biologia plantarum
an international journal for experimental botany

BIOLOGIA PLANTARUM (2023) 67: 45-53 DOI: 10.32615/bp.2023.002

The impact of cadmium stress on the ascorbate-glutathione pathway and ascorbate regeneration in tea plants

H.B. WANG[1], Y.Q. LIU[1], L.L. CHEN[1], X.Q. LI[1], N.H. HA[1,2], T.X. HOANG[2], X.H. LI[1], and X. CHEN[1,*]

[1] *Tea Research Institute, Nanjing Agricultural University, Nanjing 210095, P.R. China*
[2] *Northern Mountainous Agriculture and Forestry Science Institute, Phu Tho 35909, Vietnam*

[*]Corresponding author: E-mail: chenxuan@njau.edu.cn

Abstract

Ascorbic acid (AsA) and glutathione (GSH) contribute to defense responses under abiotic stresses. The present study explored the ascorbate-glutathione cycle and ascorbate regeneration under high concentration (30 mM) of cadmium in the tea plant (*Camellia sinensis* L.). The tea leaves showed speckles and necrosis from the third day of Cd treatment. The content of superoxide anion ($O_2^{·-}$) and hydrogen peroxide (H_2O_2) in the leaves were significantly higher until the seventh day after Cd treatment. The content of $O_2^{·-}$ and H_2O_2 were the highest on the fifth day (212.7 and 153.6 % of the control, respectively). The AsA content increased (86.9 %) on the first day after Cd treatment and decreased significantly in the subsequent days, while GSH showed a reverse trend. The enzymatic activity assays showed that dehydroascorbate reductase (DHAR) and glutathione reductase (GR) involved in AsA regeneration were downregulated considerably after Cd foliar application. In contrast, the activities of ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR) increased on the first day and then declined. Reverse-transcription quantitative PCR showed upregulation of glutathione synthetase (CsGSHS), γ-glutamylcysteine synthetase (Csγ-ECS), and CsMDHAR of the AsA regeneration pathway and downregulation of CsDHAR and CsGR. The expressions of GDP-L-galactose phosphorylase (CsGGP), L-galactose-1-phosphate phosphatase (CsGPP), and L-galactono-1,4-lactone dehydrogenase (CsGaILDH) of the L-galactose pathway were also downregulated. The results indicated that AsA, which can respond to Cd stress of plants by increasing antioxidant ability, was consumed to scavenge ROS; moreover, Cd stress inhibited AsA synthesis and regeneration, which made that tea plants suffering severe damage.

Keywords: AsA-GSH cycle, cadmium stress, *Camellia sinensis*, gene expression.

Introduction

Heavy metals in the atmosphere, land, and water cause severe damage to all organisms. Tea, one of the most important beverage crops, thrives well in acidic soils and, therefore, is vulnerable to exposure to stress associated with heavy metals, such as copper, cadmium, zinc, and lead (Fung and Wong 2002). The vulnerability is attributed to easy absorption of heavy metals from acid soils with low adsorption capacity (Elliott *et al.* 1986, Subba *et al.* 2014). Cadmium is a non-essential element that adversely affects plant growth and development (Printz *et al.* 2016). Due to acidification, the concentration of Cd in the soils of tea plantations is generally above the permissible

---

Received 9 September 2022, *last revision* 21 February 2023, *accepted* 27 February 2023.

Abbreviations: APX - ascorbate peroxidase; AsA - ascorbic acid; CK - control treatment; DHAR - dehydroascorbate reductase; f.m. - fresh mass; GR - glutathione reductase; GSH - glutathione; HPLC - high-performance liquid chromatography; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase; $O_2^{·-}$ - superoxide anion; ROS - reactive oxygen species.

Acknowledgements: This study was financially supported by the National Natural Science Foundation of China (31972457, 32172628), the China Agriculture Research System of MOF and MARA (CARS-19), Jiangsu Agriculture Science and Technology Innovation Fund (JASTIF), (CX(20)3006), Jiangsu Student Innovation Research and Entrepreneurship Training (202110307107Y), the International Joint Project (G20200010081), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD-1). The Expert Workstation of Yunnan Province (202105AF150045), the Nanjing special plan for Shangluo Leading Industry Demand (20201105). We thank Dr. Yuehua Ma (Central laboratory of College of Horticulture, Nanjing Agricultural University) for assistance in using the quantitative real-time PCR.

Conflicts of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

limit (Nejatolahi *et al.* 2014). Moreover, high content of Cd in irrigation water significantly reduce plant biomass (Murtaza *et al.* 2017). Excessive Cd reduces the yield and quality of tea. Content of selected metals (Cu, Mn, Zn, Cd) in tea leaves were investigated, and Cd content exceeded local regulations (Podwika *et al.* 2018). Growing tea plants under excessive Cd in soil has a direct impact on the safety of tea drinking. And it will affect the taste of tea, reduce its nutrition and quality, and ultimately influence people's health.

Stress associated with increased content of reactive oxygen species (ROS) and an imbalance in the antioxidant system leads to cell death (Ahmad *et al.* 2019, Kohli *et al.* 2019). Stress due to heavy metals, such as Cd, also leads to changes in gene expression, antioxidant capacity, redox balance, and leaf anatomy (Dobrikova *et al.* 2022). Meanwhile, cadmium stress resulted in membrane damage, *e.g.*, in rice and *Sedum alfredii* (Tian *et al.* 2011, Thounaojam *et al.* 2012). Cadmium at high concentrations in the soil is toxic to plants; this has been indicated by changes in chlorophyll content and gas exchange parameters (Adamczyk-Szabela *et al.* 2019). Plants respond to cadmium by reducing its absorption and transport, and maintaining redox homeostasis. $O_2^{\cdot-}$ and H_2O_2 production is increased and a situation is further complicated by the production of nitric oxide (NO·). *Arabidopsis thaliana* plants overexpressing tomato metallocarboxypeptidase inhibitor (TCMP-1) displayed a lower amount of Cd accumulation in shoots and an increased expression of heavy metal-associated isoprenylated plant protein (AtHIPP26) in comparison with wild-type plants (Manara *et al.* 2020). Few researchers indicated that different transcription factors, including C2H2, MYB, WRKY, and bZIP family members, respond to Cd stress (He *et al.* 2021). Moreover, two basic helix-loop-helix (bHLH) transcription factors (bHLH29 and bHLH38) were different between two wheat genotypes with contrasting Cd accumulation (Sabella *et al.* 2021).

