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Effect of drought stress and subsequent recovery on protein, carbohydrate contents, catalase and peroxidase activities in three chickpea (*Cicer arietinum*) cultivars

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Abstract

Drought stress is one of the major abiotic stresses in agriculture worldwide. This study was carried out to investigate the effects of drought stress and subsequent recovery on protein, carbohydrate content, catalase (CAT), and peroxidase (POX) activities in three varieties of chickpea (drought tolerant Bivaniej and ILC482 and drought sensitive Pirouz). A field experiment with four irrigation regimes was carried out in a randomized complete block design with three replications. Treatments included control (well-watering), drought stress imposed during the vegetative phase, drought stress imposed during anthesis and drought stress during the vegetative phase and anthesis. Drought stress imposed during vegetative growth or anthesis significantly decreased soluble protein content and increased water soluble carbohydrate concentration. The tolerant variety accumulated more soluble carbohydrate than the sensitive one. Drought stress at flowering stage had significantly higher POX activity compared to than that at vegetative stage. Compared with the stress, there was significantly more soluble protein after exposure to recovery conditions but POX decreased in all three varieties. These results suggest that CAT and POX activities play an essential protective role against drought stress in chickpea. Antioxidants act as a major defense against radical mediated toxicity by protecting the damages caused by free radicals. An increase was observed in POX and CAT activity of three cultivars under stress conditions throughout the experiment. Results showed that POX acts as the major antioxidant enzyme in chickpea leaves under oxidative stress condition. So activity of this enzyme in stress condition can be used as an index for chickpea cultivars tolerance assessment.

Keywords: Carbohydrate; Catalase; chickpea; *Cicer arietinum*; Drought stress; Peroxidase; Protein; Recovery.
Abbreviations: CAT- Catalase; POX- Peroxidase.

Introduction

Chickpea (*Cicer arietinum*) is valued for its nutritive seeds with high protein content, 25.3–28.9%. Among the abiotic stress factors, drought stress problem is relatively important in chickpea (Singh et al., 1994). Drought is the most severe abiotic stress factor limiting plant growth and crop production. When plants are subjected to various abiotic stresses, some reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) are produced. However, under various abiotic stresses the extent of ROS production exceeds the antioxidant defense capability of the cell, resulting in cellular damages (Almeselmani et al., 2006). One of the main reasons why environmental stress inhibits growth and photosynthetic abilities of plants is the breakdown of the balance between the production of reactive oxygen species (ROS) and the antioxidant defense (Iturbe-Ormaetxe et al., 1998) These activated oxygens injure the cellular components of proteins, membrane lipids and nucleic acids (Foyer et al., 1994). To mitigate and repair damage initiated by ROS, plants have developed a complex antioxidant system (del Rio et al., 2002). Water deficit is also known to alter a variety of biochemical and physiological processes ranging from photosynthesis to protein synthesis and solute accumulation (Hu and Schmidhalter 1998). Catalase is localized in the mitochondria, peroxisomes and cytoplasm of higher plants (Bray et al., 2000). It is instrumental in the decomposition of H_20_2, which is produced outside the chloroplasts by the H_20_2 generating oxidases present in the peroxisomes (Tolbert 1971). Catalase is a tetrameric heme protein, occurring in almost all aerobic organisms, and one of the few enzymes showing dual activity: it has hyperoxidase activity (catalytic activity) when it catalyzes the breakdown of hydrogen peroxide into water and oxygen. It also shows peroxidase activity (Luhova et al., 2003). Peroxidase (POX), an iron heme protein, accelerates the reduction of H_2O_2 with a concurrent oxidation of a substrate, mostly located in cell wall; it is also involved in oxidation of phenol compounds as the key enzyme for polymerization towards the synthesis of lignin (Gaspar et al., 1991; Ozdemir et al., 2004). POX is a major enzyme scavenging H_2O_2 in chloroplasts produced through dismutation of O_2^-catalyzed by superoxide dismutase. Several physiological processes are dramatically affected by peroxidase over-production, and severe wilting was found in transgenic plants (Arora et al., 2002). Finally, it has been frequently observed that under drought conditions

carbohydrates often accumulate (Chaves, 1991). The purpose of the present study was to contribute to a better understanding of the physiological responses of chickpea plants to drought stress. We investigated the influence of four types of drought stress on the contents of proteins, catalase (CAT), peroxidase (POX) and carbohydrates in chickpea varieties differing in drought tolerance. We also investigated how plants recovered from the drought stress.

