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Zinc Modulates Drought-Induced Biochemical Damages in Tea [*Camellia sinensis* (L) O Kuntze]

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ABSTRACT: Zinc (Zn) is an essential micronutrient that affects the growth and productivity of tea plant. Drought stress causes various biochemical and physiological damages in plants. The present study aims at understanding the role of Zn in modulating drought stress induced growth and biochemical damages in tea plant. The results of the present investigation demonstrated that drought-induced decrease in relative water content (RWC), dry mass of leaf, and antioxidants such as ascorbate and glutathione in the tested tea clones (TV-1, TV-17, and TV-29) was minimized by zinc sulfate ($ZnSO_4$) treatment before water withholding for 7 days. Increase in phenolic content with decrease in hydrogen peroxide (H_2O_2) and lipid peroxidation and differential activities of enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), polyphenol peroxidase (PPO), glutathione reductase (GR), and ascorbate peroxidase (APX) with concomitant increased Zn uptake in leaf suggested Zn modulates drought-mediated biochemical damages in tea plant.

KEYWORDS: *antioxidant, antioxidant metabolism, biochemical damages, Camellia sinensis, drought, growth, zinc*

INTRODUCTION

Drought stress is an important abiotic stress, which induces oxidative damage in tea plant and affects the antioxidant systems, altering different physiological and biochemical processes[1,2] and leading to significant crop losses. Drought induces an increase in reactive oxygen species (ROS) production resulting in various degrees of oxidative damage in different clonal varieties of tea. Drought is a significant limiting factor for agricultural productivity and generally inhibits plant growth through water absorption and nutrient uptake. Drought stress may involve accumulation of mineral elements in plant tissues by affecting root growth, nutrient mobility in soil, and nutrient uptake and hence alter physiological and antioxidative responses.[1−7]

Crop performance may be adversely affected by stress-induced nutritional disorders as a consequence of stress on nutrient availability, competitive uptake, transport, or partitioning within the plant. Therefore, many studies have tested the effects of abiotic stress on growth and mineral nutrition of crop plants[8,9] and the role of various minerals in stress amelioration[10] and enhancement of post stress recovery potential in plants.[11] Zn is an essential nutrient that plays important roles in numerous physiological processes in plants, serving as a cofactor for many enzymes and as the key structural motifs in transcriptional regulatory proteins. A deficiency of Zn decreases growth, but excess Zn is toxic to biological systems through metal-based cytotoxic reactions. Therefore, the uptake and transport of Zn must be strictly regulated. Intracellular Zn homeostasis is achieved through the coordinated regulation of specific transporters engaged in Zn influx, efflux, and intracellular compartmentalization. The level of Zn nutrition may affect plant water relations and alter stomatal conductance. Stomatal conductance and transpiration rates also declined under zinc deficiency. Gas exchange data presented by Hu and Sparks[12] and Sharma et al.[13] indicated that Zn deficiency causes a reduction in the instantaneous transpiration efficiency of leaves. However, Zn fertilization and water stress affect plant−water relationships.[10] Possible roles of Zn in protecting plant cells from damage by ROS and its effect on plant metabolism have also been well reviewed.[8,9] However, little information about the effect of zinc nutrition on drought stress induced biochemical damages in plant is available. The protective role of Zn may be an important response in drought tolerance not only simply affecting the plant−water relationship and dry matter accumulation but also changes in antioxidant balance during drought stress.

The present investigation was undertaken to understand the mechanism of drought stress induced oxidative damage on drought and the role of Zn in modulating the antioxidant function during stress conditions in selected clones of *Camellia sinensis* (L) O Kuntze. We hypothesized that Zn modulates drought-induced biochemical damages in plant cells by counteracting the adverse effect of drought stress in tea plants. In the present study, biochemical and growth responses were investigated in the leaf of *C. sinensis* (L) O Kuntze subjected to $ZnSO_4$ treatment before drought imposition.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Three clonal varieties of *C. sinensis* L. (O) Kuntze (viz. TV-1, TV-17, and TV-29) seedlings of uniform age, 1.5 years old, were procured from Rosekandy Tea Estate, Silchar. These clones were selected to elucidate the effect of Zn and 7 days of drought after treatment with Zn in understanding the

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role of Zn in drought stress amelioration in tea plants. Our previous study showed the drought-sensitive nature of TV-29, whereas TV-1 is tolerant.[2] In the present investigation a moderately tolerant TV-17 and another two clones were used to compare their responses to Zn and its interaction with drought. The seedlings grown in field soil in polythene bags were procured from the nursery of the Rosekandy tea garden and brought to the laboratory. The seedlings were potted after removal of the polythene sleeves and the addition of field soil in earthen pots. The seedlings were watered daily and fertilized with half-strength Hoagland solution once a week.[14] The plants were acclimatized for 30 days under the laboratory conditions and were grown under light and with sufficient irrigation.

After 30 days of acclimatization, experiments were conducted in two phases. In phase I, tea seedlings (TV-1, TV-17, and TV-29) were treated with 100 mL of 0 (distilled water), 100, and 500 μM $ZnSO_4$ in soil once in a week, and leaf sampling was done after 7 days of treatment. The concentration of Zn selected in this study is based on preliminary experiments. In phase II, Zn treatment is followed by water withholding for 7 days (Zn+D); that is, Zn was applied on the last day of irrigation before drought was imposed. After 7 days of drought, plants showed few morphological changes, but 14 days of drought resulted in wilting symptoms. Because this paper evaluates the effect of Zn on drought-induces biochemical damages in tea and Zn translocation is affected by the availability of water in soil, we restricted drought imposition for 7 and 14 days drought data were not considered for such study. The moisture content of the soil used for experimental purpose was 23 ± 2.5% in control conditions, whereas at the end of 7 days of drought stress it was reduced to 8.7 ± 0.5%. The average temperature range during the experimental period was noted as 25.1−32.3 and 12.5−24.7 °C maximum/minimum, respectively. The average relative humidity during the experimentation was 88−96 and 38−67% morning/afternoon, respectively. Fresh leaves of similar age were used; all sampling was done during 8:00−9:00 a.m., and samples were processed within 10 min after collection in polybags. During sampling at least two plants were used in each sampling, and each experiment was repeated three times. Data presented are the mean ± SE of three independent repeats.

Analysis of Dry Mass and RWC of Leaf. Leaf dry mass (g leaf $^{-1}$) was determined after five similarly sized leaves had been dried at 80 °C for 48 h. Leaf samples were weighed immediately after collection for relative water content (RWC) measurement according to the methods of Barrs and Weatherly.[15]

Chlorophyll and Carotenoid Pigments. Concentrations of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll, and total carotenoid (C_{x+c}) were analyzed following the methods of Shabala et al.[16] and Lichtenthaler,[17] respectively. Leaf samples (100 mg) were placed in a 25 mL glass vial, 10 mL of 95.5% acetone was added, and the glass vials were sealed with parafilm to prevent evaporation and then stored at 4 °C for 48 h in the dark. The concentrations of Chl *a*, Chl *b*, and Cx+c were measured using an spectrophotometer type 106 (Systronic, India) at 662, 644, and 470 nm, respectively. The Chl *a*, Chl *b*, total chlorophyll, and Cx+c concentrations in the leaf tissues were calculated according to the following equations:

$$\text{Chl } a = 9.784D_{662} - 0.99D_{644}$$

$$\text{Chl } b = 21.42D_{644} - 4.65D_{662}$$

$$\text{total chlorophyll} = \text{Chl } a + \text{Chl } b$$

$$\text{Cx+c} = \frac{1000D_{470} - 1.90\text{Chl } a - 63.14\text{Chl } b}{214}$$

D_i is an absorbance at the wavelength *i*.

