

BRIEF COMMUNICATION

Impact of zinc on dehydration and rehydration responses in teaH. UPADHYAYA^{1*}, B.K. DUTTA², and S.K. PANDA³*Department of Botany, Cotton University, Panbazar, Guwahati - 781001, Assam, India¹**Department of Ecology and Environmental Sciences, Assam University, Silchar - 788011, Assam, India²**Department of Life Science and Bioinformatics, Assam University, Silchar - 788011, Assam, India³***Abstract**

Zinc nutrition of tea (*Camellia sinensis*) have a great impact on acclimation to dehydration stress and on improvement of stress recovery. Dehydration stress induced by withholding water for 7 d decreased relative water content, dry mass of leaf, and content of antioxidants like ascorbate and glutathione and increased H₂O₂ content and lipid peroxidation and changed activities of superoxide dismutase, catalase, peroxidase, and glutathione reductase. A pre-treatment with 0.1 and 0.5 mM ZnSO₄ minimized these effects and increased Zn uptake. Further, foliar spray with 0.1 and 0.5 mM ZnSO₄ before rehydration lowered H₂O₂ generation, increased content of antioxidants and activities of antioxidative enzymes, and decreased lipid peroxidation. The present findings suggest that zinc regulates water stress responses and recovery after rehydration in tea.

Additional key words: antioxidants, *Camellia sinensis*, lipid peroxidation, relative water content, water stress.

Dehydration stress alters different physiological and biochemical processes leading to significant crop losses (Chakraborty 2002, Upadhyaya and Panda 2004, Upadhyaya *et al.* 2008, Singh *et al.* 2015, Rao and Chaitanya 2016). Dehydration stress tolerance is seen in almost all plant species but its extent varies from species to species. Understanding plant responses to drought and rehydration are of greater theoretical and practical importance. Dehydration stress increases production of reactive oxygen species (ROS) that oxidizes target molecules, but ROS also increases the activity of an antioxidant system.

Zinc nutrition affects plant transpiration rate and stomatal conductance. Possible roles of zinc in protecting plant cells from damage by ROS and its effect on plant metabolism has been reported (Broadley *et al.* 2007, Upadhyaya and Panda 2013). In the present study, biochemical, growth and antioxidant responses were investigated in the leaves of *Camellia sinensis* subjected to ZnSO₄ treatment before drought imposition as well as during post-drought rehydration.

Tea [*Camellia sinensis* (L.) Kuntze] seedlings (one and a half year old) were procured from *Rosekandy Tea*

Estate, Silchar, India, and were acclimatized for 30 d in a greenhouse as mentioned in the previous report (Upadhyaya *et al.* 2008). After 30 d of acclimatization, plants were subjected to different treatments. In one set of experiments, control plants were irrigated with 100 cm³ of water or 0.1 and 0.5 mM of ZnSO₄ solutions and to half of them drought was imposed by withholding water for 7 d. The moisture content of the soil of control plants was 23.35 ± 2.5 % whereas after 7 d of drought, it was 8.7 ± 0.5 %. In another experimental set, post-stress recovery was conducted (15 d drought + 14 d rehydration) where the foliar sprays with distilled water or 0.1 and 0.5 mM ZnSO₄ (twice a week) were designated as control 2, PDR 0.1, and PDR 0.5, respectively.

Leaf dry mass was determined after drying leaves at 80 °C for 48 h. A relative water content (RWC) was determined gravimetrically as per the methods of Bars and Weatherly (1962). The content of chlorophyll (Chl) *a*, Chl *b*, and total carotenoids (Car) was analyzed in acetone extract spectrophotometrically at 662, 644 and 470 nm following Shabala *et al.* (1998) and Lichtenthaler (1987) methods, respectively.

The proline content was determined following the

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Abbreviations: CAT - catalase; GR - glutathione reductase; MDA - malondialdehyde; POX - peroxidase; ROS - reactive oxygen species; RWC - relative water content; SOD - superoxide dismutase.

