ORIGINAL ARTICLE

The Responses of Ascorbate - Glutathione Cycle Enzymes in Seedlings of *Pancratium maritimum* L. under Drought Treatments

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In this study, physiological and biochemical responses of (*Pancratium maritimum* L.), desert plant which is very widespread on coastal sand dunes to drought were determined. Therefore 28 days (d) old plants were drought stressed by withholding water for 5 and 10 days. The changes in relative growth rate (RGR), relative water content (RWC) lipid peroxidation, and ascorbate-glutathione cycle enzymes activity ((ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2) dehydroascorbate reductase (DHAR, EC 1.8.5.1) and monodehydroascorbate reductase (MDAR, EC 1.6.5.4)) were investigated. Relative growth rate, relative water content were both decreased on the 5 and 10d of stress treatment while it was higher on the 10d. MDA content increased on the 10d while it did not change on the 5d. On the other hand, activities of APX, GR, DHAR and MDAR increased on the 5d but were not change on the 10d. These results suggest that ascorbate – glutathione cycle enzymes were efficient to prevent from oxidative damage under short term of drought stress in (*Pancratium maritimum* L.) plants.

Key words: Pancratium maritimum, drought stress, ascorbate- glutathione cycle

Drought is one of the most important abiotic stress which adversely affects crop growth and yield. It is characterized by reduction in water content, diminished leaf water potential and turgor, causes stomatal closure and decrease in cell enlargement and growth. Water stress caused by drought may result in the arrest of photosynthesis, disturbance of metabolism and finally the death of plant (Jaleel *et al.* 2008). Each of these processes involves a large number of genes, enzymes, hormones and metabolites (Tardieu *et al.* 2010). Understanding plant responses to drought is of

great importance and also a fundamental part for making the crops stress tolerant (Zhao *et al*. 2008).

Drought stress can lead to oxidative stress through the increase in reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^·$), which are highly reactive and may cause cellular damage through oxidation of lipids, proteins and nucleic acids (Pastori and Foyer, 2002). There are many studies that report an increased ROS accumulation and oxidative stress under drought stress (Sgherri *et al.* 1995). When plants exposure to drought stress, ROS production is enhanced through multiple ways. For instance, the limitation on CO_2 fixation will reduce $NADP^+$ regeneration through the Calvin cycle, hence provoking an over reduction of the photosynthetic electron transport chain. In fact, during photosynthesis and under drought stress there is a higher leakage of electrons to O_2 by the Mehler reaction. Also photorespiratory pathway under drought stress is enhanced, especially when RuBP oxygenation is maximal due to limitation on CO_2 fixation. The predominance of photorespiration on the oxidative load under drought stress has been recently put forward. Photorespiration is likely to account for over 70% of total H_2O_2 production under drought stress conditions (Noctor *et al.* 2002).

The accelerated generation of reactive oxygen species under drought stress leads to induction of ROS scavenging enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and peroxidase (POX, EC 1.11.1.7). Ascorbate peroxidase (APX, EC 1.11.1.11) glutathione reductase (GR, 1.8.1.7), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and monodehydroascorbate, reductase (MDAR, EC 1.6.5.4), which are in ascorbate **-** glutathione cycle.

A correlation between the antioxidant capacity and drought tolerance has been found in different plant species, including wheat, maize and rice (Lascano *et al.* 2001; Jiang and Zhang 2002; Guo *et al.* 2006). In recent years, ensure the productivity of agricultural regions under drought stress has became an important issue. So select tolerance species and identify their responses to stress conditions and clarifying the antioxidant mechanism is an efficient way to generate genetically transformed tolerant species.

