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Boosting *Beta vulgaris* L. resistance to cadmium toxicity: the protective benefits of Zinc

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Abstract

The risk of cadmium (Cd) entering the food chain makes the higher quantity of Cd in soil highly alarming for plant productivity and human health. Zinc (Zn) is an essential micronutrient that is required by plants for their proper growth and development. The study's objective was to ascertain the effectiveness of Zn in the management of Cd toxicity in spinach (*Beta vulgaris* L.), var. All green. The effective concentration of Cd ($Cd_{EC50;}$ 27.42 mg/L) dose was combined with 100, 200, 300, 400, and 500 mg/L of Zn and applied to plants at 20 d after emergence. The effect of Zn on Cd-exposed plants was studied through the response of their biochemical, physiological, and yield characteristics. Application of Zn led to an increase in the stomatal conductance (*gs*) of Cd-treated plants; however, a higher rate of photosynthesis (*Ps*) and decrease in oxidative stress, which stabilized the membrane lipids of the photosynthetic apparatus and facilitated the *Ps* of Cd + Zn treated plants. Improvement in biochemical and physiological characteristics were manifested in yield which was higher in Cd_{EC50} + Zn treated plants, compared to Cd_{EC50} treated plants. The results of the present study suggest that 300 mg/L Zn dose can be used as an efficient tool in managing Cd toxicity in spinach plants. However, more experiments are required to establish a proper Cd-Zn dose, that can be effective on plants under Cd stress.

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Introduction

The continuous interaction of cadmium (Cd) in the soil and food crop system poses a threat to agricultural production and increases risk of contamination in the food chain[1,2]. Cd toxicity in plants is displayed by inhibition of plant growth, reduced chlorophyll synthesis and carbon fixation, generation of oxidative activity, and peroxidation of membrane lipids[3,4]. A high concentration of Cd in soil generates osmotic activity in plants by disturbing the equilibrium between several physiological parameters[5]. The complementary production of reactive oxygen species (ROS) brings about irreversible damage to biomolecules[6,7]. Additionally, Cd interferes with the uptake of several elements such as calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K), and Manganese (Mn)[8]. A high concentration of Cd in agricultural soil and high mobility of Cd intensifies its toxicity potential and facilitates easy movement from one trophic level to another[9,2].

Zinc (Zn) is an essential micronutrient for plants whose deficiency results in stunted growth, delayed maturity, and reduced yield[10]. Zn plays a role in structural integrity, bio physicochemical responses such as assisting in the metabolism of carbohydrates, lipids, and proteins[11,12]. At high concentrations, toxic effects of Zn have been reported in several plants[13]. Cd and Zn are chemically similar and often compete in root uptake, xylem transport, phloem translocation, and accumulation in edible parts[14,15]. Studies have shown that Zn application decreased uptake, translocation and accumulation of Cd in edible parts[14,16,17]. Zn homeostasis is closely related to that of Cd[18]. A study indicated that Zn had no significant effect on Cd uptake and accumulation[19]. Although Zn is an essential micronutrient, its toxic effects are observed at high concentrations[13,20,21].

The objective of the study was to assess the effectiveness of Zn in mitigating Cd toxicity in spinach (*Beta vulgaris* L. var. All green). A preliminary experiment was done to assess EC_{50} Cd dose for spinach (27.42 mg/L)[22], and then the calculated EC_{50} dose was combined with a series of Zn doses. The response of EC_{50} Cd-Zn doses was studied to determine if the structural homology of Zn is useful in the management of Cd toxicity in plants; which Zn dose acts as a transitory stage and defines the differentiation between antagonistic and synergistic responses of the Cd-Zn interactions, and what is the mechanistic approach used by spinach for the management of Cd toxicity through Zn application. Antagonistic, or synergistic, impacts of Cd–Zn combination was evaluated through responses of thiol, proline, APX (ascorbate peroxidase), LPO (lipid peroxidation), and ascorbate production.