Determination of Metabolite and Antioxidants Concentration. The total phenolic content was extracted from the tea leaves in 2 N HCl. Fresh tea leaves (0.25 g) were boiled for 30 min in 5 mL of 2 N HCl. After cooling, the homogenate was filtered through a Whatman no. 1 paper and the filtrate used to determine total phenolic compounds. Samples (5 mL) were mixed with 0.75 mL of 1.9 M Na_2CO_3 plus 0.25 mL of Folin−Ciocalteu phenol reagent. This mixture was kept in the dark, at room temperature (25 °C), for 1 h, before its absorption was read at 750 nm.[18]

Proline concentration in tea leaves was determined following the method of Bates et al.[19] Fresh leaf samples (0.5 g) were homogenized with 5.0 mL of sulfosalicylic acid (3%) using a mortar and pestle and filtered through Whatman no. 1 filter paper. Two milliliters of filtrate was incubated with 2.0 mL of glacial acetic acid and 2.0 mL of ninhydrin reagent and boiled in a water bath at 100 °C for 30 min. After the reaction mixture had cooled, 4.0 mL of toluene was added and cyclomixed; the toluene phase was used to measure absorbance at 570 nm. Glutathione was extracted and estimated as per the method of Griffith.[20] Leaf tissue was homogenized in 5.0% (w/v) sulfosalicylic acid, and the homogenate was centrifuged at 10000*g* for 10 min. The supernatant (1.0 mL) was neutralized with 0.5 mL of 0.5 M potassium phosphate buffer (pH 7.5). Total glutathione was measured by adding 1.0 mL of neutralized supernatant to a standard solution mixture consisting of 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.5) containing EDTA, 0.2 mL of 6.0 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 0.1 mL of 2.0 mM NADPH, and 1.0 mL of 1 U $(mL)^{-1}$ GR (Sigma Chemicals, St. Louis, MO, USA). The change in absorbance at 412 nm was read at 25 ± 2 °C. For the extraction and estimation of ascorbate, the method of Oser[21] was used. The reaction mixture consists of 2.0 mL of 2% sodium molybdate, 2.0 mL of 0.15 N H_2SO_4, 1.0 mL of 1.5 mM Na_2HPO_4, and 1.0 mL of tissue extract. The reaction mixture was incubated at 60 °C in a water bath for 40 min and cooled and centrifuged at 3000*g* for 10 min, and then the absorbance was measured at 660 nm.

Determination of Superoxide Anion (O_2^-), H_2O_2 Concentration, and Lipid Peroxidation. The level of O^-_2 was assayed spectrophotometrically by measuring the reduction of exogenously supplied nitroblue tetrazolium (NBT) according to the method of Doke.[22] Ten leaf disks (diameter = 3 mm) were immersed in 2 mL of the mixture containing 0.01 M sodium phosphate buffer (pH 7.8), 0.05% NBT, and 10 mM NaN_3 in a beaker (volume of 25 mL). After 60 min of incubation at room temperature, 1.5 mL of the reaction solution was transferred into a test tube and heated at 85 °C for 15 min. Then the solution was cooled, and its absorbance at 580 nm was measured. NBT reducing activity (indicating O^-_2 generation) was expressed as the increase in A_{580} per hour per gram of dry weight.

H_2O_2 was extracted in 5.0% trichloroacetic acid (TCA) from tea leaves using fresh leaf samples (0.2 g). The homogenate was used for the estimation of total peroxide content.[23] The reaction mixture contained TCA (50%), ferrous ammonium sulfate (10 mM), potassium thiocyanide (2.5 M), and plant extract, and the absorbance was read at 480 nm. The level of lipid peroxidation, expressed as malondialdehyde (MDA) content, was determined as 2-thiobarbituric acid (TBA) reactive metabolites. Plant fresh tissues (0.2 g) were homogenized and extracted in 10 mL of 0.25% TBA made in 10 mL of 20% TCA. The extract was heated at 95 °C for 30 min and then rapidly cooled in ice. After centrifugation at 10000*g* for 10 min, the absorbance of the supernatant was measured at 532 nm. Nonspecific turbidity was corrected by subtracting the absorbance value taken at 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.[24]

Extraction and Estimation of Enzyme Activities. Leaf tissues were homogenized with potassium phosphate buffer (pH 6.8, 0.1 M) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1% polyvinyl pyrrolidone (PVP), and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) in a prechilled mortar and pestle. The extract was centrifuged at 4 °C for 15 min at 17000*g* in a centrifuge. For assay of ascorbate peroxidase (APX), extraction buffer was supplemented with 2 mM ascorbate. The supernatant was used for the assay of catalase (CAT), peroxidase (POX), polyphenol oxidase (PPO), superoxide dismutase (SOD), and gluathione reductase (GR). Protein content in enzyme extracts was determined according to the method of Bradford.[25] The activity of CAT was measured by the method of Aebi[26] and was determined by monitoring the disappearance of H_2O_2 at 240 nm by using the extinction coefficient 0.036 mM cm^{-1}. The CAT activity was expressed as micromoles of H_2O_2 destroyed per

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Table 1. Changes in Leaf Dry Mass, Relative Water Content (RWC %), Proline, Chl *a*, Chl *b*, Chl *a*/*b* Ratio, Total Chlorophyll, and Total Carotenoid Content in Three Clonal Varieties of *Camellia sinensis* (TV-1, TV-17, and TV-29) after 7 Days of Zinc Treatment (0, 0.1, and 0.5 mM) and Its Interaction with Drought Stress[a]