Result and discussion

Soluble protein

Protein soluble decreased significantly in all three varieties at both vegetative stage and flowering stages when they were imposed to drought stress (Table 1). Plants stressed at the vegetative stage, but not stressed subsequently, gave significantly higher protein levels than plants stressed during anthesis or during both the vegetative stage and anthesis. The interactions between variety and drought treatment were not significant. As suggested by earlier workers, protein degradation might be the result of increased activity of protease or other catabolic enzymes, which were activated under drought stress, or due to fragmentation of proteins due to toxic effects of reactive oxygen species resulting in reduced protein content (Davies 1987). A decrease in the protein concentration would be a typical symptom of oxidative stress and has frequently been observed in drought-stressed plants (Seel et al., 1992; Moran et al., 1994).

Carbohydrate

Drought stress increased water soluble carbohydrate (WSC) concentration in all three varieties at flowering (Table 1). Plants usually had the highest carbohydrate levels when grown under drought during both the vegetative phase and during anthesis. Owing to their solubility they may help plants to survive periods of osmotic stress induced by drought. Our results showed that 'Bivaniej' had the highest carbohydrate and 'Pirouz' the lowest water soluble carbohydrate levels (Table 1). The tolerant variety accumulated more soluble carbohydrate than the sensitive one. Results showed that drought stress at vegetative stage did not increase WSC, But severe drought stress during vegetative and flowering phases increased WSC in drought resistance chickpea cultivars significantly. WSC roles as a compatible solute under drought stress and might be a useful marker for selecting more drought tolerant varieties. Changes in carbohydrate, in addition to depending on severity and duration of water deficit, might also reflect genotypic differences in the regulation of carbon metabolism and partitioning at the whole plant level (Praxedes et al., 2005). During the course of drought stresses active solute accumulation of compatible solutes such as carbohydrates is claimed to be an effective stress tolerance mechanism (McKersie and Leshem 1994).

Catalase

Catalase activity (CAT) was measured in three chickpea varieties during vegetative and flowering stages (Table 1). Compared with the control, there was significantly higher CAT activity upon exposure to drought stress in all three varieties during the vegetative stage. 'ILC482' had the highest CAT activity whereas 'Bivaniej' showed the lowest CAT activity (Table 1). No significant differences were observed in 'Pirouz' and 'ILC482'. The enhanced scavenging ability for H_2O_2 in cv. ILC482 inhibited the accumulation of ROS and thus protected the plants from lipid peroxidation of membrane systems and oxidative damages under drought stress. The results showed that under drought stress condition during vegetative and flowering stages in comparison to control treatment, CAT activity increased in Bivaniej and ILC482 drought resistance cultivars significantly.Plants stressed at the vegetative stage, but not stressed subsequently, gave a significantly lower CAT enzyme than plants stressed during anthesis or during both the vegetative stage and anthesis. The interactions between variety and drought treatment were not significant. However increases in catalase activity in response to drought stress suggest a prominent role for this enzyme in the protection of leaf tissue against oxidative damage.There is a fragile balance between ROS production and scavenging that defines the normal steady‐state level of intracellular ROS. Under drought stress this balance suffers an upward shift, ROS production being enhanced due to stomatal closure and the concomitant limitation on CO2 fixation. The avoidance of ROS production during drought stress is also an important strategy that enables plants to cope with water shortage without extensive damage (de Carvalho 2008). In environmental stresses conditions such as drought, high activities of CAT enzymes are important for plants to tolerate stresses. Catalase is essential for the removal of H_2O_2 produced in the peroxisomes by photorespiration (Noctor et al., 2000). Catalase, which degrades H_2O_2 into water and oxygen, is one of the major antioxidant enzymes (Scandalios et al., 1997). AOS are highly reactive and, therefore, harmful. Aerobic organisms would not survive without antioxidant systems that counteract the detrimental effects of free radicals (Devasagayam et al., 2004). Similar results were reported in wheat cultivars (Luna at al. 2004) and in barley (Kublis 2003). On the other hand, different results were found by Türkan et al. (2005) in common bean and by Fu and Huang (2001) in cool-season grasses.