Materials and methods

Experimental plot and design

The open-air field experiment was conducted at the Botanical Garden (November 2021–January 2022) of Banaras Hindu University, Varanasi, Uttar Pradesh (25°18' N, 82°1' E; 76.19 m above sea level) in the eastern Gangetic planes of India. The site is characterized by sandy clay loam (45:28:27: sand : silt : clay), pH between 7.20–7.60 ± 0.03, organic carbon content of 0.68%, available nitrogen of 93.04 mg/kg, phosphorous of 12.71 mg/kg,

available potassium of 49.67 mg/kg, and Cd of 0.09 mg/kg. The meteorological data such as average maximum temperature (21.4 °C), minimum temperature (9.1 °C), relative humidity (75.5%), and rainfall (16.5 mm) in the study site, collected from the department of Geology, Banaras Hindu University, Varanasi (India). The experiment was arranged in three replicate pots (upper dimension: 28.5 cm, lower dimension: 18.2 cm, and height: 20.3 cm). Each pot was filled with 4 kg of alluvial soil prepared as per local agriculture practices and 10 tons per acre farmyard was added in the pots. Seeds of spinach were procured from the Indian Institute of Vegetable Research (ICAR-IIVR), Varanasi. Six to seven seeds were sown at 2 cm depth in each pot. After emergence, plants were thinned to five seedlings per pot. Water (100 mL per pot once a week) was maintained and regular manual weeding depending upon weed intensity. At 20 d after emergence (DAE), plants were treated with doses of Cd and Zn in the form of $CdCl_2$ and $ZnSO_4$, respectively. The treatments were: (1) Cd_{EC50} (2) Cd_{EC50} + Zn_{100} (3) Cd_{EC50} + Zn_{200} (4) Cd_{EC50} + Zn_{300} (5) Cd_{EC50} + Zn_{400} (6) Cd_{EC50} + Zn_{500}, and no treatment (control). Where, Cd_{EC50} represents the EC_{50} Cd dose and Zn_{100}, Zn_{200}, Zn_{300}, Zn_{400}, and Zn_{500} depict Zn doses of 100, 200, 300, 400, and 500 mg/L, respectively. Each treatment regime was replicated three times. The Cd_{EC50} treatment served as a control for studying the ameliorative effect of Zn in plants grown under treatments 2 to 6.

Plant sampling and analysis

For the estimation of biochemical and physiological parameters, triplicate samples per pot were randomly selected at 45 DAE.

Pigment contents

Chlorophyll and carotenoid contents were estimated using the formulae of MacLachlan & Zalik[23] and Duxbury & Yentsch[24]. Pigment content was determined by homogenizing 0.5 g of fresh leaves in 20 mL of 80% acetone and then centrifuged at 4,032 × *g* for 15 min. Optical densities were determined at 645 and 663 nm wavelength for chlorophyll contents, and at 480 and 510 nm for carotenoid contents using a double-beam spectrophotometer (Model-2203, Systronics, Lucknow, India).

ROS production and LPO

At 45 DAE, fresh leaf tissue was used for the determination of LPO in terms of malondialdehyde (MDA) content by thiobarbituric acid (TBA) as described by Heath & Packer[25]. For estimation of superoxide ($O_2^{\cdot-}$), At 45 DAE, 0.2 g leaf was extracted in an ice bath in 1 mL 50 mM potassium phosphate buffer containing diethyl di-thiocarbamate (pH 7.8) and then centrifugated at 22,000 × *g* for 15 min and assay mixture optical density taken at 530 nm[26], with a few modifications. Production of hydrogen peroxide (H_2O_2) levels was measured according to Alexieva et al.[27].

Enzymatic and non-enzymatic extraction and assays

Enzymatic antioxidants superoxide dismutase (SOD), APX, and catalase (CAT) were estimated with procedures described by Fridovich[28], Nakano & Asada[29] and Aebi[30], respectively. Non-enzymatic antioxidants ascorbic acid (AsA), were estimated by following the method of Keller & Schwager[31].

Metabolite contents

Total phenolic, thiol, and proline content, were estimated following Bray & Thorpe[32], Arvind & Prasad[33], and Bates et al.[34], respectively. For estimation of total phenolics, a leaf sample was extracted in acetone, using the Folin-Ciocalteu reagent and Na_2CO_3. Thiol content was determined using Ellman's reagent (DTNB)-5,5'-dithio-bis-(2-nitrobenzoic acid) and 0.02 M EDTA added to fresh leaf extract. For determination of proline content, 0.5 g of fresh leaf tissue was homogenized in 3% sulphosalicylic acid and the mix was filtered through Whatman filter paper (2). Chromophore containing toluene was extracted from the aqueous phase, and absorbance was determined at 520 nm.

