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ANTIPEROXIDATIVE ENZYMES IN RETAMA AND THEIR SEASONAL VARIATION

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The antiperoxidative enzymatic activities of ascorbate peroxidase, dehydroascorbate reductase, glutathione reductase and catalase and the content of the antioxidants ascorbate and glutathione were followed in the legume *Retama (Retama reatem)* in the desert. Antiperoxidative enzymatic activities and antioxidants content were related to seasonal variations in irradiance and precipitation.

Retama was found to possess a very efficient removal mechanism for hydrogen peroxide as was shown by the high catalase activity and the high affinity of the ascorbate-glutathione pathway enzymes to their substrates. The increase in irradiance during the spring (March to May) was accompanied by increasing antioxidative enzymatic activities and ascorbate content. A marked enhancment in catalase activity also accompanied the increased light intensity during the spring. Changes in the enzymatic activities of the ascorbate-glutathione pathway followed the increased ascorbate content. These results suggest that physiological adaptation of *Retama* involves efficient H_2O_2 removal mechanisms which respond to different seasonal and environmental stresses.

KEY WORDS: Ascorbate peroxidase, ascorbate, glutathione, catalase, hydrogen peroxide removal.

INTRODUCTION

Production of active oxygen species was reported in chloroplasts of higher plants[1] and in cyanobacteria.[2] Superoxide and peroxide are assumed to be the product of oxygen reduction by the photosynthetic apparatus.[3] Oxygen is considered to be an alternative substrate to NADP photoreduction during overproduction of reducing power by the photosynthetic apparatus. Photoproduced oxygen radicals interact with various cell constituents leading to lipid peroxidation and damages protein and DNA. Higher plants possess means to scavenge oxygen radicals by various enzymes including the well studied superoxide dismutase[4] and the ascorbate-glutathione pathway.[5] Removal of oxygen radicals also involves antioxidative compounds, such as ascorbate and glutathione.[6] The photorespiration mechanism provides means to reduce the intracellular oxygen concentrations and involves catalase activity in the peroxisomes where H_2O_2 is decomposed.[7]

Excess production of oxygen radicals is expected to take place under desert stress conditions due to the combination of high light intensity and drought. These conditions may result in high intracellular concentrations of photoproduced oxygen and low CO_2 influx due to closure of stomata. The high ratio of O_2/CO_2 will favour photorespiration and formation of oxygen radicals.[7]

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Retama (white broom) is a highly adapted evergreen legume, common in Israeli desert (Negev), which survives severe drought and irradiation stress.[8] This report correlates changes in antiperoxidative activity of retama with seasonal stresses of high light and drought.

MATERIALS AND METHODS

Plant Material. Irradiance and Precipitation

Selected retama plants were sampled once a month in two locations in the Negev, Sede Boquer and Ramat Hovav. Young shoots were cut from the plants, kept at 4°C and stored at −20°C for analysis. Precipitation and irradiance measurements were obtained from the Desert Ecosystem Research Center of the Hebrew University of Jerusalem, and the Energy Research Station at Sede Boquer.

Cell Free Extracts

Retama shoots (5 g) were homogenized at 4°C by ultra-turrax (Janke & Kunkel GmbH) in 70 ml of the appropriate buffer for determination of enzymatic activities and glutathione content, or in 70 ml of 5% trichloroacetic acid for determination of ascorbate content. The buffers used were 0.1 M potassium phosphate pH 7.5, for ascorbate peroxidase, 0.1 M potassium phosphate pH 6.5, for dehydroascorbate reductase, 0.1 M potassium phosphate pH 8.5, for glutathione reductase and 0.1 M potassium phosphate pH 8.0, plus 5 mM EDTA for glutathione determination. A 50 mM potassium phosphate buffer pH 7.0 was used for catalase assay. The homogenates were filtered through eight layers of gauze and centrifuged at 35000 g for 30 min in a beckman J2–21 centrifuge at 4°C. The supernatant obtained was used for enzymatic assays and antioxidants determination.

Biochemical Assays

Ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase were measured spectrophotometrically at 25°C, using a Uvicon 810 spectrophotometer.[9] The reaction buffer contained 0.1 M phosphate buffer plus 5 mM EDTA at the appropriate pH. Catalase activity was measured using a Clark-type oxygen electrode (YSI-5331).[9] Monodehydroascorbate reductase activity was estimated by the decrease in the rate of ascorbate disappearance from the medium, using the reaction mixture for the ascorbate peroxidase activity with the addition of 0.4 mM NADH. Ascorbate content was measured using the dinitrophenyl hydrazine thiourea method.[10] Glutathione content was measured using the O-phtalaldehyde fluorometric method,[11] with a Jasco FP-550 fluorimeter. Chlorophyll and carotenoids were determined in 80% (v/v) aceton extracts.[12] Protein content was determined according to Bradford.[13]

RESULTS AND DISCUSSION

Ascorbate Peroxidase Stability and Some Enzymatic Properties

Ascorbate peroxidase activity in retama soluble extracts was found to be stable in an ascorbate depleted medium for more than 24 hours. Shoot homogenates prepared in

TABLE I
Substrate affinities of retama antioxidative enzymes.