The ascorbate-glutathione (AsA-GSH) cycle, an important and efficient ROS-scavenging pathway, plays an essential role in the antioxidant defense system in plants (Sarker and Oba 2018a, Zhang *et al.* 2021, Hossain *et al.* 2022). The cycle comprises two non-enzymatic antioxidants, ascorbate (AsA) and glutathione (GSH). Meanwhile, APX (ascorbate peroxidase), MDHAR (monodehydroascorbate reductase), DHAR (dehydroascorbate reductase), and GR (glutathione reductase) are the main enzymatic antioxidant components of the AsA-GSH cycle. Studies have proven that plants regulate the activities of these enzymes and provide adequate protection against oxidative stress caused by Cd stress (Hasanuzzaman *et al.* 2017). AsA-GSH cycle is essential in defending oxidative damage and balancing the redox status of AsA and GSH under abiotic stresses (Sarker and Oba 2018b, 2020). Researchers have found that the high efficiency in AsA-GSH cycle and antioxidant enzyme activities provide increased tolerance to heavy metals in wheat (Shan *et al.* 2018). However, the limiting factors of alleviating tea plant oxidative damage under cadmium stress are not completely clear. Therefore, we employed physiological and molecular techniques to reveal the limiting factors of alleviating tea plant oxidative damage in resisting heavy metal stress in the AsA-GSH system. The findings will provide a useful reference for homeostatic changes in tea plants under cadmium stress.

Materials and methods

Plants, growth conditions, and treatments: One-year-old tea plants [*Camellia sinensis* (L.) O. Kuntze cv. Baiye 1] cultured in plastic containers containing 2 dm^3 of Hoagland's nutrient solution (pH 5.0) were pre-incubated in a growth chamber under a 14-h photoperiod, an irradiance of 200 μmol m^{-2} s^{-1}, a temperature of 25 °C, and a relative humidity of 80 % for one week before treatment. The nutrient solution was continuously aerated every day and changed every 3 d to maintain the pH level and regular supply of plant nutrients. The experimental plants were divided into two groups: control treatment (CK) and Cd treatment (Cd); with 10 tea plants per group, and replicated three times. Depending on the treatment type, 30 mM Cd^{2+} solutions were sprayed twice a day (10 am and 6 pm) lasting 7 d. The CK was sprayed with ultrapure water. The first and second tender leaves were harvested at 1, 3, 5, and 7 d after the last application of treatment. All samples were washed with ultrapure water and immediately frozen in liquid nitrogen, and then stored at - 80 °C for further analyses.

Determination of ROS: Hydrogen peroxide and superoxide anion were extracted using a reagent kit (*Nanjing Jiancheng Bioengineering Institute*, Nanjing, China) according to the manufacturer's instructions: 0.1-g leaf sample was homogenized to fine particles in 1 cm^3 phosphate buffer (50 mM, pH 7.0) using a mixer mill (*MM 400*, *Retsch*, Germany). To ensure sufficient extraction, the solution was mixed thoroughly on a vortex mixer for 30 s and centrifuged at 10 000 *g* for 20 min at 4°C. The reaction mixture was measured by spectrophotometer (*Thermo Scientific*, Wilmington, DE, USA) at 410 nm for H_2O_2 and at 530 nm for $O_2^{\cdot-}$.

Lipid peroxidation: The extent of lipid peroxidation in the tea seedlings was determined from the measurement of malondialdehyde (MDA) content resulting from the thiobarbituric acid (TBA) reaction. Leaf sample (0.1 g) was homogenized in 1.0 cm^3 of 50 mM precooled phosphate buffer (pH 7.2). The solution was mixed thoroughly in a vortex mixer for 30 s and centrifuged at 10 000 *g* for 2 min at 4 °C. The supernatant was separated and used for lipid peroxidase determination (Chen *et al.* 2015). The MDA content was calculated based on absorption at 532 and 600 nm, with a coefficient of absorption of 155 mM^{-1} cm^{-1}, and expressed as μmol g^{-1}(F.M.).

Ascorbate (AsA) content was determined by high-performance liquid chromatography (HPLC). Leaf samples (0.1 g) were homogenized in oxalic acid (1 %; m/v) in a 2-cm^3 centrifuge tube using a mixer mill. Sample analysis by HPLC was performed using the *Shimadzu LC-20A* series (*Shimadzu*, Kyoto, Japan) with a *Hedera ODS-2* C_{18} analytical column (250 mm × 4.6 mm i.d., 5 µm nominal particle size) at 254 nm. 10 mm^3 of extract was injected in HPLC for AsA determination. The AsA content was then quantified by external calibration, and results were recorded as µg g^{-1}(F.M.) (Noctor and Foyer 1998).

Glutathione and GSSG were determined using a reagent kit (*Solarbio Science and Technology Co.*, Beijing, China) according to the manufacturer's instructions. Glutathione was oxidized by 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and reduced by NADPH in the presence of glutathione reductase. The GSSG content was determined after removing GSH by 2-vinylpyridine derivatization. Absorbance readings were recorded at 412 nm. A standard curve was generated to calculate the content of GSH.