Protein content

Protein content was quantified using the method of Lowry et al.[35]. Fresh leaf tissue (0.5 g) was extracted in 5 mL tris buffer (0.1 M, pH 6.8) and centrifuged at 5,000 × *g* for 5 min. Five mL of TCA was added to the supernatant and re-centrifuged at 6,000 × *g* for 10 min and absorbance was taken at 650 nm on a double beam spectrophotometer (Model-2203, Systronics, Lucknow, India).

Physiological parameters

Rates of photosynthesis (*Ps*) and stomatal conductance (*gs*) were measured using a Portable Photosynthetic System (Model LI6400XT, Version 6.2, Lincoln, NE, USA). A flag leaf from three randomly selected plants per treatment were chosen for *Ps* and *gs* observation. Measurements were done on cloud-free days between 900 and 1,000 h at 45 DAE. Photosynthetically active radiation (PAR) was set at 1,200 μmol/m²/s using a known CO_2 source (510 mol/mol concentration) for calibration. Gas measurements were made at a constant flow rate of 500 mmol/s.

Yield

Five plants per treatment were selected and the yield was quantified as the fresh weight of the above-ground edible portion of plants.

Statistical analysis

All data were subjected to principal component analysis (PCA) and one-way ANOVA using IBM SPSS/PC+ (ver. 25.0, x64, TEAM EQX, MICROSOFT corporation) software. Before performing one-way ANOVA, normality of data was tested by the Shapiro-Wilk test and differences between treatments were above 0.05. The relationships between variables were evaluated using linear regression analysis. Correlation coefficients and bar and staked graphs were drawn between parameters and Cd and combined Cd, Zn dose using Origin 9.0 (Origin Lab, Northampton, MA, USA).

Results

Evaluation of toxic effects of Cd_{EC50} on plants, in which the response of plants treated with Cd_{EC50} dose was studied, as compared to the control (no treatment). While studying the effect of Zn, Cd_{EC50} dose-treated plants served as control and Cd_{EC50} + Zn_{100}, Cd_{EC50} + Zn_{200}, Cd_{EC50} + Zn_{300}, Cd_{EC50} + Zn_{400}, Cd_{EC50} + Zn_{500}.

Response of plants treated with Cd_{EC50} dose

In plants treated with Cd_{EC50}, the increment was observed in SOD (2.43%), APX (4.31%), CAT (11.32%), AsA (10.72%), and LPO (30.46%) and reduction was observed in chlorophyll a (20.43%), chlorophyll b (25.16%), protein (15.41%), and *Ps* (28.13%) (Fig. 1).

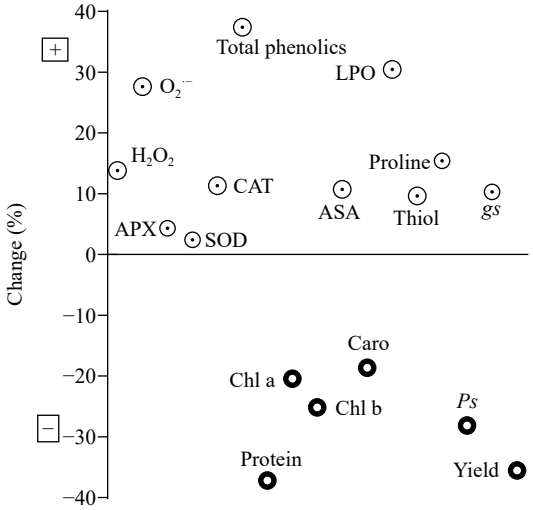

Fig. 1 Assessment of toxic effects of EC_{50} Cd dose on selected characteristics of *Beta vulgaris* L. Upper quadrant is showing a positive percentage change and lower quadrant is showing a negative percentage change in enzymatic, nonenzymatic, pigment contents, and physiological parameters during treatments. LPO: lipid peroxidation, H_2O_2: hydrogen peroxides, $O_2^{\cdot-}$: superoxide, SOD: superoxide dismutase, CAT: catalase, AsA: ascorbic acid, APX: ascorbate peroxidase, *Ps*: net photosynthetic rate, *gs*: stomatal conductance, chl a: chlorophyll a, chl b: chlorophyll b, caro: carotienoid.

