CHANGES IN SOME ANTIOXIDANT ENZYME ACTIVITIES IN SIX SOYBEAN CULTIVARS IN RESPONSE TO LONG-TERM SALINITY AT TWO DIFFERENT TEMPERATURES

***N. Çiçek*[1*] *and H. Çakırlar*[1]**

[1]*Hacettepe University, Faculty of Science, Department of Biology, 06800, Beytepe, Ankara, Turkey*

Summary. In the present study, the effect of long-term salt stress (NaCl with 0, - 0.1, - 0.4 and - 0.7 MPa)-temperature (25±2 ºC and 35±2 ºC) interaction on some antioxidant enzyme activities (ascorbate peroxidase, glutathione reductase and peroxidase) in six soybean cultivars (*Glycine max* L. Merr.) was investigated. The leaves of twenty-three-day old plants were used for the analysis of enzyme activities. Salt stress significantly increased APX activities of Mitchell, Nazlıcan and Türksoy compared to control, and decreased in other three cultivars at 25 ºC, whereas it caused differences in the APX activities depending on the salt treatment levels at 35 ºC. Under both temperatures, GR activities in the cultivars were generally increased by salt treatment, except for - 0.1 MPa. POD activities at 25 ºC decreased in all cultivars, except for - 0.7 MPa in CX-415 and Nazlıcan. However, - 0.4 MPa increased the activity only in cv. Nazlıcan at 35 ºC. Our results demonstrated that the studied cultivars exhibited discrepancies in their antioxidant enzyme activities in response to salt treatment and the interaction of salt-temperature intensified the obtained responses.

***Key words*:** Ascorbate peroxidase, glutathione reductase, peroxidase, salt stress, soybean, temperature.

*Corresponding author, e-mail: ncicek@hacettepe.edu.tr

Abbreviations: APX - ascorbate peroxidase; EDTA - ethylenediaminetetra acetic acid; GR - glutathione reductase; H_2O_2 - hydrogen peroxide; $^1O_2$ - singlet oxygen; O_2^- - superoxide radical; $OH^{\bullet}$ - hydroxyl radical; POD - peroxidase; PVP - polyvinyl polypyrrolidone; ROS - reactive oxygen species.

INTRODUCTION

Environmental stresses including salinity and temperature affect nearly every aspect of the physiology and biochemistry of plants and significantly diminish yield. Many arid and semi-arid regions in the world contain soils and water resources that are too saline for most of the common economic crops, which affect plants through osmotic effects, ion specific effects and oxidative stress (Munns, 2002; Pitman and Lauchli, 2002).

Much of the injury to plants exposed to stress is connected with oxidative damage at the cellular level (Foyer and Noctor, 2003). If there is a serious imbalance in any cell compartment between the production of reactive oxygen species (ROS) and antioxidant defense, oxidative stress and damage occurs (Mittler, 2002). Even under normal growth conditions, low amounts of ROS such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), and singlet oxygen ($^1O_2$) are metabolic by-products of plant cells (Cai-Hong et al., 2005). Plants have developed the scavenging mechanism of ROS categorized as enzymatic and non-enzymatic (Reddy et al., 2004; Demiral and Türkan, 2005). When ROS increases, chain reactions start, in which superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\cdot-}$ radicals to molecular O_2 and H_2O_2 (Meloni et al., 2003). The H_2O_2 is then detoxified in the ascorbate–glutathione cycle (Willekens et al., 1995; Asada, 1999; Mittler, 2002), which involves the oxidation and re-reduction of ascorbate and glutathione through the ascorbate peroxidase (APX) and glutathione reductase (GR) action (Foyer and Halliwell 1976; Noctor and Foyer, 1998).

Salt stress induces cellular accumulation of ROS which can damage membrane lipids, proteins and nucleic acids (Hernandez et al., 1993; 1999; 2000; Mansour et al., 2005; Alscher et al., 1997; Ben Amor et al., 2007;

Eyidoğan et al., 2007). A correlation between the antioxidant capacity and NaCl tolerance has been demonstrated in some plant species (Gossett et al., 1994; Dionisio-Sese and Tobita 1998; Hernandez et al., 1999). Several studies have pointed out that salt-tolerant species increased their antioxidant enzyme activities and antioxidant contents in response to salt treatment, whereas salt-sensitive species failed to do so (Shalata et al., 2001; Demiral and Türkan, 2005).