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method of Bates *et al.* (1973). The glutathione was extracted and estimated as per the method of Griffith (1980) as described in our previous paper (Upadhyaya *et al.* 2008). For the extraction and estimation of ascorbate, the method of Oser (1973) was used. The reaction mixture consisted of 2.0 cm³ of 2 % (m/v) Na-molybdate, 2.0 cm³ of 0.15 M H₂SO₄, 1.0 cm³ of 1.5 mM Na₂HPO₄, and 1.0 cm³ of tissue extract. The reaction mixture was incubated at 60 °C in a water bath for 40 min. It was cooled and centrifuged at 3 000 g for 10 min and the absorbance was measured at 660 nm.

From fresh leaf samples (0.2 g), H₂O₂ was extracted in 5.0 % trichloroacetic acid (TCA). The homogenate was used for the estimation of total peroxide content (Sagisaka 1976) as reported earlier (Upadhyaya *et al.* 2008). The malondialdehyde (MDA) content was determined using 2-thiobarbituric acid (TBA). The content of MDA was calculated using coefficient of absorbance (ϵ) of 155 mM⁻¹ cm⁻¹ (Zhang 1992).

Leaf tissues were homogenized with 0.1 M potassium phosphate buffer (pH 6.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 % (m/v) polyvinylpyrrolidone (PVP), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in prechilled mortar and pestle. The extract was centrifuged at 17 000 g and 4 °C for 15 min. The supernatant was used for the assay of antioxidative enzymes. Protein content in enzyme extracts was determined according to the method of Bradford (1976). The activity of catalase (CAT; EC 1.11.1.6) was measured by the method of Aebi (1974) and was determined by monitoring the disappearance of H₂O₂ at 240 nm ($\epsilon = 0.036$ mM⁻¹ cm⁻¹). One unit of CAT activity was defined as the amount of enzyme required to catalyze the decomposition of 1 μ mol of H₂O₂ per min. The

activity of peroxidase (POX; EC EC 1.11.1.7) was assayed using pyrogallol as a substrate according to Kar and Mishra (1976). The purpurogallin formed was determined at 430 nm. One unit of POX activity was defined as the amount of enzyme which forms 1 μ mol of purpurogallin per min. The activity of superoxide dismutase (SOD; EC EC 1.15.1.1) was measured using the method of Giannopolitis and Reis (1977) as described in our previous paper (Upadhyaya *et al.* 2008). One unit of SOD activity was defined as the amount of enzyme that inhibits nitroblue tetrazolium chloride photo-reduction by 50 %. The activity of glutathione reductase (GR; EC 1.6.4.2) was assayed by the method of Smith *et al.* (1988). The reaction mixture contained 1.0 cm³ of 0.2 M potassium phosphate buffer (pH 7.5), 1 mM EDTA, 0.5 cm³ of 3.0 mM DTNB, 0.1 cm³ of 2.0 mM NADPH, 0.1 cm³ of tissue extract, and distilled water to make up a final volume of 2.9 cm³. The reaction was initiated by adding 0.1 cm³ of 2 mM oxidized glutathione. The increase in absorbance was recorded at 412 nm over a period of 5 min. One unit of GR activity was defined as the amount of enzyme catalyzing reduction of 1 μ mol of GSSG per min.

Leaves from stressed and control plants were briefly rinsed with deionized water and oven dried at 80 °C for 48 h. The dried sample (100 mg) was digested in 5 cm³ of mixture of concentrated acids (HNO₃:HCl, 3:1). The sample volume was adjusted to 20 cm³ with deionized water, and zinc content was measured using an atomic absorption spectrometer (model 3110, Perkin-Elmer, USA).