Gene expression analysis: Reverse-transcription (RT) quantitative PCR was performed using genes involved in AsA biosynthesis and recycling. Total RNA was extracted from plant samples from each treatment group and controlled using *TRIzol* (*Invitrogen*, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using the *RevertAid* first strand cDNA synthesis kit (*Thermo Scientific*). To ensure the efficiency of optimal polymerization, the amplification length for each gene was restricted to 100 - 200 bp. The reaction program of RT-qPCR was performed in 20 mm^3 of reaction solution containing 1.5 mm^3 of diluted cDNA strand, 7.7 mm^3 of deionized water, 10 mm^3 of *SYBR Premix Ex Taq* (*TaKaRa*, Dalian, China), 0.4 mm^3 of each of forward and reverse primers, using *CsGAPDH* as a reference gene. The PCR amplification was conducted on *Bio-Rad* (Hercules, CA, USA) real-time thermal cycler *CFX96* at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, and 60 °C for the 30 s. All experiments were replicated three times with two biological replicates.

Antioxidant enzymes: Fresh leaves (0.1 g) were ground in 1.0 cm^3 of 50 mM precooled phosphate buffer (pH 7.2) containing 0.1 mM ethylene diamine tetraacetic acid (EDTA), 2 mM AsA, and 5.0 % (m/v) polyvinylpyrrolidone (PVPP). The homogenate was centrifuged at 12 000 *g* and 4 °C for 15 min. The supernatant was used for the determination of APX, MDHAR, DHAR, and GR. Each measurement was replicated three times. Activity of APX was determined by measuring the oxidation rate of AsA at 290 nm, and CAT activity was determined at 25 °C by the decrease in absorbance of H_2O_2 at 240 nm (Li *et al.* 2018). The activities of GR and DHAR were assayed by measuring the decline in the absorbance of NADPH at 340 nm and the increase rate of AsA at 265 nm, respectively (Chen *et al.* 2015). The MDHAR activity was determined at 340 nm. The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, and 0.5 unit ascorbic acid oxidase (AO) (Hossain *et al.* 1984).

Statistical analysis: The data were subjected to one-way analysis of variance (*ANOVA*) and post hoc multiple comparisons using *SPSS version 13.0* (Chicago, IL, USA). Differences were significant at $P < 0.05$. All values are presented as means ± SEs ($n = 3$).

Results

Compared with CK, tea leaves treated with Cd showed many speckles and necrosis; the leaves were completely withered on the seventh day (Fig. 1*A*). Examination of the individual leaves showed necrosis and speckles already on the third day of Cd treatment, which subsequently became severe (Fig. 1*B*).

Compared with CK, Cd treatment induced a significant accumulation of $O_2^{\cdot-}$ in the leaves from the first to the fifth day after treatment, which subsequently declined to initial and control levels by the seventh day (Fig. 2*A*). The content of $O_2^{\cdot-}$ and H_2O_2 in the Cd-stressed tea plants on the fifth day after treatment were 312.7 and 253.6 % higher than in the non-stressed control plants (Fig. 2*A*,*B*). Meanwhile, the MDA content in Cd-stressed plants was higher than in the CK in the first and third day after treatment; however, no significant difference was observed between the two in the fifth and seventh day (Fig. 2*C*).

Compared with the CK, foliar application of Cd significantly decreased the GSH content on the first day; however, the content increased significantly in the subsequent days and maintained higher than that in CK; GSH content was 106.4 % higher than CK on the third day after treatment (Fig. 3*A*). Meanwhile, the GSSG content in the Cd-stressed plants was significantly higher than CK from the first to the fifth day, while the content was considerably lower on the seventh day (Fig. 3*B*). Total glutathione (GSH+GSSG) content in the leaves decreased on the first day and then increased subsequently under Cd stress, reaching a maximum (390.8 µg g^{-1}) on the third day after treatment (Fig. 3*C*).

The AsA and total AsA content in the tea seedlings were 86.9 and 14.1 % higher on the first day after Cd treatment, respectively, which decreased from the third to the seventh day (Fig. 4*A*,*C*). Unlike AsA, the DHA content in the

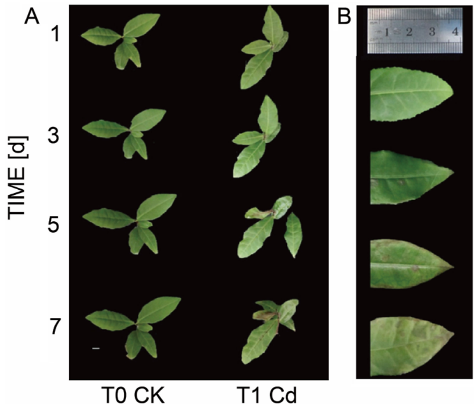

Fig. 1. Phenotypic responses of tea cultivar Baiye 1 during cadmium stress (30 mM) for 1 to 7 d. *A* - whole plants; *B* - single leaves.

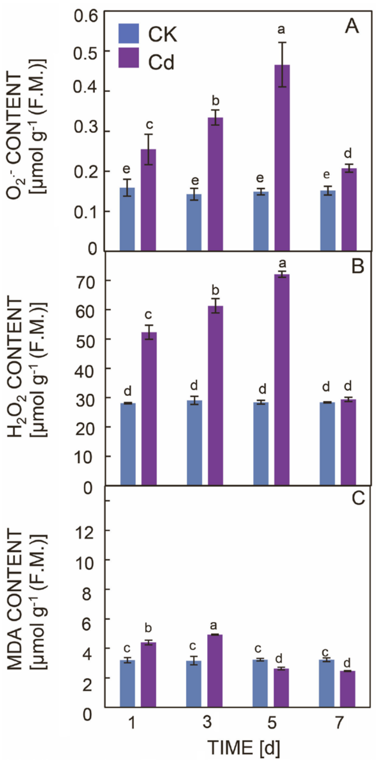

Fig. 2. Effects of Cd treatment on the $O_2^{\cdot-}$ (*A*), H_2O_2 (*B*), and MDA (*C*) content in the leaves of tea seedlings. Means ± SDs of three replicates. Different lowercase letters indicate significant differences ($P < 0.05$; Duncan's multiple range test).