To be able to endure oxidative damage under unfavorable conditions such as high/low temperatures, water deficit, and salinity etc., plants must possess efficient antioxidant system (Sairam et al., 2002). In addition, plants are subjected to the interaction of two or more environmental stress factors under natural conditions and many studies have been carried out to study the effects of these stress factors on plant metabolism separately. Therefore, the aim of the study was to investigate the effect of long-term salt stress and temperature interaction on antioxidant enzyme activities (APX, GR and POD) in the soybean plants.

MATERIALS AND METHODS

In the present study, six soybean (*Glycine max* L. Merr., A 3935, CX-415, Mitchell, Nazlıcan, SA 88 and Türksoy) cultivars grown in South and Southeastern parts of Turkey were investigated. The seeds were sown in plastic pots (14 cm in diameter and 13 cm in height) filled with perlit. They were watered with a half-strength Hoagland's solution during the first 7 days following sowing, and then subjected to salt treatments. Salinized culture solutions were prepared by adding various amounts of NaCl to the ½ Hoagland's solution (control) to create three osmotic potentials: - 0.1, - 0.4 and - 0.7 MPa. Plants were grown in a growth room with a 16 h /8 h light/dark cycle at 25±2 and 35±2 ºC, 50 ± 5 % humidity and at 200 µmol m^{-2} s^{-1} light intensity. The measurements of the antioxidant enzyme activities were done on 23-day-old plants.

For determination of antioxidant enzyme activities, fresh leaf samples (0.3 g) from control and treated plants were ground with liquid nitrogen, and suspended in specific buffer and pH for each enzyme extraction. The homogenates were centrifuged at 14.000 rpm for 20 min at 4 °C and resulting

supernatants were used for enzyme assay. The protein concentrations of leaf crude extract were determined according to Bradford (1976).

APX activity was determined according to Wang et al. (1991). APX extraction was performed in 1.5 ml of suspension solution including 50 mM Tris-HCl (pH 7.2), 2 % PVP, 1 mM Na_2EDTA, and 2 mM ascorbate. Assay solution contained 50 mM potassium phosphate buffer (pH 6.6), 2.5 mM ascorbate, 10 mM H_2O_2, and enzyme containing 100 µg protein in a final volume of 1 ml. The enzyme activity was calculated from initial rate of the reaction using the extinction coefficient of ascorbate (ε = 2.8 mM cm^{-1} at 290 nM).

GR activity was determined according to Sgherri et al. (1994). GR extraction was performed in 1.5 ml of suspension solution containing 100 mM potassium phosphate buffer (pH 7.0), 1 mM Na_2EDTA, and 2 % PVP. Assay mixture contained 200 mM potassium phosphate buffer (pH 7.5), 0.2 mM Na_2EDTA, 1.5 mM $MgCl_2$, 0.5 mM GSSG, 50 µM NADPH, and enzyme extract containing 100 µg protein in a final volume of 1 ml. Correction was made for the non-enzymatic oxidation of NADPH by recording the decrease at 340 nm without adding GSSG to assay mixture. The enzyme activity was calculated from the initial rate of the reaction after subtracting the non-enzymatic oxidation using the extinction coefficient of NADPH (ε = 6.2 mM cm^{-1} at 340 nm).

POD activity was based on the determination of guaiacol oxidation (ε = extinction coefficient 26.6 mM cm^{-1}) at 470 nm by H_2O_2. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 20.1 mM guaiacol, 12.3 mM H_2O_2, and 100 µl enzyme extract in a 3 ml volume (Bergmeyer, 1974).