Pancratium maritimum L. is an Amaryllidaceous typical species of the sandy coasts of the Mediterranean, Atlantic, Black and Caspian seas (Dothan, 1986). This plant has been reported to grow on coastal sand dunes and is thus suggested to be drought tolerant and at least relatively salt tolerant (Grassi *et al.* 2005). In a study which belongs to the vegetation of Sinop Peninsula, it is detected with *Ammophila arenaria subs. Arundinacea- Elymus elongatus subsp. Elongatus, Otanthus maritimus –Eryngium maritimum, Cionura erecta* plant group (Kılınç and Karaer, 1995). It is severely threatened in its original range due urbanization and tourism development. In literature, there are a few studies on its genetic diversity and or the vegetation in a specific area like Northern Tyrrhenian Sea, Black Sea and Tunisia (Kılınç and Karaer, 1995; Grassi *et al.* 2005; Sana and Fathel, 2010). In recent years, low MDA content under moderate stress in *P. maritimum* with efficient catalase and peroxidase acitivity were determined although strong upregulation of photorespiration rate (Abogadallah, 2011). But there is no study on ascorbate-glutathione cycle enzymes under stress in *P.maritimum* seedlings. In the light of this information, the aim of the present study was to determine the differences in relative

growth rate, water content, lipit peroxidation and to see how changes the activity of ascorbate-glutathione cycle enzymes under drought conditions.

MATERIALS AND METHODS

Seeds of *P.maritimum* L. were collected from the wild from Akliman area in Sinop is located at the most northern point of the Black Sea Region in the north of Turkey. Seeds were shaked in warm water in an hour before germination. Than the seeds were sown in plastic trays (6cm x 12cm), containing sand and soil mixture (2:1:1 compost: sand: vermiculite). Water was added every day under dark conditions until the seeds germinate. After germination, plants were grown in a growth room, at 25 °C, 16h day/8h night photoperiod, light intensity of 500 µmol $m^{-2}s^{-1}$ and for 14 days with an interval of 2 days in a full strenght Hoagland solution.

For drought experiment, four weeks old *Pancratium* seedlings were divided in two groups. Control groups were watered with Hoagland solution every 3 day and water was not given to the drought groups. Seedlings were harvested on 5th and 10th day and stored at – 80 °C for further analysis.

The fresh weights (FW) of seedlings were determined. The samples were dried in a forced draft oven at 70 ˚C for 72 h and then dry weights (DW) were determined. The relative growth rate (RGR) of seedling was calculated from the dry mass data taken at initial and final harvests, using the formula given by (Venus and Causton, 1979). The relative water content (RWC) was calculated by (Smart and Bingham, 1974). After harvest on 5d and 10d of drought treatment, shoots were obtained from plants for each species and their FW was determined. The seedlings were floated on de-ionised water for 5h under low irradiance and then the turgid tissue was quickly blotted to remove excess water and their turgid weights (TW) were determined. DW was determined after seedlings were dried in the oven.

The level of lipid peroxidation in leaf samples was determined in terms of malondialdehyde (MDA) content according to the method of (Madhava and Sresty, 2000). Content of MDA, which is an end product of lipid peroxidation, was determined using the thiobarbituric acid reaction. MDA concentration was calculated from the absorbance at 532 nm and measurements were corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 $mM^{-1} cm^{-1}$.

For protein and enzyme extractions, 0.5 g of fresh leaf samples were homogenized in 1,5 ml of 50 mM sodium phosphate buffer (pH 7.8) containing 1mM ethylenediaminetetraacetic acid (EDTA).Na_2 and 2% (w/v) polyvinylpolypyrrolidone (PVPP). All operations were performed at 4 ˚C. For APX activity determination, 2 mM ascorbate was added into homogenization buffer. Samples were centrifuged at 14,000×g for 30 min, and supernatants were used for the determination of protein content and enzyme activities. All spectrophotometric analyses were conducted on a (Shimadzu) UV visible spectrophotometer.

APX (EC 1.11.1.11) activity was measured according to (Nakano and Asada, 1981). The assay depends on the decrease in absorbance at 290nm as ascorbate was oxidized. The reaction mixture contained 50 mM Na-phosphate buffer (pH 7.0), 50 mM ascorbate, 0.1 mM EDTA.Na_2, 1.2 mM H_2O_2 and 0.1 ml of enzyme extract in a final assay volume of 1 ml. The concentration of oxidized ascorbate was

calculated by using extinction coefficient of 2.8 mM^{-1} cm^{-1}. One unit of APX was defined as 1mmol ml^{-1} ascorbate oxidized min^{-1}.