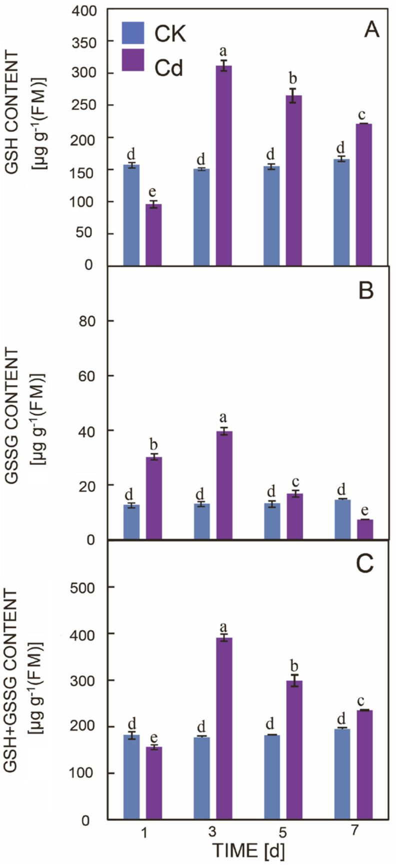

Fig. 3. Change in GSH (*A*), GSSG (*B*), and total glutathione (GSH+GSSG) (*C*) content during Cd treatment. Means ± SDs of three replicates. Different lowercase letters indicate significant differences ($P < 0.05$; Duncan's multiple range test).

Cd-treated leaves did not display a regular pattern. The Cd-treated plants, DHA gradually increased and peaked (670 μg g^{-1}) on the third day and decreased later (Fig. 4*B*).

We analyzed the expressions of 12 genes involved in the L-Gal pathway, GSH biosynthesis, and AsA-GSH cycle in the leaves of tea seedlings under Cd stress by RT-qPCR (Fig. 5). The gene sequences and the primer sequences used in this study are listed in Table 1 and Table 1 Suppl., respectively. The expression of *CsGMP* involved in the Gal-pathway increased from the first to the seventh day under Cd stress, while the expressions of *CsGME*, *CsGGP*, *CsGPP*, and *CsGalLDH* decreased. The tea plants showed high *CsGalDH* expression under Cd stress. Analysis of the GSH pathway and the AsA-GSH cycle components showed high expression of *CsGSHS* and *Csγ-ECS* in tea leaves under Cd treatment. In contrast, the expressions of *CsDHAR* and *CsGR* were remarkably inhibited. Expression of *CsMDHAR* increased steadily from the first to the seventh day after treatment, while expression of *CsAPX* increased from the first day to the third day and subsequently decreased.

Cd treatment resulted in a significant increase (81.2 and 17.2 %) in APX and MDHAR activities on the first and the third day after treatment and then decreased suddenly, compared with CK (Fig. 6*A*,*C*). Meanwhile, Cd stress markedly suppressed the activities of GR throughout the study period. DHAR activity slightly increased on the third day and then decreased under Cd treatment; the DHAR activity was remarkably inhibited on the fifth and seventh day of Cd stress (46.0 and 59.0 %, respectively, compared with CK) (Fig. 6*B*,*D*).

Discussion

Cadmium stress mainly causes ROS accumulation, leading to lipid peroxidation, enzyme inactivation, and DNA and membrane damage (Genchi *et al.* 2020). Cd amplifies free Fe^{2+} by displacing it from the active sites, enhancing Fenton reaction and ROS production (Pető *et al.* 2013). Tea plants under Cd stress produce large amounts of ROS, which increases lipid peroxidation, resulting in severe damage (Morkunas *et al.* 2018). The effects of Cd on the AsA-GSH system are not completely clear in the tea plant. We explored the ascorbate-glutathione cycle and ascorbate regeneration under high concentrations of Cd.

Cd-induced ROS accumulation is mediated and indirectly regulated by NADPH oxidase localized on the plasma membrane; it produces $O_2^{\cdot-}$ from O_2 when exposed to excess of Cd (Jakubowska *et al.* 2015). In the present study, tea plants under Cd stress initially produced more $O_2^{\cdot -}$ and H_2O_2, consequently increasing MDA content, consistent with observations in *Phaseolus vulgaris*, *Lemna minor*, and *Cucurbita pepo* (Zengin and Munzuroglu 2005, Hou *et al*. 2007, Al-Harthi *et al*. 2021). Though the MDA content under Cd stress after the fifth day was significantly lower than on the third day, tea plants were severely damaged at both stages. An increase in ROS content and a redox imbalance can lead to cell death, as reported in *Echinodorus grisebachii* and *Microsorum pteropus* (Yan *et al.* 2018).

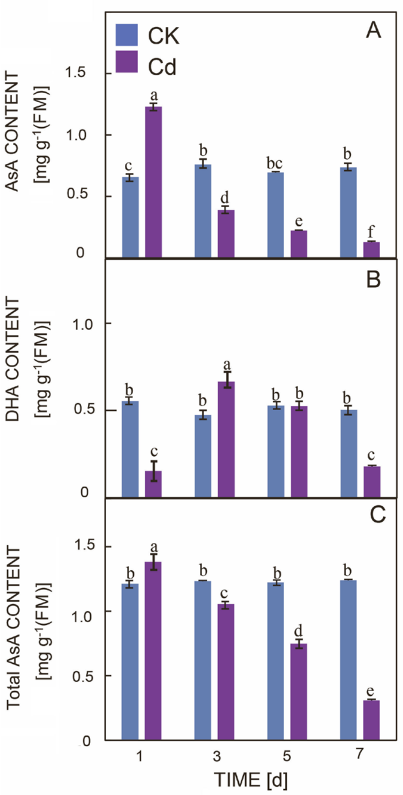

Fig. 4. Changes in content of AsA, DHA, and total AsA in tea seedlings under cadmium stress. Means ± SDs of three replicates. Different lowercase letters indicate significant differences ($P < 0.05$; Duncan's multiple range test).