clone	treatment	dry mass (mg leaf^{-1})	RWC (%)	proline (μmol g^{-1} DW)	Chl *a* (mg g^{-1} DW)	Chl *b* (mg g^{-1} DW)	Chl *a*/Chl *b* ratio	total chlorophyll (mg g^{-1} DW)	total carotenoid (mg g^{-1} DW)
TV-1	control	0.17 ± 0.002	88 ± 1.2b	5.3 ± 0.2b	2.68 ± 0.2b	1.1 ± 0.15b	2.4 ± 0.02	3.8 ± 0.12b	7.6 ± 0.20b
	drought	0.15 ± 0.001	60 ± 5.1a	16.6 ± 1.5a	1.5 ± 0.1a	0.5 ± 0.02a	2.9 ± 0.02	2.0 ± 0.12a	3.0 ± 0.01a
	0.1 mM (Zn)	0.12 ± 0.001a	83 ± 5.0b	8.5 ± 0.5ab	6.22 ± 0.2ab	5.8 ± 0.32ab	1.0 ± 0.01ab	12.1 ± 0.52ab	8.3 ± 0.51ab
	0.5 mM (Zn)	0.17 ± 0.001	87 ± 3.0b	16.9 ± 1.0a	5.54 ± 0.3ab	5.9 ± 0.32ab	0.9 ± 0.01ab	11.4 ± 0.52ab	7.9 ± 0.41ab
	0.1 mM (Zn+D)	0.19 ± 0.002b	73 ± 5.2b	14.8 ± 1.5a	1.65 ± 0.2	0.5 ± 0.02a	3.0 ± 0.12	2.2 ± 0.12a	5.4 ± 0.61ab
	0.5 mM (Zn+D)	0.25 ± 0.002ab	84 ± 3.2b	16.7 ± 1.6a	1.87 ± 0.2b	0.6 ± 0.02a	2.9 ± 0.12	2.5 ± 0.12a	7.6 ± 0.71b
TV-17	control	0.20 ± 0.002b	88 ± 3.9b	9.9 ± 1.2 b	2.45 ± 0.2	0.4 ± 0.05	5.9 ± 0.22	2.8 ± 0.12	4.2 ± 0.20b
	drought	0.11 ± 0.001a	64 ± 3.5a	17.0 ± 1.0a	2.20 ± 0.1	0.3 ± 0.03	6.1 ± 0.32	2.5 ± 0.12	2.3 ± 0.10a
	0.1 mM (Zn)	0.13 ± 0.001a	77 ± 3.0a	13.5 ± 1.5ab	6.87 ± 0.5ab	3.8 ± 0.05ab	1.8 ± 0.02ab	10.6 ± 0.42ab	9.8 ± 0.51ab
	0.5 mM (Zn)	0.24 ± 0.001ab	87 ± 3.9b	30.7 ± 2.0ab	4.74 ± 0.3ab	5.2 ± 0.05ab	0.9 ± 0.02ab	9.9 ± 0.32ab	5.1 ± 0.21ab
	0.1 mM (Zn+D)	0.17 ± 0.002b	67 ± 3.9a	27.4 ± 1.0ab	1.774 ± 0.1a	0.4 ± 0.01	4.5 ± 0.12b	2.2 ± 0.52ab	4.2 ± 0.21b
	0.5 mM (Zn+D)	0.18 ± 0.001b	71 ± 3.9a	28.6 ± 1.5ab	1.908 ± 0.2a	0.6 ± 0.02b	3.3 ± 0.32ab	2.4 ± 0.12	9.0 ± 0.51ab
TV-29	control	0.18 ± 0.002b	86 ± 5.7b	8.3 ± 1.2b	1.99 ± 0.2b	1.1 ± 0.03b	1.7 ± 0.02b	3.1 ± 0.12b	7.4 ± 0.20b
	drought	0.13 ± 0.002a	50 ± 5.0a	9.2 ± 1.5a	0.86 ± 0.2a	0.3 ± 0.04a	2.9 ± 0.52a	1.1 ± 0.12a	2.4 ± 0.25a
	0.1 mM (Zn)	0.21 ± 0.001b	73 ± 3.9b	8.3 ± 1.2b	7.04 ± 0.3ab	4.5 ± 0.05ab	1.5 ± 0.02ab	11.5 ± 0.72ab	7.8 ± 0.61b
	0.5 mM (Zn)	0.26 ± 0.002ab	81 ± 4.9b	31.8 ± 1.5ab	6.57 ± 0.5ab	8.2 ± 0.05ab	0.8 ± 0.02ab	14.8 ± 0.52ab	7.6 ± 0.51b
	0.1 mM (Zn+D)	0.17 ± 0.001	53 ± 5.1a	20.6 ± 1.5ab	1.81 ± 0.1b	0.4 ± 0.01ab	4.3 ± 0.25ab	2.2 ± 0.12ab	7.2 ± 0.51b
	0.5 mM (Zn+D)	0.19 ± 0.002b	73 ± 4.9b	24.4 ± 1.0 ab	1.98 ± 0.1b	0.6 ± 0.02ab	3.4 ± 0.32ab	2.5 ± 0.12ab	6.1 ± 0.64b

[a]Data presented are the mean ± SE (n = 3). C and D indicate control and 7 days of drought imposed on plants; 7 days of water withholding after $ZnSO_4$ treatment is denoted as Zn+D. Letters a and b indicate significant mean difference from C and D, respectively, at $p < 0.05$ in multiple comparison by LSD test.

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minute per gram of dry weight (DW). POX and PPO was assayed using pyrogallol as substrate according to the method of Kar and Mishra[27] with slight modification, where 5.0 mL of assay mixture contained 300 μM H_2O_2 and 1.0 mL of leaf extract. After the incubations at 25 °C for 5 min, the reaction was stopped by the addition of 1.0 mL of 10% H_2SO_4. The purpurogallin formed was read at 430 nm. For the PPO assay the reaction mixture was the same except H_2O_2 was not added. One unit of enzyme activity is defined as that amount of enzyme which forms 1 μmol of purpurogallin formed per minute under the assay conditions.

The activity of SOD was measured using the method of Giannopolitis and Reis.[28] Three milliliters of assay mixture for SOD contained 79.2 mM Tris-HCl buffer (pH 8.9), having 0.12 mM EDTA and 10.8 mM tetraethylenediamine, bovine serum albumin (3.3×10^{-3}%), 6 0.0 mM NBT, 600 μM riboflavin in 5.0 mM KOH, and 0.2 mL of tissue extract. The reaction mixture was illuminated by placing the test tubes between the two tube lights (Philips 20 W). Reaction was initiated by switching the light on and terminated after 10 min. The increase in absorbance due to formazan formation was read at 560 nm. The increase in absorbance in the absence of enzyme was taken as 100%, and 50% initial was taken to be equivalent to 1 unit of SOD activity. GR was assayed according to the method of Smith et al.[29] The reaction mixture contained 1.0 mL of 0.2 M potassium phosphate buffer (pH 7.5) having 1 mM EDTA, 0.5 mL of 3.0 mM DTNB in 0.01 M potassium phosphate buffer (pH 7.5), 0.1 mL of 2.0 mM NADPH, 0.1 mL of tissue extract, and distilled water to make up a final volume of 2.9 mL. Reaction was initiated by adding 0.1 mL of 2 mM oxidized glutathione (GSSG). The increase in absorbance at 412 nm was recorded at 25 °C over a period of 5 min, spectrophotometrically. The activity has been expressed as absorbance change (ΔA_{412}) g dry mass^{-1} s^{-1}.

The activity of APX was determined according to the method of Nakano and Asada[30] by the decrease in the absorbance of ascorbate at 290 nm. The assay mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2, and enzyme extract. APX activity was calculated by using the extinction coefficient 2.8 mM cm^{-1}. One unit of the enzyme is the amount necessary to decompose 1 μmol of substrate per minute at 25 °C. Phenyl ammonia-lyase (PAL) was extracted from tea leaves in 0.1 M sodium borate buffer (pH 8.8) containing 2 mM β-mercaptoethanol and assayed as described by Chakraborty et al.[31] The assay mixture consisted of 0.5 mL of enzyme extract, 0.1 mL of 30 μM L-phenylalanine, abd 0.1 mL of 300 μM sodium borate buffer (pH 6.8), which was incubated at 40 °C for 1 h, and absorbance was measured at 290 nm.

Zinc Uptake. Leaves from stressed and control plants were briefly rinsed with deionized water and oven-dried at 80 °C for 48 h. One hundred milligrams of dried sample was digested in 5 mL of acid mixture 3:1 (HNO_3/HCl). The sample volume was adjusted to 20 mL with deionized water, and zinc content (mg g^{-1} DW) was measured using an atomic absorption spectrometer (Perkin-Elmer, 3110).

Statistical Analysis. Each experiment was repeated three times, and data are presented are the mean ± standard error (SE). The results were subjected to ANOVA using GLM factorial model on all parameters. LSD test was used for comparison between pairs of treatments. To study the relationship between the changes in lipid peroxidation and ROS generation and zinc uptake, a regression analysis was done. The data analysis was carried out using the statistical package SPSS (ver. 10; SPSS Inc., Chicago, IL, USA).