GR (EC 1.6.4.2) activity was measured according to (Foyer and Halliwell, 1976). The assay medium contained 25 mM Na.phosphate buffer (pH 7.8), 0.5 mM GSSG, 0.12 mM $NADPH.Na_4$ and 0.1 ml enzyme extract in a final assay volume of 1 ml. NADPH oxidation was followed at 340 nm. Activity was calculated using the extinction coefficient of NADPH (6.2 mM^{-1} cm^{-1}). One unit of GR was defined as 1mmol ml^{-1} GSSG reduced min^{-1}. The specific enzyme activity for all enzymes was expressed as in unit mg^{-1} protein.

For the DHAR assay, a reaction mixture containing phosphate buffer (pH 7.0) 0.7 ml, reduced glutathione (GSH) 20 mmol/L 0.1 ml in the phosphate buffer (pH 7.0), 2 mmol/l DHA 0.1 ml, and crude enzyme 0.1 ml was used. DHA was freshly prepared and kept on ice until it was added to the reaction mixture in the cuvette to prevent its fast oxidation at room temperature. The reduction of DHA to ASA was monitored by the increase in absorbance at 290 nm, taking 2.8 $(mmol/l)^{-1}$ cm^{-1} as the absorbance coefficient (Krivosheeva *et al.* 1996). For the MDAR assay, the reaction mixture containing 0.9 mL of 2 mmol/l ASA in phosphate buffer (pH 7.0), 0.04 ml of ascorbate oxidase (2 units) in phosphate buffer (pH 5.6), 0.03 ml of 2 mmol/l NADPH in phosphate buffer (pH 7.6), and 0.03 ml crude enzyme was used. The consumption of NADPH was monitored by the reduction of absorbance at 340 nm taking 6.2 $(mmol/l)^{-1}$ cm^{-1} as the absorbance coefficient (Krivosheeva *et al.* 1996).

Statistical analysis

All analyses were carried out on a completely randomized design. All data obtained were subjected to non parametric test Kruskal Wallis. Each data point was the mean of six replicates (n=6) and comparisons with p values <0.05 were considered significantly different.

RESULTS

In our experiment, drought stress reduced the growth of *P. maritimum* seedlings. Fresh and dry weight of this plant were reduced on the 5 and 10d of stress treatment. Fresh weight was decreased by 13.7 % and 26.6 % according to control group. Similarly dry weight was decreased by 9.85 % and 32.7 % on the 5 and 10d of drought stress as compared to control groups respectively. As a result, the highest inhibiton was on the 10d of drought treatment.

Relative growth rate (RGR) was decreased on the 5 and 10d of drought stress by 25.21 % 33.89 % according to control groups (Table 1). This result is parallel to dry weight reduction in the seedling of *P.maritimum*. Similar to RGR, the greatest reduction in relative water content was observed on the 10d of drought stress by 14.8 % while it was 8.5 % on the 5d.

Protein content was both increased on the 5 and 10d of drought stress according to control groups. Moreover the highest increase was on the 10d of drought stress by 31.8 % (Table 2).

The level of damage related to oxidative stress was determined by monitoring the differences in lipid peroxidation referring malondialdehyde (MDA) formation (Table 2). MDA content was not change on the 5d and changed by 68.40 % on the 10d of stress treatments as compared to control groups.

APX activity was enhanced in *P.maritimum* by 17,77 % on the 5d and was not change on the 10d (Fig. 1a). Similarly GR activity was also increased by

9.85 % on the 5d as compared to control groups but was not change on the 10d (Fig.1b).

In parallel to results of GR enzyme, DHAR and MDAR activities were enhanched only on the 5d of stress treatments by 19.2 % and 8.97 % according to control groups respectively. There was no change on the 10d (Fig. 1c, 1d).