During sampling, at least two plants were used in each sampling and each experiment was repeated thrice. Data presented are means \pm standard errors (SEs). Least

Table 1. Changes in leaf dry mass, relative water content (RWC), and content of proline, chlorophyll (Chl) *a+b*, total carotenoids (Car), Zn, and malondialdehyde (MDA) in control 1 leaves of *Camellia sinensis* and after pretreatment with zinc (0.1 and 0.5 mM ZnSO₄) and drought stress lasting 7 d (D). Further, the same parameters were determined after foliar spraying with water (control 2) or 0.1 (PDR 01) and 0.5 (PDR 05) mM ZnSO₄ and 14 d rehydration after drought for 15 d. Means \pm SEs, *n* = 3. Letters 'a' and 'b' indicate significant differences from control and drought, respectively, at *P* < 0.05. In the second experiment, '*' indicates significant differences from control at *P* < 0.05 (LSD test).

Treatments	Dry mass [g leaf ⁻¹]	RWC [%]	Proline [μ mol g ⁻¹ (d.m.)]	Chl <i>a+b</i> [mg g ⁻¹ (d.m.)]	Car [mg g ⁻¹ (d.m.)]	Zn [mg g ⁻¹ (d.m.)]	MDA [μ mol g ⁻¹ (d.m.)]
Drought							
Control 1	0.180 \pm 0.002b	86.04 \pm 5.7b	8.27 \pm 1.2b	3.12 \pm 0.12b	7.54 \pm 0.2b	3.45 \pm 0.02b	1.29 \pm 0.02b
Drought	0.130 \pm 0.002a	50.27 \pm 5.0a	9.20 \pm 1.5a	1.15 \pm 0.12a	2.48 \pm 0.25a	1.71 \pm 0.02a	5.78 \pm 0.00a
0.1 mM Zn	0.214 \pm 0.001b	73.76 \pm 3.9b	8.33 \pm 1.2b	11.55 \pm 0.72ab	7.81 \pm 0.61b	4.10 \pm 0.02b	1.19 \pm 0.02b
0.5 mM Zn	0.258 \pm 0.002b	81.19 \pm 4.9b	31.80 \pm 1.5ab	14.82 \pm 0.52ab	7.59 \pm 0.51b	5.60 \pm 0.02b	1.16 \pm 0.01b
0.1 mM Zn + D	0.170 \pm 0.001ab	53.08 \pm 5.1a	20.64 \pm 1.5ab	2.23 \pm 0.12ab	7.23 \pm 0.51b	2.40 \pm 0.01ab	2.86 \pm 0.02ab
0.5 mM Zn + D	0.187 \pm 0.002b	73.91 \pm 4.9b	24.45 \pm 1ab	2.56 \pm 0.12ab	6.09 \pm 0.64b	2.26 \pm 0.02ab	2.42 \pm 0.02ab
Rehydration							
Control 2	0.105 \pm 0.002	90.45 \pm 5.0	5.39 \pm 1.2	1.46 \pm 0.02	1.06 \pm 0.005	2.06 \pm 0.02	0.50 \pm 0.002
PDR 0.1	0.120 \pm 0.002*	91.25 \pm 5.0	5.62 \pm 1.5	1.47 \pm 0.02	1.23 \pm 0.005*	4.21 \pm 0.02*	0.36 \pm 0.002*
PDR 0.5	0.168 \pm 0.001*	95.00 \pm 3.0*	12.98 \pm 1.2*	1.54 \pm 0.006	1.42 \pm 0.001*	6.06 \pm 0.02*	0.17 \pm 0.001*

significance difference (LSD) test was used for comparison between pairs of treatments. The data analysis was carried out using a statistical package, *SPSS v. 10* (SPSS Inc., Chicago, IL, USA).

The effect of Zn and its interaction with dehydration stress on growth of tea was significant. Dry mass of leaves decreased by 27.77 % due to dehydration. Dry mass increased in 0.1 and 0.5 mM ZnSO₄ treated plants by 18.9 and 38.9 % relative to control. Zinc also helped

to minimize dehydration induced dry mass loss by 30.76 and 43.8 % at RWC of 53.08 and 73.91 %. Dehydration stress significantly decreased content of Chl *a+b* and Car (Table 1) and Zn enhanced Chl *a+b* and Car content relative to control and to the stressed plant. Thus Zn nutrition protects tea plants from drought injury by reducing dry mass and photosynthetic pigment loss and maintaining RWC.