RESULTS

Growth and Pigments. The effect of Zn and its interaction with drought on three clonal varieties of tea was found to be significant. As indicated in Table 1, dry mass of tea leaf decreased uniformly due to drought; minimum decrease was shown by TV-1 (11%) after 7 days of water withholding. In camparison to control, dry mass was not only increased in Zn-treated plants but it also minimized dry mass loss when plants were treated with Zn before drought stress was imposed. The imposed drought affects photosynthetic pigments significantly. Chl *a*, Chl *b*, total chlorophyll, and Cx+c contents decreased in all of the selected tea cultivars. The decrease in Chl *a*, and Chl *b* was maximum in TV-29 (56 and 74%). The Chl *a*/Chl *b* ratio also increased after 7 days of drought stress. Total chlorophyll and Cx+c content in TV-1, TV-17, and TV-29 were reduced by 46, 10, and 63% and by 60, 43, and 66%, respectively, in drought-stressed plants. Leaf growth depends on its photosynthetic activity, where chlorophyll pigment plays important roles. In comparison with control and stressed plants, Zn treatment enhanced Chl *a*, Chl *b*, total chlorophyll, and Cx+c content in all three tested tea cultivars. With respect to control, total chlorophyll and Cx+c contents increased in TV-1, TV-17, and TV-29, respectively, at 0.1 and 0.5 mM $ZnSO_4$ treatment. As depicted in Table 1, Zn treatment also reduced drought stress induced photosynthetic pigment loss, where maximum increase in total chlorophyll content was maintained in TV-29 (94 and 122%), but total Cx+c content was highest in TV-17 (277%) relative to only drought-stressed plants.

RWC and Proline. Drought-induced decrease in RWC was observed in all of the tested clones of *C. sinensis*. There was a reduction in leaf RWC from 88, 88, and 86% (control plants) to 60, 64, and 50% (stressed plants) in TV-17, TV-17, and TV-29, respectively. Thus, apparently maximum decrease in RWC was shown by TV-29, whereas TV-17 showed the least decrease (Table1). Although RWC did not show significant changes in Zn-treated plants when compared with control, the same is significantly increased in the interaction study, where RWC was higher in all of the clones due to Zn treatment before drought stress imposition relative to only drought-stressed plants (D). There was an increase in RWC of plants subjected to 7 days of drought stress (D) after Zn treatment (Zn+D) when compared to only drought-stressed plants.

In the present study, proline content is increased not only by Zn treatment, but the increase in the same was greater during stress when treated with Zn in all of the tested cultivars. As depicted in Table 1, and increase in leaf proline content in tea plants was observed in Zn, Zn+D, and D stressed plants. Relative to only drought-stressed plants, Zn treatment increases proline content significantly in TV-17 (61 and 68%) and TV-29 (124 and 165%), but TV-1 showed significant proline content relative to control plants only.

Antioxidant Metabolites. Drought stress affects antioxidant metabolism in plants. In the present study, drought stress caused decreased glutathione and ascorbate contents. Such a decrease was checked to some extent due to Zn treatment before drought imposition (Figure 1). The glutathione content was highest in TV-1 in both Zn treatment (38 and 7%) and drought stress after Zn treatment (64 and 60%) relative to control. Also due to Zn treatment, glutathione content increased by 348 and 336% in TV-1 relative to drought-stressed plants. The role of Zn in enhancing the nonenzymic antioxidant function may be attributed to the drought stress amelioration effect. Thus, this nutrient helps in drought tolerance in woody plants by altering the nonenzymic antioxidants. As depicted in Figure 1B, decrease in total ascorbate content in stressed plants was 9, 59, and 9% in TV-1, TV-17, and TV-29, respectively. The effect of zinc treatment before drought also showed some protective role of Zn in regulating the nonenzymic antioxidant level in plants.

Total phenolic content in tea leaves decreased with increasing drought stress. The phenolic content of Zn treated plants decreased relative to control plants, but the effect of Zn

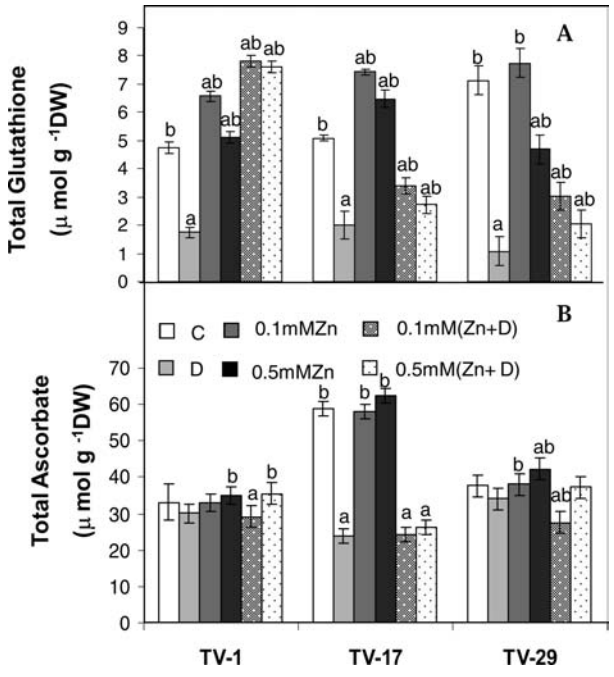

Figure 1. Changes in total glutathione (A) and total ascorbate content (B) of three tea cultivars after 7 days of zinc treatment [0.1 mM (dark gray bars) and 0.5 mM (black bars)] and its interaction with drought stress. Data presented are the mean ± SE (n = 3). C (white bars) and D (light gray bars) indicate control and 7 days drought imposed on plants; 7 days water withholding after $ZnSO_4$ treatment is denoted Zn +D [0.1 mM (gray bars with white dots) and 0.5 mM (white bars with gray dots)]. Letters a and b over the bars indicate significant mean difference from control (C) and drought (D), respectively, at $p < 0.05$ in multiple comparison by LSD test.

treatment helps in maintaining higher phenolic content as compared to stressed plants (Figure 2B). In the stressed plants, decreases in total phenolic content of TV-1, TV-17, and TV-29 were 67, 68, and 47%, respectively. Drought stress induced decreased in total phenolic content was minimized by Zn treatment, where the highest content was shown by TV-1 (810%) when compared with stressed plants only.

ROS and Lipid Peroxidation. Drought stress induced increased ROS generation in plants and caused membrane lipid peroxidation and oxidative damage. Drought stress prompted the production of ROS, namely, O_2^- and H_2O_2 (Figure 2A,C). H_2O_2 and other active oxygen species $OH^•$, $^1O_2$, and O_2^- may be responsible for lipid peroxidation and oxidative damage, leading to disruption of metabolic function and loss of cellular integrity at sites where it accumulates.[32] Drought stress results in increased O_2^- and H_2O_2 content in plants as reported earlier.[2] O_2^- and H_2O_2 contents in leaves of TV-1, TV-17, and TV-29 were 37, 207, and 402% and 37, 241, and 200% more after 7 days of drought, respectively, when compared to control. Zn treatment reduces drought stress induced increased in O_2^- and H_2O_2 contents of different tested tea cultivars (Figure 2A,C). After Zn treatment, the O_2^- contents of TV-1, TV-17, and TV-29 were decreased by 56 and 65, 65 and 46, and 13 and 16%, respectively, relative to only drought-stressed plants. Also, drought-induced H_2O_2 accumulation in tea plants was reduced by 4 and 35, 21 and 40, and 26 and 45% in TV-1, TV-17, and TV-29, respectively, relative to drought-stressed plants (Figure 2C). Lipid peroxidation was measured in terms of MDA. Lipid peroxidation was increased in stressed plants relative to control. As depicted in Figure 2D, stress-induced increased in MDA content was minimized in TV-1 (50 and 58%) and TV-17 (28 and 41%) due to Zn treatment at 0.1 and 0.5 mM concentration, whereas TV-29 (75%) showed a positive effect