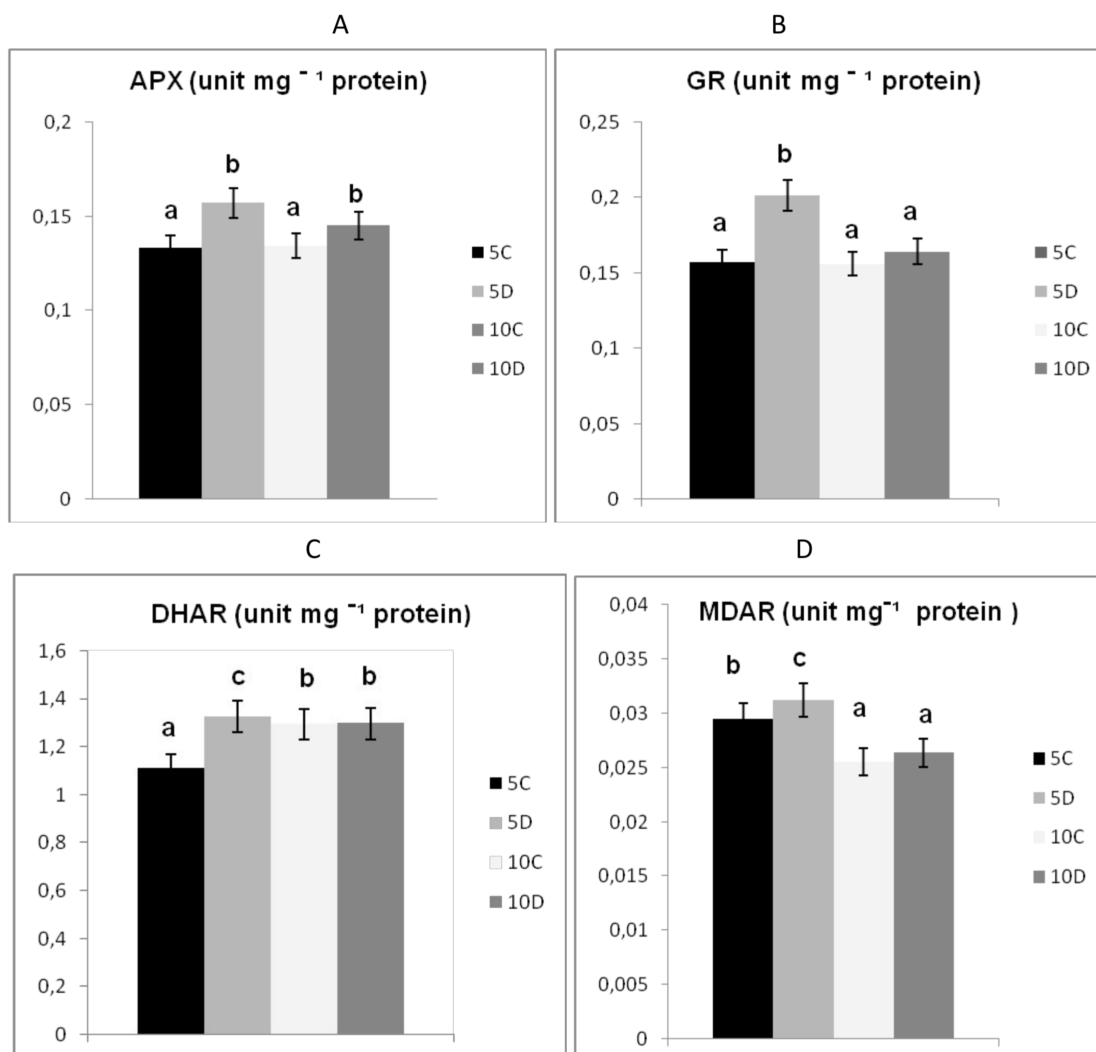

Figure 1: Changes in A (APX), B (GR), C (DHAR), D (MDAR) activities in seedlings of *P. maritimum* under drought stress (on 5d and 10d). The different letters are significantly different ($p < 0.05$) values. 5C: 5d control; 5D: 5d drought stress; 10C: 10d control; 10D: 10d drought stress.