Response of plants treated with Cd_{EC50} and different Zn doses

Leaf pigment content

In plants treated with Cd_{EC50} and Zn doses leaf pigments chlorophyll a, b, total chlorophyll, and carotenoid varied (Fig. 2). Chlorophyll a and chlorophyll b increased by 18%, 42%, 48%, 14%, and 4% and 25%, 42.86%, 46.43%, 32.14%, and 7.14% under Cd_{EC50} + Zn_{100}, Cd_{EC50} + Zn_{200}, Cd_{EC50} + Zn_{300}, Cd_{EC50} + Zn_{400}, and Cd_{EC50} + Zn_{500} mg/L, respectively, compared to the control (Cd_{EC50}). A similar pattern occurred for carotenoid which increased 12.12%, 21.21%, 39.39%, and 3.03% under Cd_{EC50} + Zn_{100}, Cd_{EC50} + Zn_{200}, Cd_{EC50} + Zn_{300}, and Cd_{EC50} + Zn_{400} mg/L whereas reduced by 9.09% at Cd_{EC50} + Zn_{500} mg/L, respectively compared to the control (Cd_{EC50}) (Fig. 2).

ROS production and LPO

There was a decrease in ROS production and LPO. The H_2O_2 decreased by 7.51%, 10.79%, and 49.76% at Cd_{EC50} + Zn_{100}, Cd_{EC50} + Zn_{200}, and Cd_{EC50} + Zn_{300} mg/L, respectively, there were increases at Cd_{EC50} + Zn_{400} (5.16%) and Cd_{EC50} + Zn_{500} mg/L (14.55%) over the control (Cd_{EC50}). The $O_2^{\cdot-}$ decreased by 14.71%, 29.41%, 32.35%, and 8.82% at Cd_{EC50} + Zn_{100}, Cd_{EC50} + Zn_{200}, Cd_{EC50} + Zn_{300}, and Cd_{EC50} + Zn_{400} mg/L, respectively. There was an increase at Cd_{EC50} + Zn_{500} mg/L by 1.47%. The LPO decreased by 23.23%, 26.38%, and 29.92% at Cd_{EC50} + Zn_{100}, Cd_{EC50} + Zn_{200}, and Cd_{EC50} + Zn_{300} mg/L. There was an increase at Cd_{EC50} + Zn_{400} mg/L (4.33%), and Cd_{EC50} + Zn_{500} mg/L (31.89%) compared with the control (Cd_{EC50}) (Fig. 3a).

Antioxidative response

Enzymatic antioxidants increased up to Cd_{EC50} + Zn_{300} mg/L of APX (3.28%, 7.38%, and 16.40%) and CAT (2.28%, 18.24%, 59.61%); for SOD there was an increase up to Cd_{EC50} + Zn_{400} mg/L (2.28%, 18.24%, 59.61%, and 6.84%). There were lessened activities of APX (2.46%, 16.39%), CAT (0.66%, 2.5%) and SOD

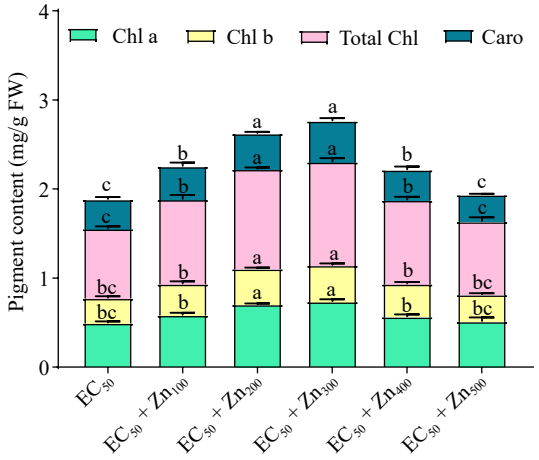

Fig. 2 Variations in pigment contents (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids) of *Beta vulgaris* L. plants treated with combined EC_{50} Cd and doses of Zn at 45 DAE. Bars represent Mean ± S.E. Different colors represent photosynthetic pigments (green; chlorophyll a, yellow; chlorophyll b, pink; total chlorophyll and teal blue; carotenoid) ($p < 0.05$).