RESULTS

Antioxidant enzyme activities in the leaves of the soybean cultivars studied exhibited discrepancies depending on inter-specific characteristics under different NaCl and temperature conditions (Figs. 1, 2 and 3). Salt treatment decreased remarkably APX activity in the A 3935, CX-415 and SA 88 at the 25 °C (about 29-43 %), whereas it increased the activity in the Mitchell, Nazlıcan and Türksoy compared to their controls (approximately

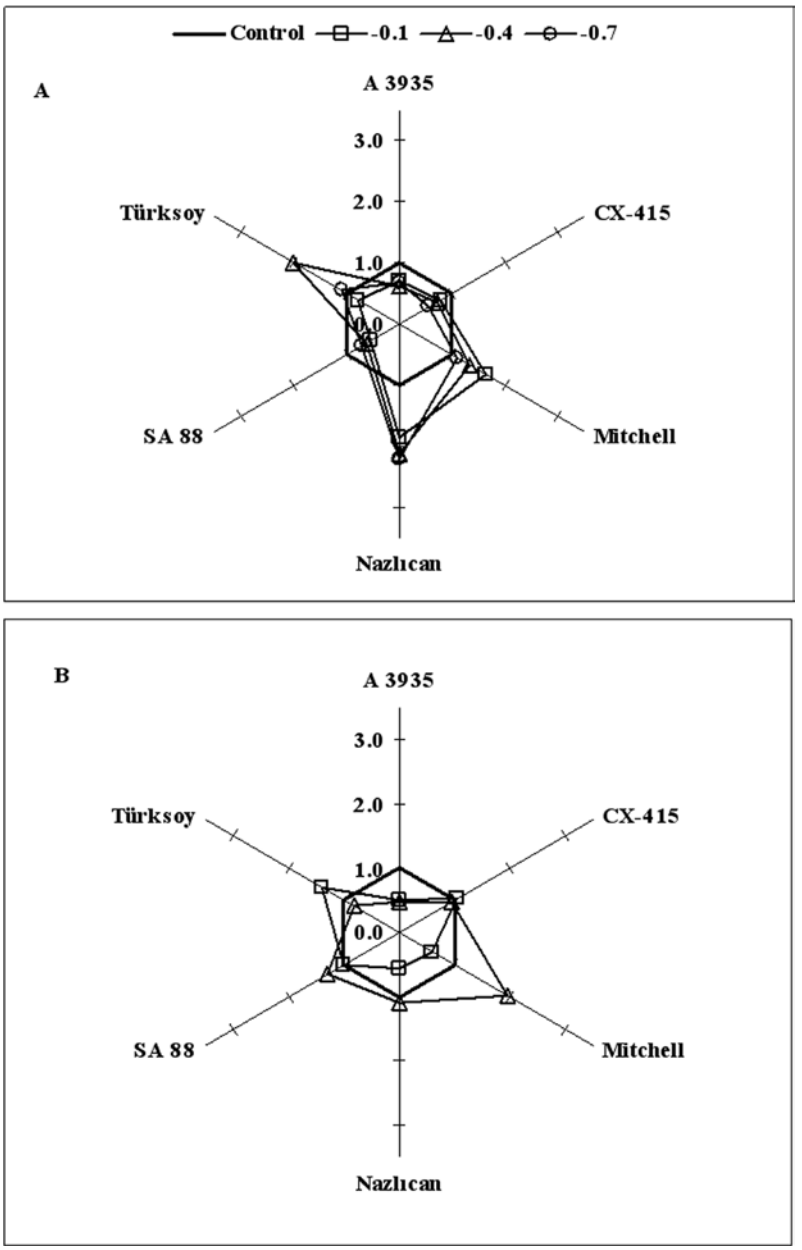

Fig. 1. Effect of salt stress-temperature interaction on the APX activity in soybean cultivars (a, 25 °C; b, 35 °C). The activity determined in the stress treated seedlings is plotted relative to that of control seedlings utilizing Biolyzer Software Program (Maldonado-Rodriguez, 1999-2002).

1.3-2.2 folds) (Fig. 1a). Similarly, the highest salt treatment (- 0.4 MPa) decreased significantly the activity in the A 3935, CX-415 and Türksoy (between 4-52 %), while it increased the activity in the other three soybean cultivars growing at the 35 °C (max. 1.3 folds) (Fig. 1b). In addition, - 0.1 MPa increased significantly the activity in the CX-415 and Türksoy, but it reduced in cultivars A 3935, Mitchell and Nazlıcan. Furthermore, the activity of APX in soybean cultivars growing at 35 °C was higher (avg. 3 times) than that at 25 °C.