Enzyme:	Substrate:	Apparent Km value (mM):
Ascorbate Peroxidase	Ascorbic Acid	0.030
	Hydrogen peroxide	0.005
	Cummene hydroperoxide	0.500
	t-Butyl hydroperoxide	2.000
Dehydroascorbate Reductase	Dehydroascorbate	0.070
	Glutathione (red)	0.400
Glutathione Reductase	Glutathione (Ox)	0.016
	NADPH	0.007
Catalase	Hydrogen peroxide	25.000

Activities were determined as described in materials and methods. No activity of glutathione reductase with NADH could be detected.

the presence and absence of ascorbate and sorbitol, in an attempt to stabilize the enzyme,[14] exhibited similar activities (data not shown). These results are in agreement with the results obtained with the enzyme from soybean nodules[15] and suggest that ascorbate peroxidase of retama is similar to the more stable cytosolic type ascorbate peroxidase.[16]

Ascorbate peroxidase of retama and the ascorbate-regenerating enzymes were found to have a very high affinity to their substrates (Table 1). The affinity of ascorbate peroxidase for H_2O_2 and ascorbate is very high compared to spinach and *Euglena* ascorbate peroxidases.[14,17] Affinities of dehydroascorbate reductase and glutathione reductase for their substrates were also higher than previously reported for spinach and *Euglena*.[18,19] The high affinity of ascorbate peroxidase and its related enzymes in retama under desert stress conditions may be related to the need to scavenge H_2O_2 and prevent membrane damage under drought conditions.[20]

The activity of monodehydroascorbate reductase was only one fifth of that of dehydroascorbate reductase and would account for a minor role in the ascorbate regenerating capacity (data not shown).

Seasonal Variation in The Activities of Antiperoxidative Enzymes

Seasonal variation in the activities of antioxidative enzymes were previously reported for peas grown under greenhouse conditions.[21] We have determined the activities of retama enzymes in the desert during the season between October to May. The accumulated information on precipitation and irradiance in the Sede Boquer area (Figure 1) demonstrates the dry nature of this ecosystem and the drastic changes in irradiance during the spring. The content of chlorophyll and carotenoids was not affected throughout the season between October and April, and was only slightly decreased in May (Figure 2). Therefore drought and high light intensity did not cause pigment bleaching. Figure 3 demonstrates that the activities of catalase and ascorbate peroxidase have a similar pattern of induction that follows the high irradiance changes during the spring (Figure 1) though the increase in ascorbate peroxidase activity was not significant. Catalase was reported to be photoinactivated,[22] therefore the induced catalase activity under high light intensity, in the desert, may suggest that retama catalase is characterized by a high turnover rate, or a low susceptibility to

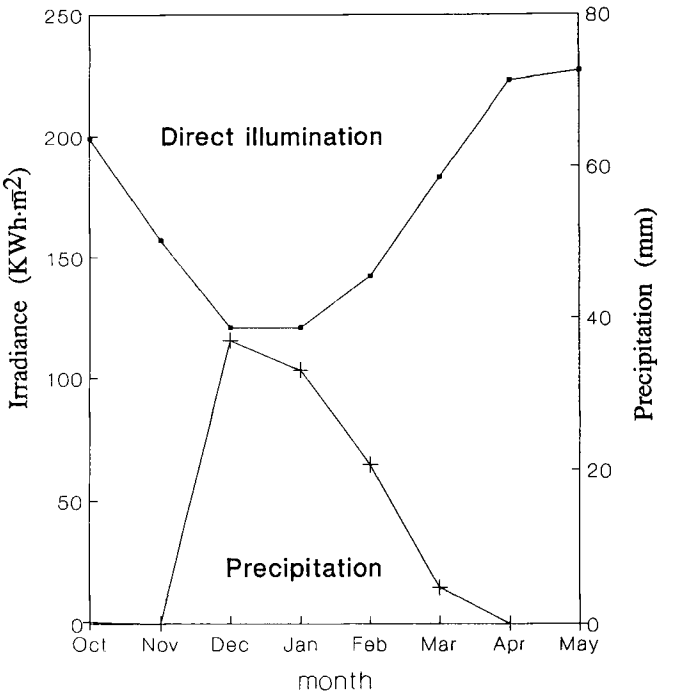

FIGURE 1 Precipitation and irradiance at the Sede Boquer area. Measurements were obtained as described in materials and methods.