(12.70%, 14.90%) at Cd_{EC50} + Zn_{400}, and Cd_{EC50}+Zn_{500} mg/L, respectively, over the control (Cd_{EC50}). Maximum increases occurred in all enzymatic antioxidant pools (APX, CAT, and SOD) at Cd_{EC50} + Zn_{300} mg/L. The AsA contents decreased in all treatments by 2.33%, 44.19%, 30.23%, and 36.05% at Cd_{EC50} + Zn_{100} Cd_{EC50} + Zn_{200}, Cd_{EC50} + Zn_{400}, and Cd_{EC50} + Zn_{500} mg/L, respectively, compared with control (Cd_{EC50}) plants. The highest decrease was for Cd_{EC50} + Zn_{300} mg/L treatments (50%) (Fig. 3b).

Metabolite contents

Total phenolic contents declined by 13.19%, 15.35%, and 17.32% at Cd_{EC50} + Zn_{100} Cd_{EC50} + Zn_{200}, and Cd_{EC50} + Zn_{300} mg/L. They increased by 10.03% and 33.86% at Cd_{EC50} + Zn_{400} and Cd_{EC50} + Zn_{500} mg/L, respectively, compared with control (Cd_{EC50}) plants. Thiol content declined by 8.55%, 12.82%, 16.24%, 24.79%, and 74.36% at Cd_{EC50} + Zn_{100}, Cd_{EC50} + Zn_{200}, and Cd_{EC50} + Zn_{300}, Cd_{EC50} + Zn_{400}, and Cd_{EC50} + Zn_{500} mg/L, respectively, compared with the control (Cd_{EC50}). Proline increased by 0.77%, 24.58%, and 32.14% at Cd_{EC50} + Zn_{100}, Cd_{EC50} + Zn_{400}, and Cd_{EC50} + Zn_{500} mg/L and declined by 0.13% and 0.38% at Cd_{EC50} + Zn_{200}, Cd_{EC50} + Zn_{300} mg/L, respectively, compared with the control (Cd_{EC50}) (Fig. 3c).

Protein content

Protein content increased by 17.63%, 35.81%, 39.75%, and 7.66% at Cd_{EC50} + Zn_{100}, Cd_{EC50} + Zn_{200}, Cd_{EC50} + Zn_{300}, and Cd_{EC50} + Zn_{400} mg/L, but decreased by 14.85% at Cd_{EC50} + Zn_{500} mg/L, respectively, compared to the control (Cd_{EC50}) (Fig. 3c).

Rate of photosynthesis and stomatal conductance

The photosynthesis and stomatal conductance (*Ps* and *gs*) increased, with greater increases at Cd_{EC50} + Zn_{300} mg/L. *Ps* showed by 3.32%, 9.90%, 22.64%, and 1.93% at Cd_{EC50} + Zn_{100}, Cd_{EC50} + Zn_{200}, Cd_{EC50} + Zn_{300}, and Cd_{EC50} + Zn_{400}, but decreased at Cd_{EC50} + Zn_{500} mg/L (4.47%) treatment. Values of *gs* increased by 5.71%, 7.86%, and 10.71% at Cd_{EC50} + Zn_{100}, Cd_{EC50} + Zn_{200}, and Cd_{EC50} + Zn_{300}, but decreased at Cd_{EC50} + Zn_{400} (8.57%) and Cd_{EC50} + Zn_{500} mg/L (14.29%), respectively, compared with the control (Cd_{EC50}) (Table 1).