Glutathione (GSH), as a ROS scavenger, plays a key role in plant survival under salt stress (Wingate *et al.* 1988, Noctor and Foyer 1998). Meanwhile, Cd tolerance of the pepper plants improved with silicon application due to the enhanced antioxidant defense system, plant growth, and the NO, proline, and H_2S content (Kaya *et al.* 2020). Interestingly, NO increases the metal tolerance of plants *via* various key mechanisms, such as increasing antioxidant activity and limiting root-to-shoot metal translocation (Emamverdian *et al.* 2021). Studies have detected an acceptable content of reduced glutathione in plants for normal cell functioning under varying heavy metal stress conditions. In this study, the content of GSH increased from the third day to the seventh day under Cd stress. The transcriptions of glutathione biosynthetic genes, such as *γ-ECS* and *GSHS*, were upregulated under Cd stress. These results are consistent with earlier reports (Hartmann *et al.* 2003) and the changes in GSH content under high temperature (Li *et al.* 2016). GSH decreased significantly on the first day, while GSSG content increased. Several studies have shown that heavy metal stress, including Cd stress, increases GSSG content (Gajewska *et al.* 2013, Qin *et al.* 2018). Glutathione reductase significantly regulates oxidative stress by converting GSSG to reduced glutathione (GSH). However, the GR activity was remarkably inhibited under heavy metal stress, which may be related to higher GSSG content. The results in this study indicate that the synthesis of GSH may have a hysteresis effect on the reaction of copper or cadmium stress in tea plants. A lower GSH content may stimulate the expression of GSH synthetic genes, which in turn regulates GSH content and activates various GSH-mediated defense mechanisms.

Ascorbic acid (AsA) plays a pivotal role in different physiological processes in plants; it acts as a cofactor in the xanthophyll cycle, peptidyl-prolyl and -lysyl hydroxylases, and ethylene production (Eskling *et al.*

Table 1. Primers sequences of the related genes and reference gene used for RT-qPCR.

Name	Forward primer (5'–3')	Reverse primer (5'-3')
CsGGP	TCCAGAAGTGTTGCCTTCCAG	CCTCCTGGAATTACCTTGGT
CsGME	AACTACGGAGCATACACCTATGAGAAC	CTAGCAATGTGCGAGGCAATGAATC
CsGMP	CTTAGTGTCCCAAAGCCTC	TACCAAGTGGCTCAGTCTC
CsGPP	GCTGCTGGTGCTGTGGTAGAAT	CTAGAAGTGACTGCTCCACCTTATCG
CsGalLDH	GGCGGCATTGTTCAGGTTGGT	GTCCACAGCGAGCAAGATAGAATAG
CsGalDH	AAACTAAAGGAAGCAGGGAA	CCATTGAAAGAGGAGAAGCA
CsAPX	AGCAAGGTCACGAAGCCAACAAT	GCAACAACTCCAGCCAACTGATAGA
CsMDHAR	ATTTGCAAGCACAGAAGGGA	CCTTCCCGTCTTTGATCCAG
CsDHAR	ATGATGGAACCGAGCAAGCATTACT	GACAAGTCCGCAGCAGATACTCTT
CsGR	ACCCTGATGGCTAATAAGAATGCTGAA	TAGTATGTGCCTTGCCGAGTAGAGT
Csr ECS	ACATAGGGAGCGGTTTCGAG	TGACAATTGCCCGGTGTCTT
CsGSHS	GAGTTCGCTTCATGGGCTTG	TGTCTCCATTGCCTTTCGGG
CsGAPDH	TTGGCATCGTTGAGGGTCT	CAGTGGGAACACGGAAAGC