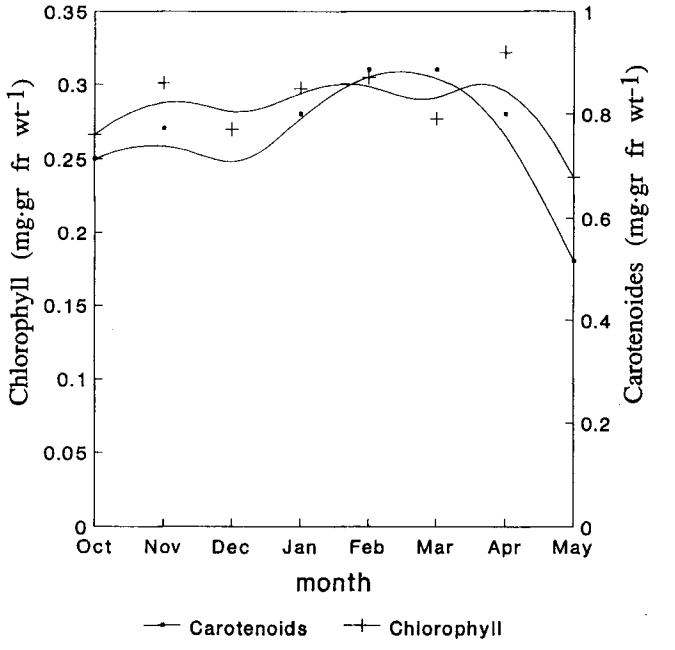

FIGURE 2 Chlorophyll and carotenoids content. Pigments were determined as described in materials and methods.

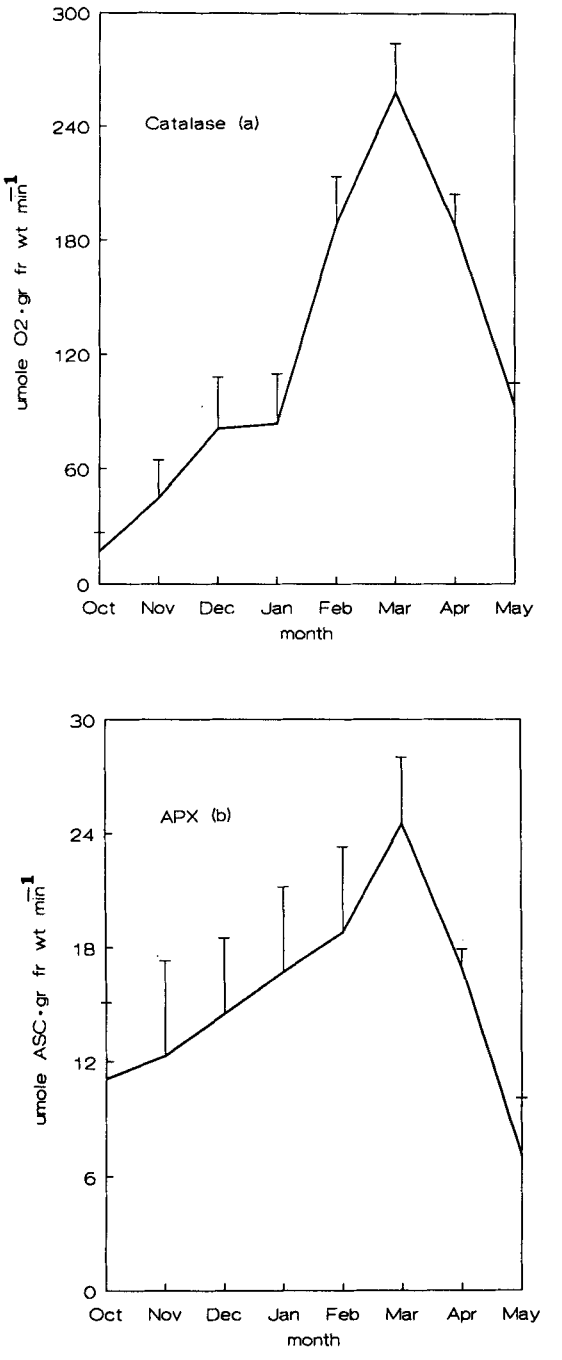

FIGURE 3 Seasonal variations of retama catalase (a) and ascorbate peroxidase (b). Catalase and ascorbate peroxidase activity were determined as described in materials and methods.