Peroxidase

In order to analyze the changes of POX enzymes in chickpea under drought stress, POX activity was increased significantly in both stages for all drought stress treatments (Table 1). Drought stress imposed at anthesis, had significantly higher POX activity at flowering, whereas restricted water supply during both the vegetative and anthesis stages had a mild effect on this activity. Drought stress imposed during the vegetative only did not influence POX activity at flowering. No differences between tolerant and sensitive chickpea cultivars were observed. The interactions between variety and drought treatment were not significant either. It is widely accepted that AOS are responsible for various stress-induced damages to macromolecules and ultimately to cellular structures. Consequently, the role of antioxidative enzymes, such as POX and CAT becomes very important.

Even under normal growth conditions, many metabolic processes produce ROS in plants, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (^-OH) (Sudhakar et al., 2001). Meanwhile, plants possess efficient antioxidant defense systems for scavenging ROS (Zhu et al.,

Table 1. Drought stress induced changes in POX, CAT, Soluble protein (mg g^{-1} FW) and carbohydrate (mg g^{-1} DW) of three varieties of chickpea

Treatment	Variety	CAT (mg protein min^{-1})		POX (mg protein min^{-1})		Soluble protein (mg g^{-1} FW)		Soluble carbohydrate (mg/g DW)
		Vegetative	Flowering	Vegetative	Flowering	Vegetative	Flowering	flowering
Control	Bivaniej	0.06c	0.34cde	0.27b	0.55cd	0.96a	1.08ab	0.049d
	ILC482	0.11bc	0.32de	0.31b	0.41d	0.92a	1.15a	0.050cd
	Pirouz	0.08bc	0.40cde	0.37b	0.69bcd	0.94a	1.00ab	0.044d
Drought during vegetative stage	Bivaniej	0.14b	0.28e	1.07a	0.64cd	0.44b	0.99ab	0.054bcd
	ILC482	0.21a	0.38cde	0.90a	0.60cd	047b	0.89bc	0.056bcd
	Pirouz	0.18a	0.42bcd	0.73a	0.71bcd	0.52b	0.73cd	0.045d
Drought during anthesis	Bivaniej	-	0.42bcd	-	1.10ab	-	0.46f	0.066bcd
	ILC482	-	0.50abc	-	0.90abc	-	0.61def	0.065bcd
	Pirouz	-	0.49abcd	-	1.19a	-	0.51ef	0.048d
Drought during vegetative phase and during anthesis	Bivaniej	-	0.58ab	-	0.87abc	-	0.69cde	0.100a
	ILC482	-	0.60a	-	0.64cd	-	0.72cde	0.079ab
	Pirouz	-	0.44abcd	-	0.91abc	-	0.68cde	0.065bcd

Data represent the mean values of three replicates. Within a column, mean values followed by different letters are statistically different based on Duncan's range test at $P \leq 0.05$