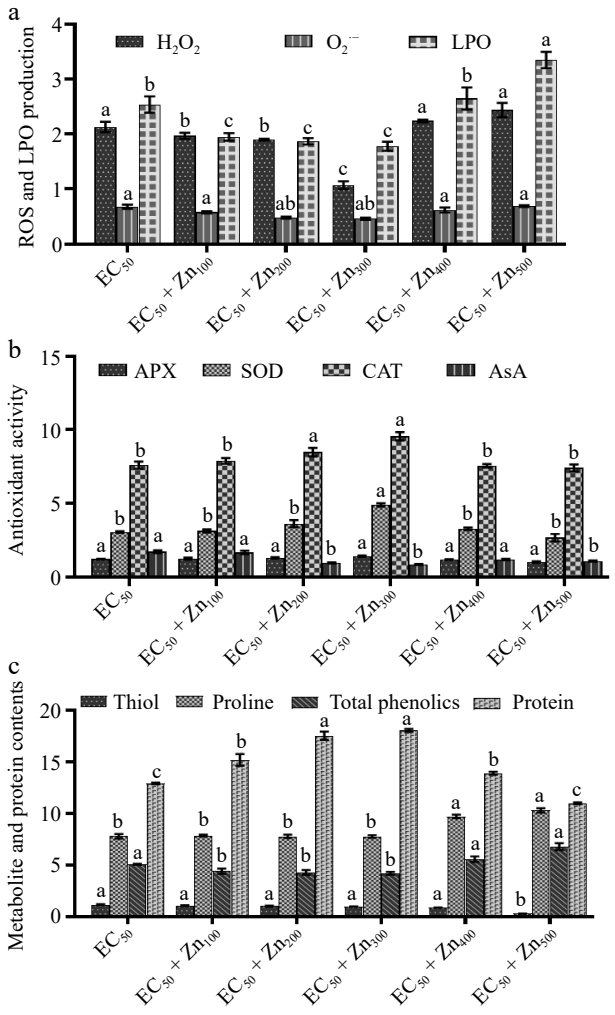

Fig. 3 Bar graph showing variations in the response of (a) ROS (H_2O_2; µmol/g FW and $O_2^{\cdot-}$; nmol/min/g), malondialdehyde (MDA) content; nmol/mL. (b) Antioxidant (ascorbate peroxidase; APX; nmol/min/g FW catalase; CAT; µM H_2O_2 oxidized min^{-1} g^{-1} FW, superoxide dismutase; SOD; g^{-1} FW) and ascorbic acid; AsA; mg/g FW). (c) Protein; mg/g DW, and metabolites (thiol; µM DW, proline; mg/g FW and phenol; mg/g FW) of *Beta vulgaris* L. plants treated with combined EC_{50} Cd and doses of Zn at 45 DAE. X-axis shows doses of Zn with EC_{50} Cd dose. Bar are mean ± S.E. Bars with different letters indicate significant variation at $p < 0.05$.

Yield

Plant yield increased by 4.01%, 23.06%, 26.57%, and 3.25% at $Cd_{EC50} + Zn_{100}$, $Cd_{EC50} + Zn_{200}$, $Cd_{EC50} + Zn_{300}$, and $Cd_{EC50} + Zn_{400}$ mg/L, respectively, but decreased by 12.03% at the higher dose of Zn ($Cd_{EC50} + Zn_{500}$ mg/L), compared to the control (Cd_{EC50}) (Fig. 4).

Discussion

Absorption of Cd from the soil by plants expedites toxic effects on plants but results in its inclusion in the food chain. The persistent and bio-accumulative nature of Cd further augments the problem of Cd toxicity in biological systems[36]. Cd toxicity in plants has become a topic of investigation because of its continuously increasing accumulation in agricultural soils owing to the extensive use of fertilizers and

Table 1. Variation in rate of photosynthesis (*Ps*; mmol $CO_2/m^2/s$) and stomatal conductance (*gs*; mmol $CO_2/m^2/s$) of *Beta vulgaris* L. plants treated with combined EC_{50} Cd and doses of Zn at 45 DAE.

Treatment	Rate of photosynthesis (*Ps*; mmol $CO_2/m^2/s$)	Stomatal gas conductance (*gs*; mmol $CO_2/m^2/s$)
EC_{50}	$16.56^{cd} \pm 0.376$	$1.40^{c} \pm 0.25$
$EC_{50} + Zn_{100}$	$17.11^{c} \pm 0.664$	$1.48^{b} \pm 0.05$
$EC_{50} + Zn_{200}$	$18.20^{b} \pm 0.459$	$1.51^{b} \pm 0.25$
$EC_{50} + Zn_{300}$	$20.31^{a} \pm 0.344$	$1.55^{a} \pm 0.09$
$EC_{50} + Zn_{400}$	$16.88^{cd} \pm 0.485$	$1.28^{d} \pm 0.11$
$EC_{50} + Zn_{500}$	$15.82^{e} \pm 0.437$	$1.20^{d} \pm 0.05$

Values are mean ± S.E. Different letters indicate significant variation at $p < 0.05$.