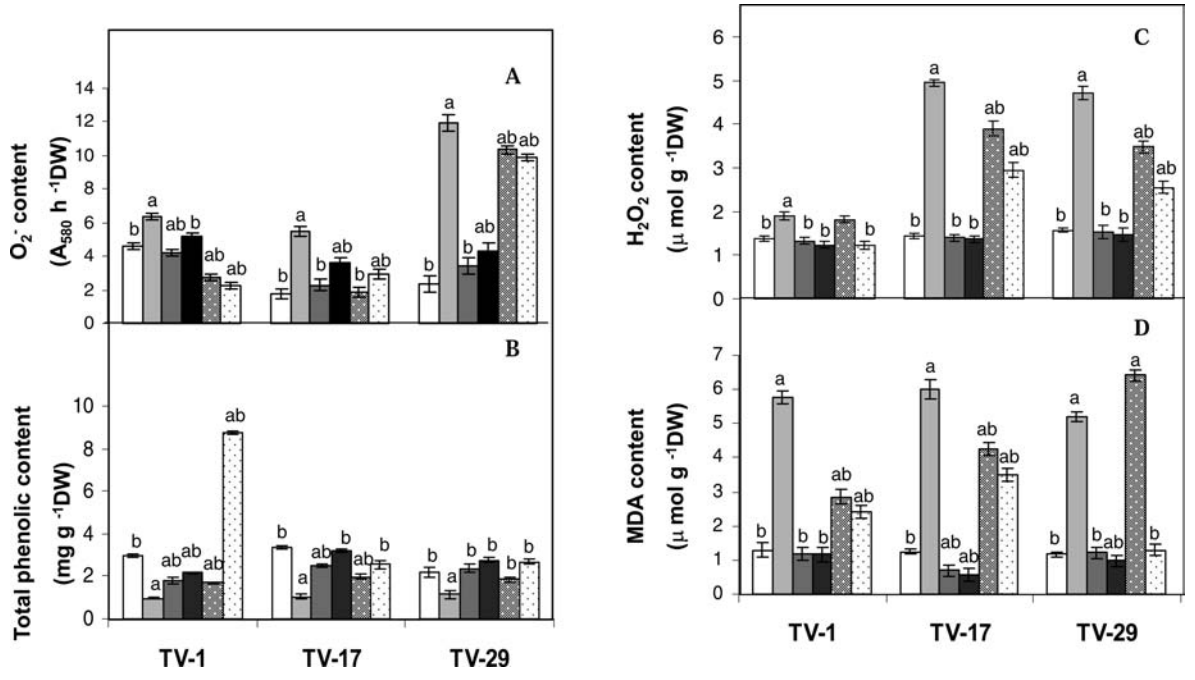

Figure 2. Changes in superoxide anion (A), total phenolics (B), H_2O_2 (C), and MDA (D) contents of three tea cultivars after 7 days of zinc treatment [0.1 mM (dark gray bars) and 0.5 mM (black bars)] and its interaction with drought stress. Data presented are the mean ± SE (n = 3). C (white bars) and D (light gray bars) indicate control and 7 days drought imposed plant; 7 days water withholding after $ZnSO_4$ treatment is denoted Zn+D [0.1 mM (gray bars with white dots) and 0.5 mM (whtie bars with gray dots)]. Letters a and b over the bars indicate significant mean difference from control (C) and drought (D), respectively, at $p < 0.05$ in multiple comparison by LSD test.

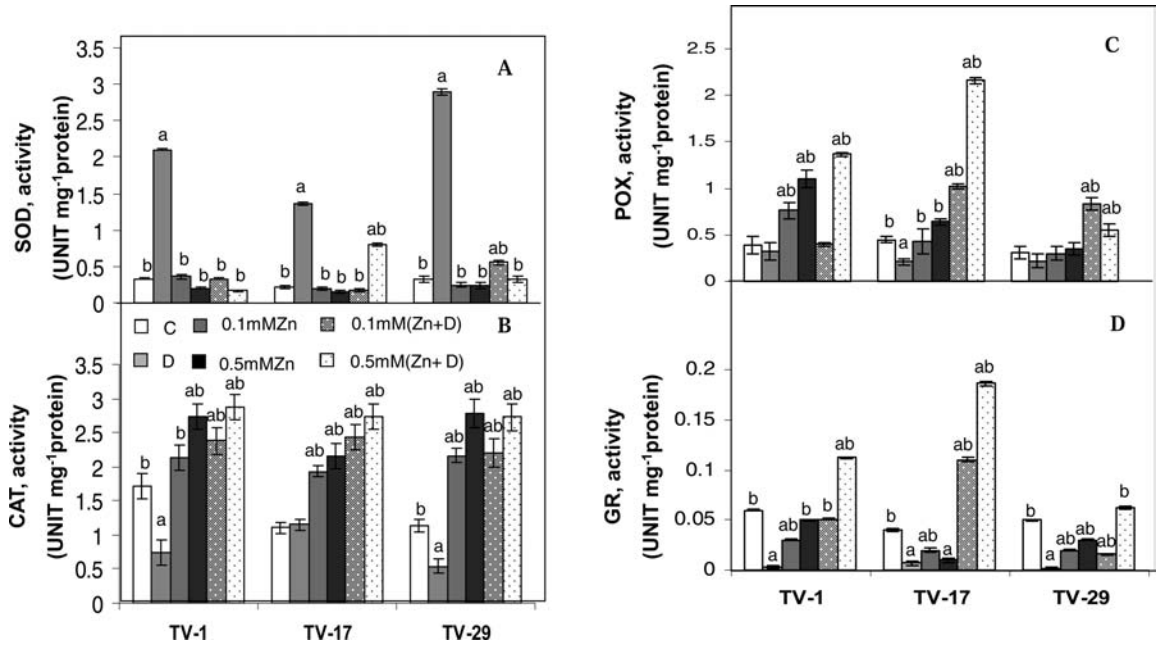

Figure 3. Changes in SOD (A), CAT (B), POX (C), and GR (D) activities of three tea cultivars after 7 days of zinc treatment [0.1 mM (dark gray bars) and 0.5 mM (black bars)] and its interaction with drought stress. Data presented are the mean ± SE (n = 3). C (white bars) and D (light gray bars) indicate control and 7 days drought imposed plant; 7 days water withholding after $ZnSO_4$ treatment is denoted Zn+D [0.1 mM (gray bars with white dots) and 0.5 mM (white bars with gray dots)]. Letters a and b over the bars indicate significant mean difference from control (C) and drought (D), respectively, at $p < 0.05$ in multiple comparison by LSD test.