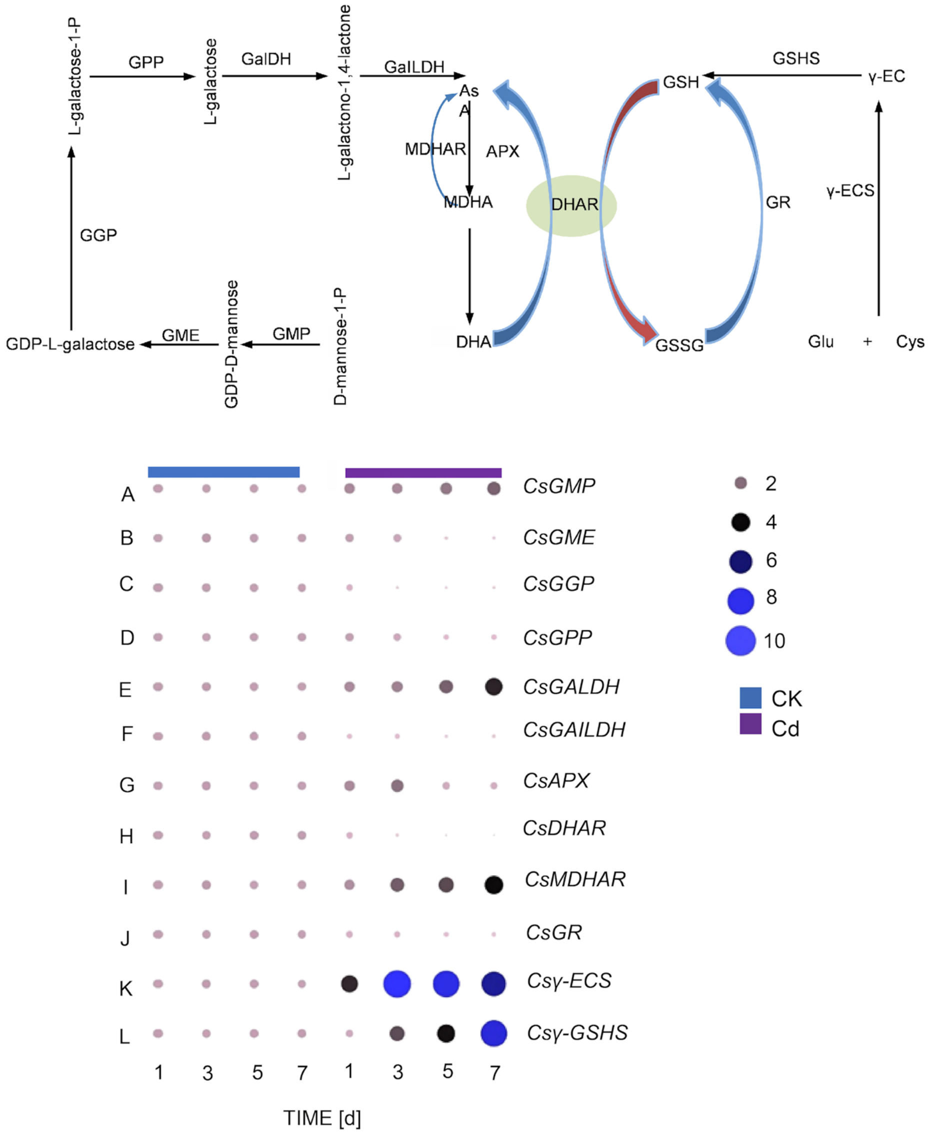

Fig. 5. Biosynthetic pathways and expressions of genes involved in the AsA-GSH system under Cd stress. Different dot size and colors indicate transcription degree.

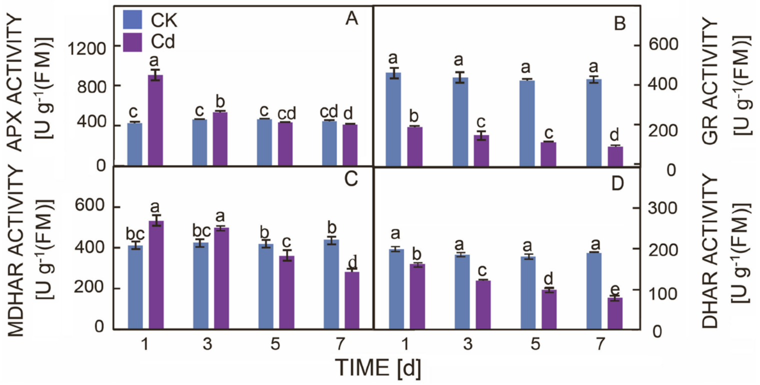

Fig. 6. Effects of Cd treatments on the activities of APX (*A*), GR (*B*), DHAR (*C*), and MDHAR (*D*) involved in the AsA-GSH cycle. Means ± SDs ($n = 3$). Different lowercase letters indicate significant differences ($P < 0.05$; Duncan's multiple range test).

1997). In addition, AsA acts as a potent water-soluble ROS scavenger that converts H_2O_2 to H_2O by the activity of APX, which participates in antioxidation under heavy metal stress (Hasanuzzaman *et al*. 2017, Sarker and Oba 2020). Reduced AsA in tea plants significantly increased on the first day under Cd stress. However, the AsA content was substantially lower than in the control group after three days of treatment. The decrease in AsA content caused damage to tea plants. Meanwhile, the content of DHA increased from day one to day five and then decreased on the seventh day.

The L-Gal pathway is essential for AsA biosynthesis in higher plants (Li *et al*. 2017). The L-Gal-pathway-related genes showed different expression patterns under Cd stress. GDP-D-mannose 3',5'-epimerase (GME) is crucial to ascorbate accumulation and cell wall biosynthesis (Tao *et al*. 2018). Plant GME releases two epimerization products from GDP-D-mannose, namely GDP-L-galactose *via* the L-galactose pathway and GDP-L-gulose *via* an alternative path of L-gulose. Though the expression of the *GME* gene slightly increased on day one of Cd stress, the transcription of the *GGP* gene decreased. The transcriptions of *GPP* and *GalLDH* genes were also significantly inhibited after three days. The results indicated that the L-Gal pathway was inhibited considerably under Cd stress. Meanwhile, MDHAR and DHAR participate in AsA regeneration, using NADPH and GSH, respectively (Noctor and Foyer 1998). The *MDHAR* gene limits the formation of MDHA free radicals *via* enzymatic disproportionation, thereby producing oxidized ascorbic acid (DHA), which is harmful to plant cells. However, GSH-dependent DHAR catalyzed AsA regeneration from DHA. Contrary to DHAR inhibition, the enzyme activities and transcriptions of *MDHAR* and *APX* significantly increased on the first day. *SlRING1* overexpression increased the transcriptions of *CAT*, *DHAR*, and *MDHAR*, which contribute to the antioxidant and detoxification system (Ahammed *et al*. 2021). Moreover, applications of low indole-3-acetic acid (10 µM) showed enhanced tolerance against Cd-induced oxidative stress by up-regulation of the ascorbate glutathione cycle (Bashri and Prasad 2016). In tea plants under Cd stress, the activities of APX and MDHAR and transcriptions of *APX* and *MDHAR* on the first day of heavy metal treatment may be related to the higher content of AsA, which enhanced the ability of cells to scavenge ROS in tea plants. Thus, AsA, which can scavenge H_2O_2, was significantly inhibited at the synthesis pathway, and AsA regeneration from DHA was interrupted due to the suppressed DHAR-mediated AsA-GSH cycle under Cd stress. These finally led to cause irreversible damage to tea plants.

Conclusions

Cadmium stress (30 mM) induced excessive accumulation of ROS and in consequence MDA in tea plants which caused irreversible damage to tea plants. Generally, plants synthesize free AsA and GSH to reduce ROS. However, the present study found that inhibition of the L-galactose pathway and DHAR-mediated AsA-GSH cycle were the limiting factors of alleviating tea plant oxidative damage and compromised the antioxidant capacity of tea plants under continuous Cd treatment. AsA may play a crucial role under Cd stress. The findings of this study provide a theoretical basis for further research on the role of AsA in Cd stress and help breed heavy metal-resistant tea cultivars with high AsA and GSH content. Moreover, the effect of Cd stress on the proteome of *Camellia sinensis* could be analyzed in the future to have a clear view of the effect of Cd on the gene and protein levels.

References

Adamczyk-Szabela D., Lisowska K., Romanowska-Duda Z., Wolf W.M.: Associated effects of cadmium and copper alter the heavy metals uptake by *Melissa officinalis*. - Molecules **24**: 2458, 2019.