At 25 °C, GR activity increased significantly in A 3935, CX-415 and Mitchell under - 0.4 and - 0.7 MPa salt treatments (about 1.1-1.9 folds) (Fig. 2a). - 0.1 MPa of NaCl treatment decreased GR activity in almost all cultivars compared to controls (max. 55 %). Conversely, the GR activity decreased in the leaves of A 3935 and Nazlıcan at both salt levels (- 0.1 and - 0.4 MPa) at 35 °C (Fig. 2b). - 0.1 MPa salt treatment also reduced the activity in the SA 88 and Türksoy, but it was not found significant in the latter. The highest salt level (- 0.4 MPa) increased significantly GR activity in the Mitchell and Türksoy by 170 and 135 % respectively, whereas the changes in CX-415 were not remarkable (Fig. 2b). It was observed that constitutive GR activity was higher in all cultivars, except for Mitchell and Nazlıcan at 35 °C than 25 °C.

POD activity also was found higher at 35 °C than 25 °C. The activity was decreased by NaCl treatment in all cultivars by approx. 32-80 %, except for CX-415 and Nazlıcan at 25 °C, it increased at the highest salt level (- 0.7 MPa) in these two cultivars by 130 % (Fig. 3a). The responses in the POD activity of cultivars against salt treatment at 35 °C were determined similar to 25 °C, except for Nazlıcan. Salt treatment decreased significantly POD activity in all cultivars, whereas the activity of Nazlıcan was remarkably increased by NaCl treatment (about 1.5-fold) (Fig. 3b).

DISCUSSION

Plants resort to a range of distinct acclimation strategies in response to abiotic environmental stresses such as high salt, dehydration, cold, heat, and excessive osmotic pressure (Pasternak et al., 2005). Salinity stress is an intricate phenomenon which includes osmotic stress, specific ion effect,