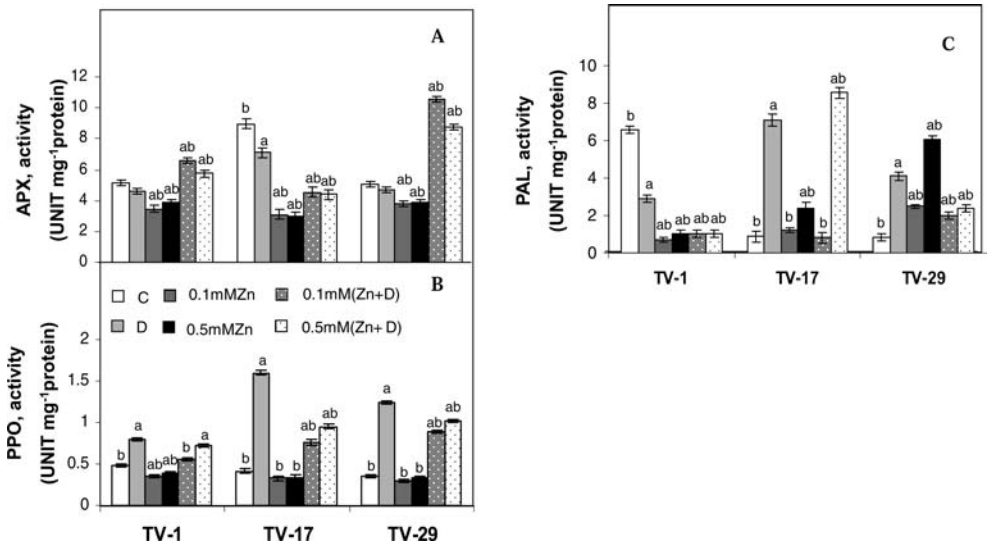

Figure 4. Changes in APX (A), PPO (B), and PAL (C) activities of three tea cultivars after 7 days of zinc treatment [0.1 mM (dark gray bars) and 0.5 mM (black bars)] and its interaction with drought stress. Data presented are the mean ± SE (n = 3). C (white bars) and D (light gray bars) indicate control and 7 days drought imposed plant; 7 days water withholding after $ZnSO_4$ treatment is denoted Zn+D [0.1 mM (gray bars with white dots) and 0.5 mM (white bars with gray dots)]. Letters a and b over the bars indicate significant mean difference from control (C) and drought (D), respectively, at $p < 0.05$ in multiple comparison by LSD test.

at 0.5 mM Zn treatment. This suggests possible roles of Zn in protecting plant cells from damage by ROS.[8]

Antioxidant Enzymes. In the present study, drought stress, Zn, and Zn treatment before drought showed significant effects on antioxidant enzymes. SOD activities were increased in tested tea cultivars due to drought stress, which was decreased by Zn treatment (Figure 3A). The SOD activity of TV-1, TV-17, and TV-29 increased by 534, 507, and 784%, respectively, relative to control after 7 days of drought stress (Figure 3A). CAT activities as depicted in Figure 3B were decreased in TV-1 (56%) and TV-29 (52%), whereas TV-17 (3%) showed no significant changes after 7 days of drought. The effect of Zn in well-irrigated and drought-stressed plants was significant. CAT activity was increased not only in well-irrigated plants, but the same is enhanced in stressed plants due to Zn treatment. After Zn treatment, increases in CAT activities were 221 and 289, 111 and 137, and 307 and 405% in TV-1, TV-17, and TV-29 at 0.1 and 0.5 mM $ZnSO_4$ treatment, respectively, when compared to only drought-stressed plants. The ability of tea clones to enhance CAT activities mediated by

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Table 2. Changes in Zn Uptake (Milligrams per Gram DW) of Three Tea Cultivars after 7 Days of Zn Treatment (0, 0.1, and 0.5 mM) and 7 Days of Drought Stress after Treatment with Zn[a]

			treatments			
			Zn		Zn+D	
clone	C	D	0.1 mM	0.5 mM	0.1 mM	0.5 mM
TV-1	3.5 ± 0.02b	1.71 ± 0.02a	4.1 ± 0.01b	5.6 ± 0.03ab	2.44 ± 0.02b	2.26 ± 0.02ab
TV-17	4.3 ± 0.03b	0.75 ± 0.002a	5.5 ± 0.04b	8.10 ± 0.02ab	15.52 ± 0.02ab	16.69 ± 0.22ab
TV-29	3.45 ± 0.02b	1.0 ± 0.01a	5.3 ± 0.02b	5.7 ± 0.02b	9.35 ± 0.01ab	10.45 ± 0.05ab

[a]Data are presented are the mean ± SE (n = 3). C and D indicate control and 7 days drought imposed plant, respectively; 7 days water withholding after $ZnSO_4$ treatment is denoted Zn+D. Letters a and b in rows indicate significant mean difference from control (C) and drought (D), respectively, at $p < 0.05$ in multiple comparison by LSD test.

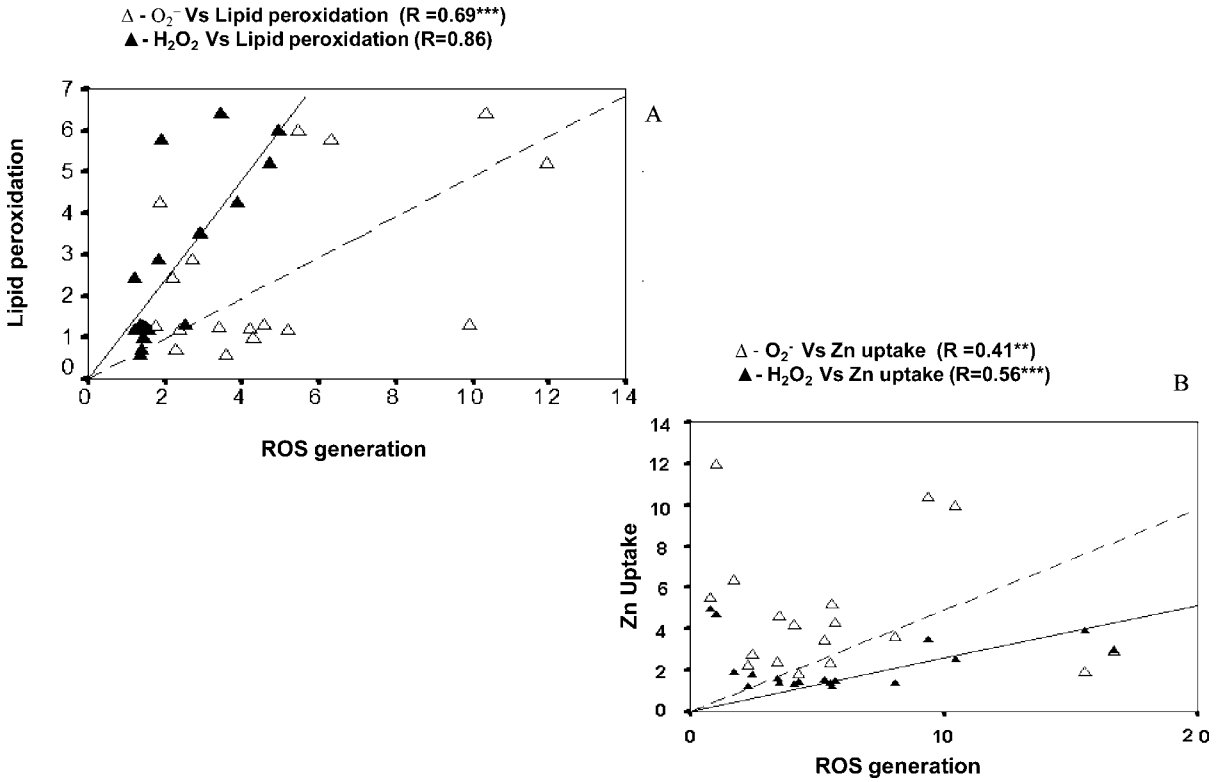

Figure 5. Relationship between the changes in ROS (superoxide anion and H_2O_2) generation and lipid peroxidation (A) and Zn uptake (B) of three tea cultivars after 7 days of zinc treatment and its interaction with drought stress. Data presented are the mean ± SE (n = 3). ** and *** indicate significant correlation at $p < 0.01$ and 0.001, respectively.