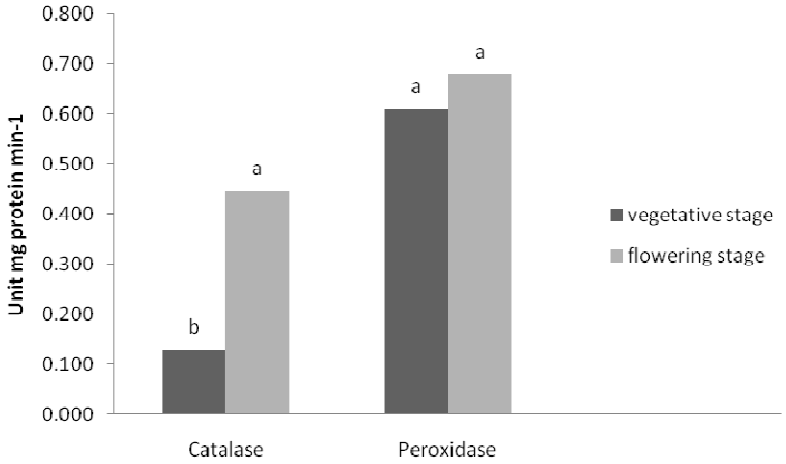

Fig 1. Effect of stage on catalase and peroxidase of three chickpea cultivars grown under control and drought stressed conditions. Values with different letters are statistically significantly different at $P \leq 0.05$.

2004). CAT and POX are the major antioxidant enzymes. Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to this oxidative damage (Sudhakar et al., 2001). Increase in CAT and POX activity is supposed to be an adaptive trait possibly helping to overcome the damage to the tissue metabolism by reducing toxic levels of H_2O_2 produced during cell metabolism and protection against oxidative stress (Sudhakar et al., 2001).

Growth Stage

Our results demonstrated that the CAT activities were significantly increased at flowering. In contrast to CAT, the constitutive level of POX activities remained unchanged in comparison with the vegetative stage. Our found an increase of catalase activity in chickpea leaves with an increase in plant age. However at flowering of the chickpea CAT activity was about 3.4 times higher than at the vegetative stage (Fig. 1). These differences might be related to plant age but might also be contributed to the higher temperature at flowering than during the vegetative stage. At flowering, POX activity was about 15 times higher than CAT activity. The high activity of POX may reflect the important antioxidant role of POX in chickpea leaves under stress condition. Under drought conditions, production of ROS depends on species, intensity of stress, duration of stress and developmental stage.

Recovery

Table 2 shows the recovery of CAT, POD and Protein by re-watering after 40 days without watering. After 20 days of exposure to re-watering POX was lower than during the stress. The oxidative damage to cellular components is limited under normal growing conditions due to efficient processing of ROS through a well coordinated and rapidly responsive antioxidant system consisting of several enzymes. Compared with stress treatments, the activities of CAT after recovery were clearly increased (Table 2). Increased levels of CAT activity in plants during recovery suggest that the re-watered plants suffered an oxidative stress. Compared with the stress, there was significantly higher protein soluble upon exposure to recovery in all three varieties. Highest protein under recovery was observed in cv. Bivaniej. pirouz drought sensitive cultivar showed the lowest protein content after recovery. Lower protein content of this sensitive cultivar may be the result of damaged biochemical process under drought stress.

Material and methods

Plant materials

The research was carried out with three chickpea (*Cicer arietinum* L.) varieties contrasting in crop cycle duration, type (desi or kabuli), growth habit, and response to drought: Bivaniej (kabuli), ILC482 (kabuli) and Pirouz (desi). The first two are considered relatively drought tolerant, the latter is drought sensitive (mafakheri et al., 2010). Seeds of these varieties were obtained from the International Centre for Agricultural Research in the Kurdistan of Iran.

Treatments

The experiment was carried out in a field of the Kurdistan University (47°1' N and 35°16' E, 1375 m above sea level) in Iran in 2008. The soil type was a sandy loam (pH until a depth of 30 cm was 7.6). The experiment was of a split-plot block design with three replications. The factors were variety (see above) and drought treatment. To realize the drought treatments, plants were subjected to one of the following four irrigation regimes:

1. Control; a well irrigated treatment (control).
2. Drought stress imposed during the vegetative stage by withholding irrigation and re-watering at and after flowering (early water shortage).
3. Drought stress imposed during anthesis by withholding irrigation (late water shortage).
4. Drought stress imposed at both the vegetative and the anthesis stage by withholding irrigation (early and late water shortage).