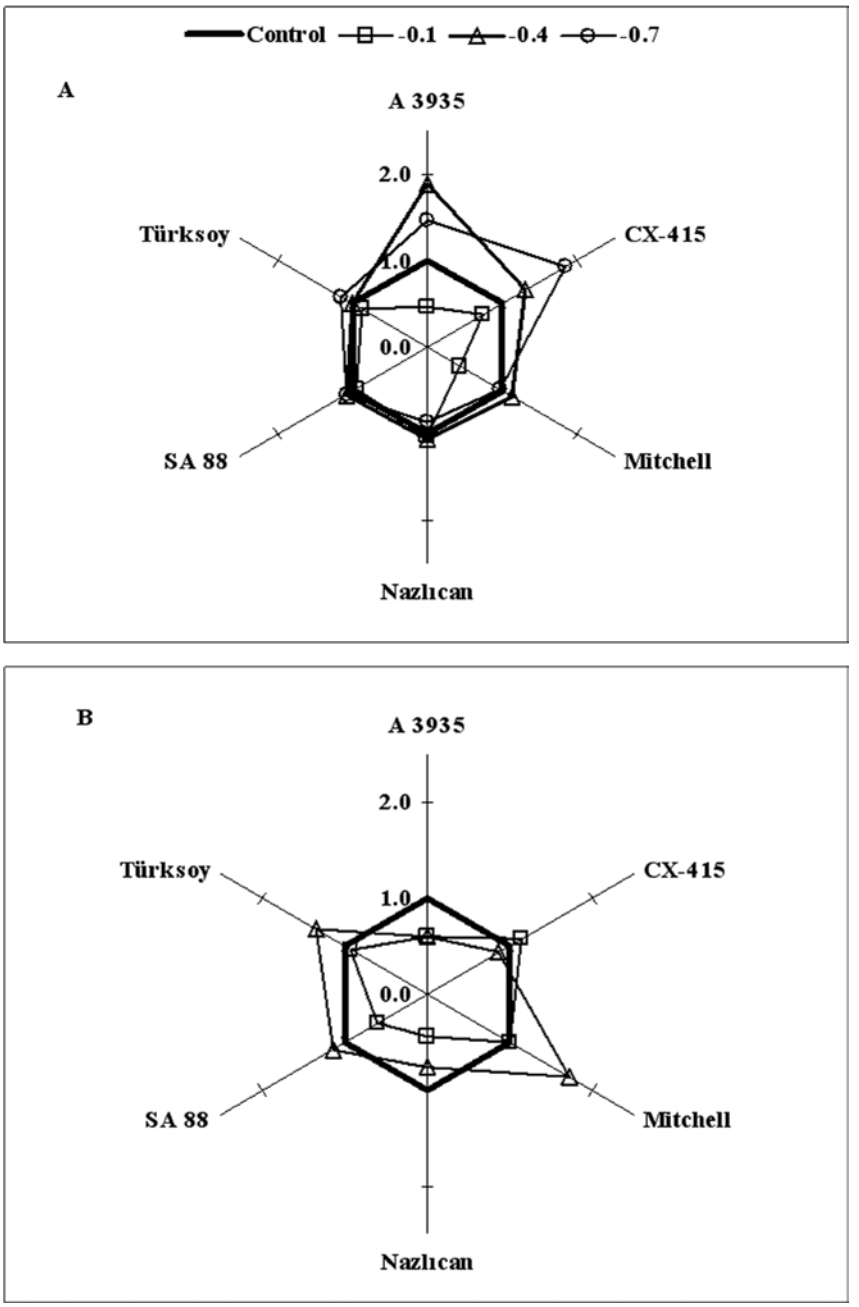

Fig. 2. Effect of salt stress-temperature interaction on the GR activity in soybean cultivars (a, 25 °C; b, 35 °C). See Figure 1. for explanation of the legend.

nutrient deficiency thereby affecting various physiological and biochemical mechanisms associated with plant growth and development (Sairam et al., 2002). In this context, plants with higher levels of antioxidants, either constitutive or induced, have been reported to possess greater resistance to these stress conditions (Dionisio-Sese and Tobita, 1998; Sreenivasulu et al., 2000). It has been suggested that salinity causes oxidative stress by inhibition of the CO_2 assimilation, exposing chloroplasts to excessive excitation energy, which in turn increases the generation of ROS from triplet chlorophyll (Gossett et al., 1994).

H_2O_2 can be removed using the ascorbate-glutathione cycle [ascorbic acid (ASA)-GSH cycle] which APX and GR are the key enzymes in this cycle (Noctor and Foyer, 1998). In the present study, salinity and high temperature led to a significant increase in the APX activities of Mitchell, Nazlıcan and Türksoy by approx. 1.3-2.2 fold, and GR activities of A 3935, CX-415, Mitchell and Türksoy by about 1.1-1.9 fold compared to the respective controls, although there were some variations among soybean cultivars and temperature (Figure 1 and 2). The diverse responses of the APX and GR enzyme activities in the plants subjected to saline conditions suggest that oxidative stress is an important component of salt stress (Stepien and Klobus, 2005). These results are in agreement with those of Stepien and Klobus (2005), who have propounded that the APX and GR action suggests that the more active ascorbate–glutathione cycle may be related to the development of relatively higher salt tolerance in maize. The constitutive and the salt-induced APX and GR activities were remarkably higher in the cultivars grown at 35 °C compared to 25 °C. These results may point out that the high temperature provokes antioxidant enzyme responses. Several researchers have suggested that salt tolerance is often correlated with a more efficient antioxidative system (Gossett et al., 1994; Dionisio-Sese and Tobita, 1998; Bor et al., 2003; Ashraf and Harris, 2004). Some soybean cultivars increased their enzyme activities as a consequence of stress, however, these responses might not be enough to overcome the detrimental effects of long-term stress or to allow survival of the plants as it was observed that all soybean cultivars lost their vitality under the highest stressful conditions at the end of experiment. These results are consistent with other growth parameters of these six soybean cultivars i.e. stress