Zn during well-irrigated and stress conditions could improve drought stress tolerance. POX activity was decreased in the stressed plant as compared to controls, but Zn-treated plants showed increased POX activity not only in stress conditions but also with sufficient irrigation (Figure 3C). Drought resulted in decreases in POX activities by 18, 53, and 29% in TV-1, TV-17, and TV-29, respectively, relative to control, but Zn-treated plants showed increased activities of the same by 251, 380, and 77 and 328, 928, and 150% at 0.5 mM $ZnSO_4$ treatment in TV-1, TV-17, and TV-29 when compared to both control and control and drought-stressed plants, respectively (Figure 3C). GR activity in stressed plant was decreased by 95, 82, and 96% relative to control (Figure 3D), but in response to both Zn treatment in irrigated and Zn treatment prior to drought imposed in plants, GR activities increased by several-fold in all three tested tea cultivars relative to drought-stressed plants. Increased GR activities in drought condition due to Zn treatment also suggest improvement of antioxidative responses of plants during stressed conditions. APX activity also decreased in stressed plants. APX activity of TV-1, TV-17, and TV-29 decreased by 10, 20, and 6% relative to control (Figure 4A). After Zn treatment before drought imposition, TV-1, TV-17, and TV-29 showed significantly enhanced APX activity by 43 and 24, 36 and 38, and 124 and 86% at 0.1 and 0.5 mM $ZnSO_4$ treatment, respectively, relative to stressed plants. PPO activity increased due to stress in TV-1 (66%), TV-17 (290%), and TV-29 (254%) as compared to control (Figure 4B). Although Zn treatment in well-watered plants did not change PPO activities significantly when compared to control plants, decrease in PPO activities relative to stressed plants was observed in tested tea cultivars. PAL activities in TV-17 and TV-29 increased due to stress by 706 and 400% when compared to control, but, interestingly TV-1 showed a significant decrease in PAL activity by 55% during stress, which is further reduced by Zn treatment as depicted in Figure 4C. The effect of Zn on PAL activities of TV-17 and TV-29 was also different. Zn caused increased PAL activities in both irrigated and drought conditions in both TV-17 and TV-29,

unlike TV-1, where it lowered enzyme activities relative to both controlled and drought conditions.

Zn Uptake. Zn uptake study in the present investigation shows that leaf Zn content decreased due to drought stress by 51, 82, and 71% in TV-1, TV-17, and TV-29, respectively, relative to control. Zn uptake increased in Zn-treated plants grown in well-irrigated condition. Interestingly, Table 2 also shows several-fold increased leaf Zn content in stressed plants due to Zn treatment. Thus, the higher leaf Zn content during drought stress improves stress tolerance in plants.

Correlation between ROS Generation, Lipid Peroxidation, and Zinc Uptake. As indicated in Figure 5, changes in O_2^- are significantly correlated with changes in lipid peroxidation ($R = 0.69$, $p < 0.001$) and changes in leaf Zn content ($R = 0.41$, $p < 0.01$) due to drought, Zn treatment, and Zn treatment before 7 days of drought stress. Although changes in H_2O_2 content did not show significant correlation with changes in lipid peroxidation ($R = 0.86$, NS), it is significantly correlated with changes in leaf Zn content ($R = 0.56$, $p < 0.001$) during drought, Zn treatment, and their interaction in growing seedlings of tea.

DISCUSSION

The dry mass of tea leaf decreased due to drought, suggesting growth arrest in plant. In comparison to control, not only was dry mass increased in Zn-treated plants, but Zn also minimized dry mass loss when plants were treated before drought stress was imposed. The imposed drought affects photosynthetic pigments significantly. Chl *a*, Chl *b*, total chlorophyll, and Cx+c contents decreased in all of the selected tea cultivars. The marked reduction of total Chl in drought-imposed plants was due to the decrease of both Chl *a* and Chl *b* contents. Chl *b* was degraded more than Chl *a* due to drought stress. These results are in contradiction with earlier reports.[33,34] Such drought stress induced reduction in Chl content has been ascribed to loss of chloroplast membranes, excessive swelling, distortion of the lamellae vesiculation, and the appearance of lipid droplets. Drought induced degradation of both total chlorophyll and Cx+c in tea plant. Decrease in chlorophyll and Cx+c in response to drought stress was reported earlier.[1] Such degradation of chlorophyll pigments may eventually decrease photosynthetic efficiency in plants, which might be one of the potent causes of reduction in growth of plant. However, a study by Sheshshayee et al.[35] also provides evidence for a significant positive relationship between transpiration efficiency and leaf chlorophyll concentration in plants. In comparison with both control and stressed plants, Zn treatment enhanced Chl *a*, Chl *b*, total chlorophyll, and Cx+c contents in all three tested tea cultivars. Zn treatment also reduced drought stress induced photosynthetic pigment loss, where maximum increase in total chlorophyll content was maintained in TV-29 (94 and 122%) but total Cx+c content was highest in TV-17 (277%) relative to only drought-stressed plants. Thus, Zn affects plant growth by reducing photosynthetic pigment lost during drought stress.

Drought-induced decrease in RWC was observed in all tested clones of *C. sinensis*. Although RWC did not show significant changes in Zn-treated plants when compared with control, the same is significantly increased in an interaction study, where RWC was higher in all of the clones due to Zn treatment before drought stress imposition relative to only drought-stressed plants. Maintenance of high RWC in drought-resistant cultivars has also been reported to be an adaptation to drought stress in several crop species.[36] A decrease in dry mass of leaf was observed in drought-stressed plants. In comparison to control, not only was dry mass increased in Zn-treated plants but dry mass loss was also minimized when plants were treated with Zn before drought stress was imposed.

An earlier paper suggested an increase in proline content of tea cultivars due to drought stress.[2] Proline acts as an osmoprotectant, and greater accumulation of proline in drought-stressed tea cultivars due to Zn treatment suggested the role of Zn in improving genotypic tolerance of tea to drought stress as proline accumulation helps in maintaining water relations, prevents membrane distortion, and acts as a hydroxyl radical scavenger.[2,37]

Increased total phenolic content during stress condition caused by Zn treatment in tea plants improved the antioxidant potential by influencing the biosynthesis of phenolics. However, plant phenolic metabolites are also reported to function as free radical scavengers and mutagenesis inhibitors.[38,39] In the case of tea, different catechins may be the important phenolic compounds contributing to free radical protection and the antimutagenesis property of the plant.

In the present study, drought stress caused decreases in glutathione and ascorbate contents. Such decreases were checked to some extent due to Zn treatment before drought imposition. The glutathione content was highest in TV-1 in both Zn-treated and Zn-treated condition relative to the other two clones. Glutathione is widely used as a marker of oxidative stress to plants, although its part in plant metabolism is a multifaceted one. As it is a nonprotein sulfur-containing tripeptide, glutathione acts as a storage and transport form of reduced sulfur. Glutathione is related to the sequestration of xenobiotics and heavy metals and is also an essential component of the cellular antioxidative defense system, which keeps ROS under control.[40,41] Antioxidative defense and redox reactions play a central role in the acclimation of plants to their environment, which made glutathione a suitable candidate as a stress marker.[42] The role of Zn in enhancing the nonenzymic antioxidant function may be attributed to its drought stress amelioration effect. Thus, this nutrient helps in drought tolerance in woody plants by altering the nonenzymic antioxidants.