Table 1. Changes in relative water content (%), relative growth rate (mg $mg^{-1}d^{-1}$) in seedlings of *P. maritimum* under drought stress (on 5d and 10d). The different letters are significantly different ($p < 0.05$) values. 5C: 5d control; 5D: 5d drought stress; 10C: 10d control; 10D: 10 d drought stress.

Groups	Relative water content (%)	Relative growth rate (mg $mg^{-1}d^{-1}$)
Control (5C)	82,36 ^{A}	0,119 ^{A}
Drought stress (5D)	75,12 ^{C}	0,089 ^{C}
Control (10C)	81,27 ^{A}	0,118 ^{A}
Drought stress (10D)	69,93 ^{B}	0,078 ^{B}

Table 2 Changes in protein content (mg/ml), MDA content (µmol g^{-1}) in seedlings of *P. maritimum* under drought stress (on 5d and 10d). The different letters are significantly different ($p < 0.05$) values. 5C: 5d control; 5D: 5d drought stress; 10C: 10d control; 10D: 10d drought stress.

Groups	Protein content (mg/ml)	MDA content (µmol g^{-1})
Control (5C)	1,144 [A]	5.04 [A]
Drought stress (5D)	1,282 [A]	5.08 [A]
Control (10C)	1,151 [A]	5.95 [A]
Drought stress (10D)	1,518 [B]	10.02 [C]

DISCUSSION

The ascorbate-glutathione cycle, an efficient antioxidant system in the detoxification of H_2O_2, involves four enzymes: APX, GR, DHAR and MDAR (Asada, 1993). The cycle maintains a ratio of a reduced per oxidized ascorbic acid and glutathione for proper scavenging reactive oxygen species (ROS) in plant cells (Mittler, 2002). In recent years, there are many studies in plants on the antioxidant enzymes under stress conditions. The measure of specific antioxidant enzyme activities during stress treatments has been generally accepted as an approach to assess the involvement of the scavenging system during stress (Carvalho, 2008). It can differ according to intensity of the stress treament and plant age or tolerance. *P. maritimum* has been reported to grow on coastal sand dunes and is thus suggested to be drought tolerant an at least relatively salt tolerant (Grassi *et al.* 2005). Also there is a few study on this plant about its tolerance mechanisms of environmental stress like drought and salinity (Krivosheeva, 1996; Abogadallah, 2011). To the best of our knowledge, this is the first study investigating the cahanges in ascorbate-glutathione cycle enzymes under stress conditions in the seedlings of *P.maritimum*. Therefore ascorbate-glutathione cycle enzymes activities were determined of this plant in the present study.

It's well known that drought stress has negative effects on plant growth. In our experiment, the growth was reduced in *Pancratium* seedlings on the 5 and 10d of drought treatments. The fresh and dry weight and also relative growth rate (RGR) of *Pancratium* seedlings were inhibited on the 5 and 10d of stress treatments. But the inhibition was more remarkable on the 10d. In agreement with our results, (Schubert *et al.* 2005; França *et al.* 2005) reported that drought stress inhibited the growth of alfafa and bean cultivars respectively. Inhibition of growth is related with decreasing water content in plants under stress conditions (Mittler *et al.* 2001). In parallel with this information, the relative water content was inhibited on the 5 and more significiant on the 10d in *Pancratium* seedlings. Similarly, (Ping *et al.* 2006; Hernandez *et al.* 2000) reported that under drought stress, relative water content (RWC) of maize and wheat cultivars were decreased respectively. As Khedr *et al*. (2003) mentioned, exogenous proline was alleviated the growth reduction in *Pancratium* seedlings by increasing the water content in the cells under salt stress (Khedr *et al*. 2003). Similarly, because of the water deficit, seedlings can not grow well under drought conditions and show less development. When the signal reaches the leaves of plant under drought stress, stomatal closure occurs to keep the water content. As a result of this, limitation on CO_2 fixation will reduce NADP regeneration through the Calvin cycle, hence provoking an over reduction of the photosynthesis