Ahammed G.J., Li C.-X., Li X., Liu A., Chen S., Zhou J.: Overexpression of tomato RING E3 ubiquitin ligase gene *SlRING1* confers cadmium tolerance by attenuating cadmium accumulation and oxidative stress. - Physiol. Plantarum **173**: 449-459, 2021.

Ahmad P., Tripathi D.K., Deshmukh R., Singh V.P., Corpas F.J.: Revisiting the role of ROS and RNS in plants under changing environment. - Environ. Exp. Bot. **161**: 1-3, 2019.

Al-Harthi M.M., Bafeel S.O., El-Zohri M.: Gibberellic acid and jasmonic acid improve salt tolerance in summer squash by modulating some physiological parameters symptomatic for oxidative stress and mineral nutrition. - Plants-Basel **10**: 2768, 2021.

Bashri G., Prasad S.M.: Exogenous IAA differentially affects growth, oxidative stress and antioxidants system in Cd stressed *Trigonella foenum-graecum* L. seedlings: toxicity alleviation by up-regulation of ascorbate-glutathione cycle. - Ecotox. Environ. Safe. **132**: 329-338, 2016.

Chen Z.X., Lan Q.Y., Zheng L., Bao Y.X., Luo Y.L.: Effects of dehydration and temperature on seed viability and antioxidative enzymes activities on three kinds of cultivars of *Camellia sinensis*. - Braz. J. Bot. **38**: 497-504, 2015.

Dobrikova A., Apostolova E., Adamakis I.-D.S., Hanć A., Sperdouli I., Moustakas M.: Combined impact of excess zinc and cadmium on elemental uptake, leaf anatomy and pigments, antioxidant capacity, and function of photosynthetic apparatus in clary sage (*Salvia sclarea* L.). - Plants-Basel **11**: 2407, 2022.

Elliott H.A., Liberati M.R., Huang C.P.: Competitive adsorption of heavy metals by soils. - J. Environ. Qual. **15**: 214-219, 1986.

Emamverdian A., Ding Y.L., Barker J., Mokhberdoran F., Ramakrishnan M., Liu G.H., Li Y.: Nitric oxide ameliorates plant metal toxicity by increasing antioxidant capacity and reducing Pb and Cd translocation. - Antioxidants **10**: 1981, 2021.

Eskling M., Arvidsson P.O., Åkerlund H.-E.: The xanthophyll cycle, its regulation and components. - Physiol. Plantarum **100**: 806-816, 1997.

Fung K.F., Wong M.H.: Effects of soil pH on the uptake of Al, F and other elements by tea plants. - J. Sci. Food. Agr. **82**: 146-152, 2002.

Gajewska E., Owacki R., Mazur J., Skłodowska M.: Differential response of wheat roots to Cu, Ni and Cd treatment, oxidative stress and defense reactions. - Plant Growth Regul. **71**: 13-20, 2013.

Genchi G., Sinicropi M.S., Lauria G., Carocci A., Catalano A.: The effects of cadmium toxicity. - Int. J. Environ. Res. Public Health **17**: 3782, 2020.

Hartmann T.N., Fricker M.D., Rennenberg H., Meyer A.J.: Cell-specific measurement of cytosolic glutathione in poplar leaves. - Plant Cell Environ. **26**: 965-975, 2003.

Hasanuzzaman M., Nahar K., Anee T.I., Fujita M.: Exogenous silicon attenuates cadmium-induced oxidative stress in *Brassica napus* L. by modulating AsA-GSH pathway and glyoxalase system. - Front. Plant Sci. **8**: 1061, 2017.

He Q., Zhou T., Sun J., Wang P., Yang C., Bai L., Liu Z.: Transcriptome profiles of leaves and roots of goldenrain tree (*Koelreuteria paniculata* Laxm.) in response to cadmium stress. - Int. J. Environ. Res. Public Health **18**: 12046, 2021.

Hossain M.A., Nakano Y., Asada K.: Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. - Plant Cell Physiol. **25**: 385-395, 1984.

Hossain M.N., Sarker U., Raihan M.S., Al-Huqail A.A., Siddiqui M.H., Oba S.: Influence of salinity stress on color parameters, leaf pigmentation, polyphenol and flavonoid contents, and antioxidant activity of *Amaranthus lividus* leafy vegetables. - Molecules **27**: 1821, 2022.

Hou W.H., Xiao C., Song G.L., Wang Q.H., Chang C.C.: Effects of copper and cadmium on heavy metal polluted waterbody restoration by duckweed (*Lemna minor*). - Plant Physiol. Biochem. **45**: 62-69, 2007.

Jakubowska D., Janicka-Russak M., Kabała K., Migocka M., Reda M.: Modification of plasma membrane NADPH oxidase activity in cucumber seedling roots in response to cadmium stress. - Plant Sci. **234**: 50-59, 2015.

Kaya C., Akram N.A., Ashraf M., Alyemeni M.N., Ahmad P.: Exogenously supplied silicon (Si) improves cadmium tolerance in pepper (*Capsicum annuum* L.) by up-regulating the synthesis of nitric oxide and hydrogen sulfide. - J. Biotechnol. **316**: 35-45, 2020.

Kohli S.K., Khanna K., Bhardwaj R., Abd Allah E.F., Ahmad P., Corpas F.J.: Assessment of subcellular ROS and NO metabolism in higher plants: multifunctional signaling molecules. - Antioxidants **8**: 641, 2019.

Li H., Huang W., Wang G.L., Wang W.L., Cui X., Zhuang J.: Transcriptomic analysis of the biosynthesis, recycling, and distribution of ascorbic acid during leaf development in tea plant (*Camellia sinensis* (L.) O. Kuntze). - Sci. Rep.-UK **7**: 46212, 2017.

Li H., Huang W., Wang G.L., Wu Z.J., Zhuang J.: Expression profile analysis of ascorbic acid-related genes in response to temperature stress in the tea plant, *Camellia sinensis* (L.) O. Kuntze. - Genet. Mol. Res. **15**: 15048756, 2016.