Individual plots were 6 rows (with a row distance of 0.30 m) of 6 m long. Plant distance within a row was 0.13 m. Plots were irrigated once immediately after sowing to ensure uniform emergence. Thereafter, plants were watered with tap water about once a week depending on treatment. The plots were kept weed free by hand weeding. Surface application and incorporation of 18 kg N ha^{-1} and 20 kg P ha^{-1} was carried out at the onset of the experiment. Seeds were inoculated with fungicide protection before sowing.

Biochemical analysis

Assessing total soluble protein content

Leaves were homogenized in ice-cold extraction buffer (50 mM potassium phosphate, pH 7.4, 1 mM EDTA). The extracts were centrifuged at 15,000×*g* for 20 min, and the resulting supernatants were used for estimation of soluble protein contents. Protein contents were assayed following Bradford's method (1976) with a standard curve prepared using bovine serum albumin.

Antioxidant enzymes assay

Frozen leaf samples were ground in liquid nitrogen and homogenized in 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA and 5 mM mercaptoethanol and 4% (w/v) polyvinylpyrrolidine-40 (PVP-40). The homogenate was centrifuged at 20,000×*g* for 30 min at 4ºC. The supernatant was used to assess antioxidant enzymes (CAT, POX).

Catalase

CAT activity was assayed by the method described by Aebi (1984). A reaction mixture consisting of 666 µL supernatant and 334 µl of 73 mM H_2O_2 (Fisher Scientific) was then assayed for 3 min at 240 nm. Activity was measured as disappearance of H_2O_2. One unit of enzyme activity was defined as a decrease in absorbance of 1 min at 240 nm.

Peroxidase

Peroxidase activity (POX; EC 1.11.1.7) was based upon the method as described by Herzog and Fahimi (1973) which measures the increase in absorbance at 470 nm. The reaction mixture contained DAB solution and 0.6% H_2O_2. The increase in A_{470} was followed for 3 min. One enzyme unit was defined as µmol mL^{-1} destroyed H_2O_2 per min. The specific enzyme activity for all enzymes was expressed as in unit mg^{-1} protein.

Table 2. Drought stress induced changes in POD, CAT and soluble protein soluble of three varieties of chickpea

Treatment	Variety	Soluble protein (mg g^{-1} FW)	Catalase (mg protein min^{-1})	Peroxidase (mg protein min^{-1})
Stress	Bivaniej	0.44c	0.133c	1.074a
	ILC482	0.47c	0.203bc	0.902ab
	Pirouz	0.51c	0.183bc	0.730b
Recovery*	Bivaniej	0.99a	0.273b	0.644b
	ILC482	0.89a	0.383a	0.604b
	Pirouz	0.73b	0.420a	0.706b

Data represent the mean values of three replicates. Within a column, mean values followed by different letters are statistically different based on Duncan's range test at $P \leq 0.05$

*recovery: following the drought stress treatment plants were re-watered and maintained for 20 days.

Water-soluble carbohydrate

Water-soluble carbohydrate content was determined in lyophilized plant material. Samples of 40 mg dry weight were extracted. The extracts were then boiled for 20 min and centrifuged for 5 min at 20,000 ×*g* to pellet insoluble material. The supernatant was removed and the pellet was extracted twice as above. Total water soluble carbohydrate determination was based on the phenol-sulfuric-acid method (Dubois et al., 1956), which measures the increase in absorbance at 535 nm.

Statistical analysis

Data were subjected to analysis of variance (*ANOVA*), and means were compared using Duncan's range test at $P \leq 0.05$. All calculations were performed using SAS software, version 9.1.

Conclusion

Reactive oxygen species (ROS) play an important role in oxidative stress related to the drought stress. Antioxidants act as a major defense against radical mediated toxicity by protecting the damages caused by free radicals. An increase was observed in POX and CAT activity of three cultivars under stress conditions throughout the experiment. A combination of characteristics like higher antioxidant activity will result in lower oxidative stress. The concentrations of water soluble carbohydrate were higher in drought stress during both the vegetative and anthesis stages. It can be concluded that severe drought stress increased WSC in drought resistance chickpea cultivars. CAT activity increased in drought resistance cultivars under drought stress, however, POX acts as the major antioxidant enzyme in chickpea leaves.

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