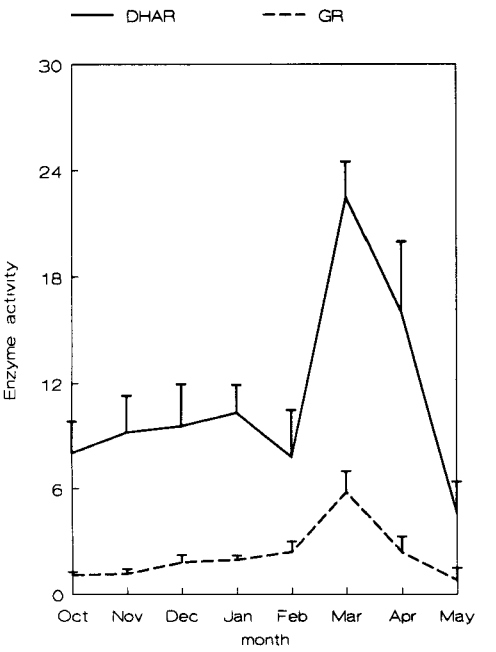

FIGURE 4 Seasonal variation in the activities of dehydroascorbate reductase and glutathione reductase. Enzymatic assays were performed as described in materials and methods. Key: DHAR – dehydroascorbate reductase, GR – glutathione reductase. Enzyme activities are expressed as follows:
DHAR: umole ascorbate reduced gr fr $wt^{-1} min^{-1}$,
GR: umole NADPH gr fr $wt^{-1} min^{-1}$.

photoinactivation. The ascorbate regenerating enzymes, dehydroascorbate reductase and glutathione reductase were also induced in a similar pattern to the increasing light intensity, as shown in Figure 4. It was recently reported that ascorbate peroxidase activity is induced by oxidative stresses in a cyanobacterial system.[23] The results presented in this report demonstrate that increase in ascorbate peroxidase activity is accompanied by the ascorbate regenerating enzymes. The induced antiperoxidative enzymatic activities during the spring suggest that removal of H_2O_2 is essential for retama growth and productivity in the desert.

The ten fold catalase activity over ascorbate peroxidase activity (Figure 3) may imply that most of the peroxide removal activity in retama is extrachloroplastic and is conducted by catalase. The ascorbate-glutathione pathway is a secondary route for hydroperoxide removal in retama desert stress conditions. Nevertheless the ascorbate-glutathione pathway is of great significance due to its potential to remove low concentrations of hydrogen and lipid peroxides and thus prevent membrane damage under drought conditions.[20]

SEASONAL VARIATION IN ANTIOXIDANT CONTENT

Figure 5 demonstrates the seasonal varation in ascorbate and glutathione content of retama plants in the desert. Biosynthesis of ascorbate is shown to precede the increase of antiperoxidative enzyme activity in response to the high irradiance changes.

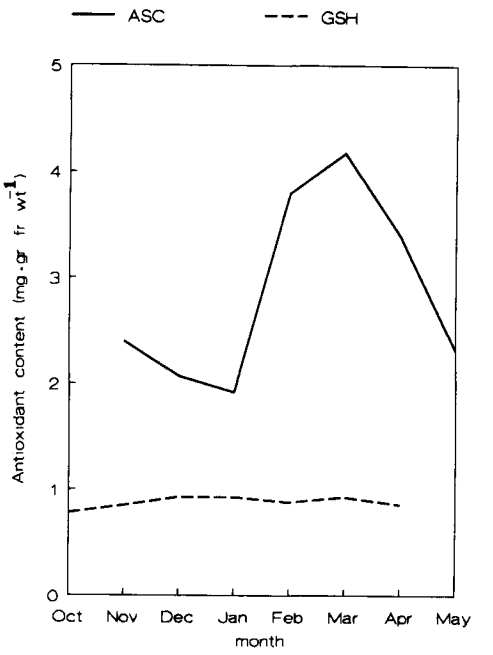

FIGURE 5 Seasonal variation in ascorbate and glutathione content of retama. Antioxidant's content was assayed as described in materials and methods. Key: ASC – ascorbate, GSH-glutathione.

High ascorbate content in February was followed by maximum enzymatic activity in March. No seasonal changes were found in the glutathione content which suggests a minor role for this antioxidant in preventing oxidative damage during the season. The high ascorbate content under the high light intensities in the desert and the low ascorbate-regenerating capacity of glutathione reductase and monodehydroascorbate reductase suggest that retama plants have a very high capacity for ascorbate biosynthesis. Ascorbate is considered to play a major role in the chloroplastic scavenging system[23] and increased ascorbate content was also reported in illuminated spinach chloroplasts[24] and *Euglena*.[25]

The high antiperoxidative enzymatic activities and ascorbate content during the spring (February–April) correlate with retama's high productivity. Retama plants produce buds, leaves and fruits between December and April (data not shown, see also [8]). Therefore it is suggested that peroxide removal supports plant productivity and vegetative growth during the season (February–April).

The correlations reported are based on data collected throughout a single growth season of retama, however, precipitation and irradiance were similar to the multiannual average, hence, this study represents typical pattern of retama development.

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