Table 2. Changes in content of total glutathione, total ascorbate, and H₂O₂ and activities of superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), and glutathione reductase (GR) in control 1 leaves of *Camellia sinensis* and after pretreatment with zinc (0.1 and 0.5 mM ZnSO₄) and drought stress lasting 7 d (D). Further, the same parameters were determined after foliar spraying with water (control 2) or 0.1 (PDR 01) and 0.5 (PDR 05) mM ZnSO₄ and 14 d rehydration after drought for 15 d. Means ± SEs, *n* = 3. Letters 'a' and 'b' indicate significant differences from control and drought, respectively, at *P* < 0.05. In the second experiment, '*' indicates significant differences from control at *P* < 0.05 (LSD test). Several fold higher CAT activity in the second experiment indicates its induction during post stress recovery.

Treatments	Glutathione [μmol g ⁻¹ (d.m.)]	Ascorbate [μmol g ⁻¹ (d.m.)]	H ₂ O ₂ [μmol g ⁻¹ (d.m.)]	SOD [U mg ⁻¹ (prot.)]	POX [U mg ⁻¹ (prot.)]	CAT [U mg ⁻¹ (prot.)]	GR [U mg ⁻¹ (prot.)]
Drought							
Control 1	4.74±0.02b	33.20±2.7b	1.38±0.02b	0.331±0.02b	0.39±0.002b	1.72±0.02 b	0.060±0.002 b
Drought	1.74±0.02a	30.05±2.0a	1.90±0.01 a	2.100±0.05a	0.32±0.002a	0.74±0.01 a	0.003±0.001a
0.1 mM Zn	6.57±0.02ab	33.02±2.9b	1.32±0.01 b	0.361±0.01b	0.76±0.002b	2.13±0.02 b	0.030±0.001 ab
0.5 mM Zn	5.11±0.02ab	35.01±2.9b	1.23±0.01 b	0.203±0.01b	1.10±0.002ab	2.73±0.02ab	0.050±0.001 b
0.1 mM Zn + D	7.80±0.03ab	29.15±2.1a	1.82±0.02 a	0.331±0.01b	0.40±0.001ab	2.38±0.02ab	0.051±0.002 b
0.5 mM Zn + D	7.60±0.02ab	35.54±2.9b	1.23±0.02 b	0.171±0.01b	1.37±0.002ab	2.88±0.02ab	0.112±0.002 ab
Rehydration							
Control 2	12.64±0.02	9.72±0.02	0.99±0.002	0.224±0.001	0.97±0.002	152.23±5.01	0.013±0.001
PDR 0.1	14.86±0.03*	17.20±0.02*	0.65±0.002*	0.521±0.002*	1.23±0.003*	875.02±4.02*	0.035±0.001*
PDR 0.5	18.97±0.05*	19.80±0.03*	0.52±0.001*	0.860±0.002*	1.63±0.002*	959.14±5.02*	0.053±0.001*

Proline acts as an osmoprotectant during stress, and its content increased in tea seedlings during dehydration (Chakraborty *et al.* 2002, Upadhyaya *et al.* 2008). In the present study, proline content in tea leaves increased during water stress (Table 1). The proline content of tea leaves increased by 149.57 and 195.64 % in comparison with control when primed with 0.1 and 0.5 mM ZnSO₄, respectively. Greater accumulation of proline due to ZnSO₄ application can help in maintaining water content, prevent membrane distortion, and act as a hydroxyl radical scavenger (Xu *et al.* 2009). Kaur and Asthir (2015) also reported that proline is a key player in abiotic stress tolerance in plants.

