (2009) also observed increase in peroxidase activity in flag leaves of four wheat varieties differing in drought tolerance under field conditions under severe drought conditions. They suggested that increase was in accordance with the membrane-stabilizing function of this enzyme. The present investigation also revealed that activity of POX was significantly increased at vegetative stages (I and II) but reduced at pre-flowering and seed filling stages under irrigated and rainfed conditions (Fig. 4). However, higher activity was also observed at seed filling stage in tolerant cultivars under the drought condition compared to control condition and thereby, would be helping in detoxification of hydrogen peroxide produced due to higher SOD activity at this critical stage of chickpea development.

Kalefetoglu - Macar and Ekmekci (2009) reported that proline content in the leaves under drought condition was higher in tolerant chickpea cultivar than in a sensitive cultivar. However, such a generalization could not be observed in present study though percentage increase in the mean proline content of tolerant cultivars with exception to GPF 2 was more than other cultivars (Fig. 8). Increase in leaf proline content with decrease in water supply means that an efficient mechanism for osmotic regulation, stabilizing sub-cellular structures and cellular adaptation to water stress is available (Khamssi *et al.*, 2010). Proline may act both as a metabolic substrate to sustain the needs of rapidly dividing cells and, in turn, as a feedback signal molecule to fine-tune developmental processes such as flower transition. Raut *et al.* (2003) reported that in five drought tolerant chickpea cultivars which when subjected to artificial drought stress showed 30 times

more mean content of proline in stressed plants as compared to control.

Phenolics are the main class of secondary metabolites with important roles in abiotic stress tolerance due to their antioxidant properties (Ruiz and Romero, 2001; Hura *et al.*, 2009). Drought stress caused significant increase in total phenolics in all chickpea cultivars in present study. However, higher accumulation of these solutes in tolerant ones might be the possible reason for their enhanced capacity to resist drought stress (Fig. 9). The accumulation of total phenol in tolerant plants might act synergistically with enzymatic and non-enzymatic antioxidative defence system to confer higher degree of stress tolerance. Wahid and Ghazanfar (2006) observed that enhanced synthesis of soluble phenolics is directly correlated with salt and heat tolerance in sugarcane. Chakraborty and Pardhan (2012) reported increased proline and total phenol contents in leaves of four wheat varieties under drought stress conditions.

One of the reactions that speed up in the presence of ROS is peroxidation of membrane lipids which lead to production of aldehydes like malondialdehyde and other products like ethylene (Liu and Hoang, 2000; Jiang and Huang, 2001). The peroxidation of lipids in the cell membrane is one of the most damaging cellular responses observed in response to drought stress (Thankamani *et al.*, 2003) and the amount of lipid peroxidation is considered to be one of the determinants which indicate the extremity of stress experienced by a plant. In the present investigation, it was observed that although MDA content increased in all chickpea cultivars during drought stress, MDA content in susceptible cultivars was more than that of tolerant cultivars (Fig. 7). Tatar and Gevrek (2008) reported that MDA content in wheat leaves increased with severity of drought stress.

Increased concentrations of hydrogen peroxide, a strong oxidant, and cause localized oxidative damage, disruption of metabolic functions and lipid peroxidation (Zlatev *et al.*, 2006). However, besides being an ROS, hydrogen peroxide is also a signal molecule, which is involved in signal transduction mechanisms for several processes in plants such as stomatal closure, root growth and responses to pathogen challenge (Neill *et al.*, 2002; Laloi *et al.*, 2004; Desikan *et al.*, 2005). Thus, levels of hydrogen peroxide are efficiently controlled to maintain balance between production and breakdown. In this study, although hydrogen peroxide accumulation increased during drought stress in tolerant and susceptible cultivars (Fig. 6), there was a lower hydrogen peroxide levels in tolerant cultivars, indicating greater antioxidant activity and also higher proline and total phenol contents. Kellos *et al.* (2008) reported accumulation of higher H₂O₂ content in leaf tissue of sensitive genotype of maize as compared to tolerant ones under drought stress induced by polyethylene

glycol.

In general, with the age of plant, the activities of superoxide dismutase (SOD) and catalase (CAT) increased but the activities of glutathione reductase (GR), ascorbate peroxidase (APX) and peroxidase (POX) decreased. Both APX and POX are actively involved in detoxification of ROS by breaking down hydrogen peroxide; therefore, it seems that these enzymes have greater role in tolerance during initial drought stress conditions. Chakraborty *et al.* (2002) also reported that POX activities increased initially in leaves of all tea cultivars following drought stress. The relatively low activity of APX and POX during seed filling stage could be because old leaves generally contain low concentration of antioxidants and that is why old leaves are more prone to enhanced oxidative injury than the young leaves. Chickpea is especially vulnerable to soil drought at flowering and seed filling stages and the activities of GR and SOD and CAT at these respective stages significantly increased under drought stress compared with irrigated condition. SOD contributes to later accumulation of hydrogen peroxide and the hydrogen peroxide produced is metabolized by CAT and also by POX. It has also been reported that ABA treatment or drought increased the activity of SOD in mature leaves of *Arabidopsis* (Jung, 2004) and wheat (Keles and Oncel, 2002). Thus, combination of activities of the different enzymes is essential for tolerance. At vegetative stage II and pre-flowering stage, hydrogen peroxide and malondialdehyde were noticed to be lowered compared to other stages, thereby, suggesting GR would be playing major role in providing tolerance against drought conditions in chickpea cultivars.

Conclusion

It was concluded that the changes in the activities of antioxidant enzymes might be due to differential expression of corresponding genes at different stages of growth and might be responsible for providing tolerance to drought tolerant cultivars. Thus, status of antioxidant enzymes could be a very useful tool for depicting drought tolerance of chickpea, which could be useful to plant breeders for developing drought tolerant cultivars. However, further studies are needed to confirm the role of antioxidant enzymes for depicting drought tolerance in a large number of genotypes.

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