electron transport chain (Hernandez *et al.* 2003). Beside this, photorespiratory pathway is also enhanched, especially when RuBP oxygenation is maximal due to limitation on CO_2 fixation. Morever mitochondrial electron transport chain is also responsible for ROS generation under normal conditions, although to a lesser extent than chloroplasts and peroxisomes in the light. Production of reactive oxygen species (ROS) by mitochondria and chloroplast cause damage to membranes, proteins and lipids. As a result of this damage, lipid peroxidation product MDA, is an indicator of the prevalence of free radical reaction in tissues (Halliwell and Gutterdge, 1989).

In the present study, malondialdehyde content was increased on the 10d of drought treatment but did not change on the 5d. But it was well marked by the exposure to 10d of drought treatment. Similarly (Abogadallah, 2011) reported that levels of MDA were highest under severe drought stress in *Pancratium* seedlings. In agreement with our result, (Bian and Jiang 2009; Sharma and Dubey, 2005) reported that malondialdehyde content was increased in the root of (*Poa protensis* L.) and in rice seedlings under drought conditions respectively. As a result, it can be said that 10d of drought stress increased oxidative damage in this plant by increasing the MDA content by 68.40 %. Also the highest increase in the protein content on the 10d can be related with this oxidative damage.

APX plays a more crucial role in the management of ROS in higher plants during stress due to its higher affinity for H_2O_2 than CAT. In our experiment, APX activity was increased only on the 10d of stress treatment. Similarly, it was shown that APX activites were also enhanced during drought stress in cotton and wheat (Ratnayaka *et al.* 2003; Keles and Oncel, 2003).

GR also plays a key role in oxidative stress by converting the oxidized glutathione, GSSG to GSH maintaining a high GSH/GSSG ratio (Alscher *et al.* 2003). Increased GR activity in maize, wheat and poplar has been reported to be related with drought tolerance of these plants. In the present study, GR activity enhanched on the 5d but did not change on the 10d. This result suggests that APX and GR activity were not efficient to protect *Pancratium* seedlings from oxidative damage on the 10d of stress treatment by ascorbate-glutathione cycle.

In ascorbate-glutathione cycle, monodehydroascorbate is regenerated by NADPH in a reaction catalyzed by MDAR and DHAR catalysis the reduction of DHA to AsA by oxidizing GSH. In our experiment, similar to GR enzyme, MDAR and DHAR activities were induced on the 5d but were not change on the 10d of stress treatment. In aggrement with our results (Sharma and Dubey, 2005; Yua-Hua, 2011) reported that MDAR and DHAR activities increased under drought stress in rice seedlings and apple leaves respectively. It's well known that APX enzyme uses ascorbic acid and oxidizes it to monodehydroascorbate. Dehydroascorbate and monodehydroascorbate will then be reduced to regenerate the ascorbate pool (Ramachandra *et al.* 2004). In our results, it can be suggested that this ascorbate regeneration in that cycle was not enough on the 10d of drought stress for the APX, GR, DHAR and MDAR activities.

In this study, the role of enzymes of ascorbate-glutathione cycle in mitigating oxidative damage and some physiological parameters under drought stress in *Pancratium* seedlings were examined. Results of this experiments clearly indicate enhanced activities of all the enzymes of ascorbate-glutathione cycle, signifying a potential role of

these enzymes in providing antioxidative defense under drought stress in *Pancratium* seedlings only on the 5d but not on the 10d of drought treatment by keeping in a certain level of MDA. More biochemical and molecular studies are needed in *Pancratium* seedlings under drought stress to clarify the drought tolerance of this desert plant.

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