The increased production of H₂O₂ and other ROS cause lipid peroxidation and oxidative damage disrupting metabolic function and cell membrane integrity. Water stress resulted in increased H₂O₂ content in tea plants similarly as reported earlier (Broadley *et al.* 2007, Cakmak, 2000, Upadhyaya *et al.* 2008, Upadhyaya and Panda 2013). Zinc (0.1 and 0.5 mM of ZnSO₄) reduced the stress-induced H₂O₂ content rise by 4.2 and 35.2 % and thus apparently lowered the lipid peroxidation by 50.5 and 58.1 % (Table 2). These results suggests possible roles of zinc in protecting plant cells from

damage by ROS (Cakmak 2000).

Antioxidative defense and redox reactions play a central role in the acclimation of plants to stress, which make glutathione a suitable candidate as a stress tolerance marker in plants (Xu *et al.* 2009, Kao 2015, Nahar *et al.* 2016). In tea plants, dehydration stress caused decrease in glutathione (63.3 %) and ascorbate (9.5 %) content but due to Zn priming, the loss in ascorbate content was ameliorated (Table 2) and glutathione content even increased. Glutathione has a role in sequestration of xenobiotics, heavy metals, *etc.*, and together with ascorbate constitutes the essential component of the ascorbate-glutathione cycle that regulates ROS in the cell. Superoxide dismutase (SOD) regulates the cellular concentration of O₂⁻ forming H₂O₂. In the present study, the SOD activity was increased during dehydration stress indicating enhanced O₂⁻ production. On the other hand, a decrease in the SOD activities by Zn priming in the stressed plant could be due to the lower O₂⁻ production. The CAT activity was reduced by 57 % in tea after 7 d of water stress (Table 2). Dehydration induced decrease in CAT activity may be due to inactivation of enzymes that could lead to the rise in endogenous H₂O₂ content. Zinc application caused increased CAT activities in both well

irrigated (23.8 and 58.7 %) and dehydrated plant (38.4 and 67.4 %). Higher CAT activity could scavenge the hydrogen peroxide formed in the photorespiratory pathway in a stressed C3 plant like tea. Zinc primed plant also showed increased POX activity during both control and water stress conditions (Table 2). POX oxidizes tea catechins to form theaflavin type compounds in presence of H₂O₂ (Sang *et al.* 2004). Zinc resulted in increased activities of both catalase and peroxidase, which efficiently detoxify H₂O₂, and consequently it lowered lipid peroxidation. Decreased GR activity in the stressed plant was observed in the present study (Table 2), but it was enhanced in Zn primed stressed plant. This was also apparent from dehydration induced reduced Zn uptake (50.43 %) that shows 40.3 and 32.16 % increased due to Zn priming (0.1 and 0.5 mM) maintaining higher leaf Zn content during stress.

During post-stress recovery ROS and antioxidants play critical roles (Upadhyaya and Panda 2013). The Zn treatments (spraying with 0.1 and 0.5 mM ZnSO₄) during rehydration resulted in increased leaf dry mass, proline content, and photosynthetic pigments with concomitant

increase in Zn content in leaves by 104 and 194 % in PDR 0.1 and PDR 0.5 plants, respectively (Table 1). Application of Zn reduced H₂O₂ content by 34.3 and 47.5 % in PDR 0.1 and PDR 0.5, respectively, and consequently lowered membrane lipid peroxidation and increased content of total ascorbate and glutathione in tea leaves during recovery (Table 2). Such lower H₂O₂ content and lipid peroxidation could be also attributed to activities of SOD, CAT, POX, and GR during post-stress recovery. They were mostly enhanced due to a foliar spray with ZnSO₄ solutions. Thus, Zn regulated responses of tea to dehydration stress and recovery after rehydration by modulating growth and antioxidant system.

In conclusion, a dehydration imposed during water stress caused several damages in tea leaves, however these damages were ameliorated due to Zn application before stress. Further, post stress recovery after rehydration was improved by foliar Zn application. The present findings suggests that zinc can regulate dehydration and rehydration mediated physiological functions in tea plants.

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