Li X., Wei J.P., Scott E.R., Liu J.W., Guo S., Li Y., Zhang L., Han W.Y.: Exogenous melatonin alleviates cold stress by promoting antioxidant defense and redox homeostasis in *Camellia sinensis* L. - Molecules **23**: 165, 2018.

Manara A., Fasani E., Molesini B., DalCorso G., Pennisi F., Pandolfini T., Furini A.: The tomato metallocarboxypeptidase inhibitor I, which interacts with a heavy metal-associated isoprenylated protein, is implicated in plant response to cadmium. - Molecules **25**: 700, 2020.

Morkunas I., Woźniak A., Mai V.C., Rucińska-Sobkowiak R., Jeandet P.: The role of heavy metals in plant response to biotic stress. - Molecules **23**: 2320, 2018.

Murtaza G., Javed W., Hussain A., Qadir M., Aslam M.: Soil-applied zinc and copper suppress cadmium uptake and improve the performance of cereals and legumes. - Int. J. Phytoremediat. **19**: 199-206, 2017.

Nejatolahi M., Mortazavi S., Ildoromi A.: Levels of Cu, Zn, Pb, and Cd in the leaves of the tea plant (*Camellia sinensis*) and in the soil of Gilan and Mazandaran farms of Iran. - J. Food Meas. Charact. **8**: 277-282, 2014.

Noctor G., Foyer C.H.: Ascorbate and glutathione: keeping active oxygen under control. - Annu. Rev. Plant Physiol. Plant Mol. Biol. **49**: 249-279, 1998.

Pető A., Lehotai N., Feigl G., Tugyi N., Ördög A., Gémes K., Tari I., Erdei L., Kolbert Z.: Nitric oxide contributes to copper tolerance by influencing ROS metabolism in *Arabidopsis*. - Plant Cell Rep. **32**: 1913-1923, 2013.

Podwika W., Kleszcz K., Krośniak M., Zagrodzki P.: Copper, manganese, zinc, and cadmium in tea leaves of different types and origin. - Biol. Trace Elem. Res. **183**: 389-395, 2018.

Printz B., Lutts S., Hausman J.-F., Sergeant K.: Copper trafficking in plants and its implication on cell wall dynamics. - Front. Plant Sci. **7**: 601, 2016.

Qin S.Y., Liu H.G., Nie Z.J., Gao W., Li C., Lin Y.H., Zhao P.: AsA-GSH cycle and antioxidant enzymes play important roles in Cd tolerance of wheat. - Bull. Environ. Contam. Tox. **101**: 684-690, 2018.

Sabella E., Luvisi A., Genga A., De Bellis L., Aprile A.: Molecular responses to cadmium exposure in two contrasting durum wheat genotypes. - Int. J. Mol. Sci. **22**: 7343, 2021.

Sarker U., Oba S.: Catalase, superoxide dismutase and ascorbate-glutathione cycle enzymes confer drought tolerance of *Amaranthus tricolor*. - Sci. Rep.-UK **8**: 16496, 2018a.

Sarker U., Oba S.: Drought stress effects on growth, ROS markers, compatible solutes, phenolics, flavonoids, and antioxidant activity in *Amaranthus tricolor*. - Appl. Biochem. Biotech. **186**: 999-1016, 2018b.

Sarker U., Oba S.: The response of salinity stress-induced *A. tricolor* to growth, anatomy, physiology, non-enzymatic and enzymatic antioxidants. - Front. Plant Sci. **11**: 559876, 2020.

Shan C., Zhang S., Ou X.: The roles of H_2S and H_2O_2 in regulating AsA-GSH cycle in the leaves of wheat seedlings under drought stress. - Protoplasma **255**: 1257-1262, 2018.

Subba P., Mukhopadhyay M., Mahato S.K., Bhutia K.D., Mondal K.M., Ghosh S.K.: Zinc stress induces physiological, ultra-structural, and biochemical changes in mandarin orange (*Citrus reticulata* Blanco) seedlings. - Physiol. Mol. Biol. Pla. **20**: 461-473, 2014.

Tao J.J., Wu H., Li Z.Y., Huang C.H., Xu X.B.: Molecular evolution of GDP-D-mannose epimerase (*GME*), a key gene in plant ascorbic acid biosynthesis. - Front. Plant Sci. **9**: 1293, 2018.

Thounaojam T.C., Panda P., Mazumdar P., Kumar D., Sharma G.D., Sahoo L., Panda S.: Excess copper induced oxidative stress and response of antioxidants in rice. - Plant Physiol. Biochem. **53**: 33-39, 2012.

Tian S., Lu L., Zhang J., Wang K., Brown P., He Z., Liang J., Yang X.: Calcium protects roots of *Sedum alfredii* H. against cadmium-induced oxidative stress. - Chemosphere **84**: 63-69, 2011.

Wingate V.P.M., Lawton M.A., Lamb C.J.: Glutathione causes a massive and selective induction of plant defense genes. - Plant Physiol. **87**: 206-210, 1988.

Yan Y.-Y., Wang J.-J., Lan X.-Y., Wang Q.-M., Xu F.-L.: Comparisons of cadmium bioaccumulation potentials and resistance physiology of *Microsorum pteropus* and

Echinodorus grisebachii. - Environ. Sci. Pollut. Res. **25**: 12507-12514, 2018.

Zengin F.K., Munzuroglu O.: Effects of some heavy metals on content of chlorophyll, proline and some antioxidant chemicals in bean (*Phaseolus vulgaris* L.) seedlings. - Acta Biol. Cracov. Bot. **47**: 157-164, 2005.

Zhang P., Liu L., Wang X., Wang Z., Zhang H., Chen J., Liu X., Wang Y., Li C.: Beneficial effects of exogenous melatonin on overcoming salt stress in sugar beets (*Beta vulgaris* L.). - sPlants-Basel **10**: 886, 2021.