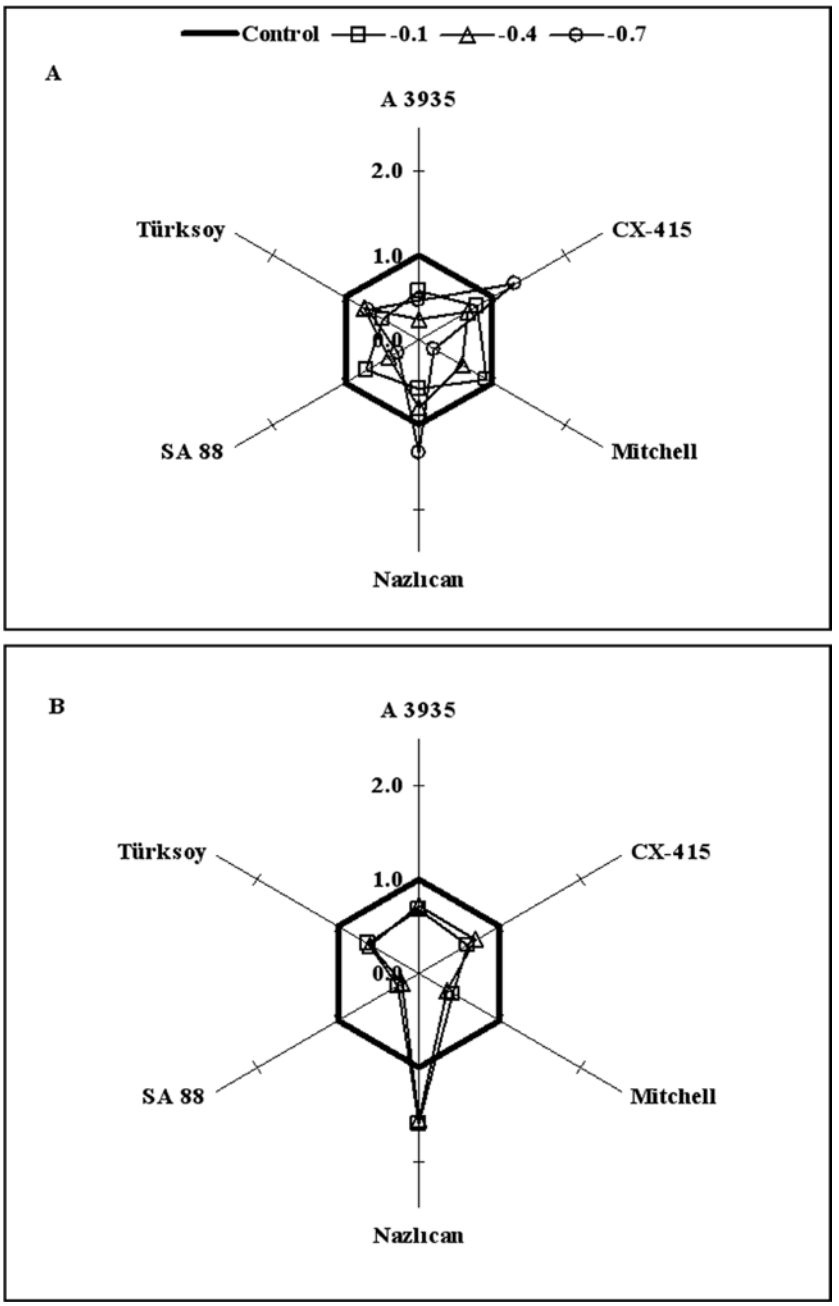

Fig. 3. Effect of salt stress-temperature interaction on the POD activity in soybean cultivars (a, 25 °C; b, 35 °C). See Figure 1. for explanation of the legend.

caused a decline in the K^+/Na^+ ratio, plant height, fresh and dry biomass of the shoot and an increase in the relative leakage ratio and the contents of proline and Na^+ (Çiçek and Çakırlar, 2008).

POD activity decreased considerably upon NaCl treatments under both temperatures in all cultivars except for Nazlıcan (Figure 3). Salt and temperature treatment increased the activity in this soybean cultivar by 1.5-fold. Conversely, Ben Amor et al. (2007) found that peroxidase activity in the *Cakile maritime* increased gradually with time and with increasing NaCl concentrations up to 400 mmol/L, whereas POD unexpectedly started to decrease in plants treated with 400 mmol/L NaCl. Foyer et al. (1994) proposed that the absence of a rapid increase in the level of transcripts of the antioxidant enzymes could be related to the role of ROS in signal transduction. This difference between transcript levels and enzyme activities during NaCl treatment may result from a higher turnover of these enzymes and/or an increase of their inactivation by H_2O_2 (Scandalios, 1993).

In conclusion, the results of the present study clearly showed that there was differential accumulation of H_2O_2 as well as genotypic variations in H_2O_2-scavenging enzymes in soybean cultivars grown under different salt stress and high temperature conditions. The soybean plant which is considered moderately salt tolerant (Pitman and Lauchli, 2002) might have inadequate ROS scavenging system, in addition to other tolerance mechanisms, to cope with stress.

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