H_2O_2 and other active oxygen species, $OH^{\bullet}$, $^1O_2$, and O_2^-, may be responsible for lipid peroxidation and oxidative damage leading to disruption of metabolic function and loss of cellular integrity at sites where it accumulates.[32] Drought stress results in increased O_2^- and H_2O_2 contents in plants, as reported earlier.[2] Zn treatment prevents the increase in ROS so that concentrations are similar to those in control plants. Lipid peroxidation was increased in stressed plant relative to control. With the exception of cultivar TV-29, Zn treatment minimized the increase in MDA caused by drought stress (Figure 2), which suggests a possible role of Zn in protecting plant cells from damage by ROS.[8]

ROS plays a crucial role in causing cellular damage under drought stress. Drought stress results in increased production of ROS, which oxidizes target molecules. ROS increases the expression of antioxidant genes, leading to increases in the levels of antioxidants and thus enhancing ROS scavenging capacity at the cellular level, conferring tolerance against the stress. Secondary products of ROS in plant cells during stress include lipid peroxides and thiol radicals. Although a series of regulatory mechanisms have evolved within the plant cell to limit the production of these toxic molecules, oxidative damage remains a potential problem, because it causes perturbations in

metabolism, such as a loss of coordination between energy production (source) and energy utilization (sink) processes during photosynthesis in green leaves in stressful environments. Plants have evolved complex protective mechanisms to prevent the damage initiated by the ROS. The primary constituents include antioxidant enzymes such as SOD, CAT, APX, and POD, GR and monodehydroascorbate reductase (MDAR), and free radical scavengers such as carotenoids, ascorbate, tocopherols, and oxidized and reduced glutathione (GSSG and GSH), respectively. SOD regulates the cellular concentrations of O^{-}_{2} and H_2O_2. In the present study, drought stress, Zn, and their interaction revealed significant effects on antioxidant enzyme activities. SOD activities were increased in tested tea cultivars due to drought stress, which was decreased by Zn treatment in stressed plants. However, Zn treatment in unstressed plants did not show significant changes from control and thus protects the level of SOD. Increases in SOD activities in stressed plants were indicative of enhanced O_2^{-} production,[43] but decreases in SOD activities by Zn treatment in stressed plant could be an adaptation to improve growth during stress by regulating the ROS level in plants. However, the exact mechanism of how Zn regulates ROS in stressed plants needs to be understood. Such decreases in SOD activities during post stress recovery in tea clones were also reported earlier.[1] There are many cases of plants growing in hostile environments exhibiting increased oxidative stress enzyme activities to combat the deleterious effect of ROS.[44,45] Although many stress conditions cause an increase in the total foliar antioxidants, little is known of the coordination and control of various antioxidant enzyme activities in tea under drought stress.[2] SOD is reported to play an important role in cellular defense against oxidative stress, as its activity directly modulates the amounts of O^{-}_{2} and H_2O_2, the two substrates of the metal catalyzed site specific Haber−Weiss reaction resulting in generation of the highly reactive OH. SOD catalyzes the conversion of the superoxide anion to H_2O_2 and oxygen. CAT activity was decreased in TV-1 and TV-29, whereas TV-17 showed no significant changes after 7 days of drought. Not only was CAT activity increased in well-irrigated plants, but the same was enhanced in stressed plants due to Zn treatment. The ability of tea clones to enhance CAT activities mediated by Zn during well-irrigated and stress conditions could improve drought stress tolerance. As tea is a C_3 plant, higher CAT activity could scavenge the hydrogen peroxide formed in the photorespiratory pathway and thereby reduce the photorespiration rate.[34]

APX activity, on the other hand, also decreased in stressed plants, but after Zn treatment, APX activity was significantly enhanced relative to stressed plants in all of the tested cultivars. APX appears to play such an essential role in the ROS scavenging process, the processes within the chloroplast associated with oxygen uptake and APX function and the reduction of molecular oxygen have been named "Mehler−ascorbate peroxidase photorespiration".[46] POX activity was decreased in the stressed plant as compared to controls, but after Zn treatment, plants showed increased POX activity not only in stress conditions but also under sufficient irrigation. The role of POX in oxidation of tea catechins to form theaflavin type compounds in the presence of H_2O_2 has also been reported.[47] PPO activity increased due to stress in tea plants. Although Zn treatment in well-watered plants did not change PPO activities significantly when compared to control plants, decrease in PPO activities relative to stressed plants was observed in tested tea cultivars. PPO is a copper-containing enzyme localized in thylakoids of plastids and catalyzes the oxidation of phenolics into toxic substances quinines. It is also involved in defense mechanism of plants against environmental stresses. Furthermore, PPO plays an important role in the production of theaflavins in tea. PPO is widely distributed in plants and plays a role in oxygen scavenging and defense against stress. PPO catalyzes the O_2-dependent oxidation of mono- and *o*-diphenols to *o*-diquinones, where secondary reactions may be responsible for the defense reaction and hypersensivity response. GR also plays a key role in oxidative stress by converting the GSSG to reduced glutathione (GSH) and maintaining a high GSH/GSSG ratio.[48,49] Decreased GR activity in stressed plants was observed in the present study. Increased GR activity in tea leaves due to Zn treatment in stressed plants (TV-1 and TV-17) may be closely related with the stress tolerance capacity of tea plants. However, Zn treatment in unstressed plants did not show significant changes from control and thus protects the level of GR. Glutathione is maintained in a reduced state by GR. However, higher GR activity induced in Zn-treated plant could be an adaptive advantage for plant growth during stress. The activity of PAL, a key enzyme involved in the metabolism of phenolics and lignification of cell wall, showed differential response. PAL activities in TV-17 and TV-29 increased due to stress relative to control, but, interestingly TV-1 showed a significant decrease in PAL activities during stress, which was further reduced by Zn treatment. Zn caused increased PAL activities in both rehydrated and dehydrated conditions in both TV-17 and TV-29, unlike TV-1, in which it lowered enzyme activities relative to both controlled and stressed conditions. PAL is the first and committed enzyme of phenyl propanoid pathways that channels L-Phe from the primary metabolic pool to synthesis of *trans*-cinnamic acid, which is further transformed in plants into phenyl propanoid compounds, which fulfill many essential functions such as mechanical support and protection against stress. Various functions of PAL have been reviewed by McDonald and D'Cunha.[50]

A Zn uptake study revealed Zn content increased in Zn-treated plants grown in well-irrigated conditions. Interestingly, leaf Zn content in stressed plant also shows several-fold increases due to Zn treatment. This indicates that under 7 days of drought condition plants maintain their Zn level, which ameliorates drought-induced biochemical damages. Thus, the higher leaf Zn content during drought stress improves stress tolerance in plants.

In conclusion, treating clones of tea plants with $ZnSO_4$ protected plants from changes caused by drought stress in antioxidant content and levels of antioxidant enzyme activities. As a consequence, the drought-induced increase in H_2O_2 and lipid peroxidation was less in the Zn-treated plants. Data support a role for Zn in improving the potential for tolerance to drought in selected clones of *C. sinensis*. Future genomic analysis of these plants is needed to elucidate the molecular mechanisms by which Zn may mediate drought stress in tea plants.

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Notes

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