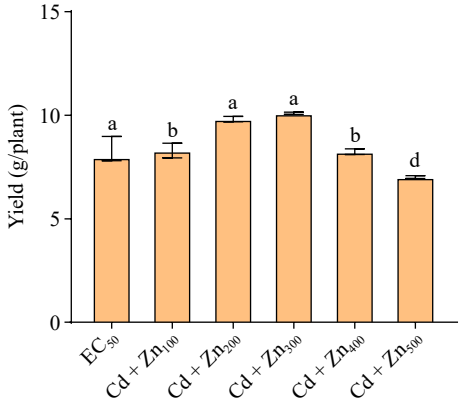

Fig. 4 Bar graph showing variations in yield of *Beta vulgaris* L. plants treated with combined EC_{50} Cd dose and different doses of Zn at 45 DAE. Bar are mean ± S.E. Bars with different letters indicate significant variation at $p < 0.05$. The Y-axis showed the unit of yield (g/plant).

pesticides[37,38]. The major cause of Cd toxicity is increased oxidative activity[39], which affects plants at morphological, physiological, biochemical, and molecular levels[40,38]. When applied above a particular dose Cd can produce irrepealable damage to plants[41]. Before establishing strategies aimed at bringing about mitigation of Cd toxicity, it is important to estimate the Cd dose preceding which the repair of plant metabolic functioning can be achieved. Under similar experimental circumstances using biomass measurements EC_{50} (effective concentration of Cd causing 50% inhibition of plant growth compared to control) was calculated to be 26 mg/L[22]. The inhibitory effect of Cd toxicity on biomass is largely the manifestation of alteration in plant physiological and metabolic activities[42]. The results of the present study indicate that spinach treated with Cd_{EC50} experienced oxidative activity and showed a negative response compared to plants with no treatment. The structural homology between Cd and Zn has been utilized in promoting Zn as a tool for ameliorating Cd toxicity, which, to the best of our knowledge, is the first effort of its kind. Oxidative activity generated by Cd is through enhanced production of important reactive oxygen species like $O_2^{\cdot-}$ and H_2O_2 in Cd_{EC50} treated plants. Studies have shown that Cd-induced oxidative activity is attributed to inhibition of cellular metabolic reactions[43]. Reduction in the ROS content by Zn indicates abatement of Cd toxicity, which is a result of restoration of disruptive metabolic activities under Cd. Since Zn has no

direct role in regulating ROS production[44], the reduction of ROS contents in Cd + Zn treated plants is attributed to the abatement of Cd-induced oxidative activity. The MDA levels, which are a potential biomarker of oxidative activity indicated a significant reduction in the Cd_{EC50} + Zn treated plants compared to Cd_{EC50} treated plants, which strengthens the curative property of Zn toward Cd. Reduced MDA contents in plants supplemented with Zn indicates stability to the membrane lipid and protein which supports increased photosynthesis and higher protein content in Cd_{EC50} + Zn treated plants. Higher Zn concentrations (above 300 mg /L) increased MDA in plants indicating the synergistic effect of Zn and Cd at a higher Zn dose. At high Zn concentrations, peroxidation of the lipid component of membranes occurs which results in activation of membrane-localized NADPH oxidase, generating more ROS[45]. The fluctuation in MDA content of Cd and Cd-Zn treated plants can be negatively correlated to their respective ROS contents (H_2O_2 and $O_2^{\cdot-}$). Whereas Cd_{EC50}-treated spinach plants had a higher concentration of H_2O_2 (28.81%) and $O_2^{\cdot-}$ (17.61%), compared to untreated plants; ROS declined substantially in Cd_{EC50} + Zn treated plants, compared to Cd_{EC50} plants. A similar correlation between ROS generation and membrane integrity was also established in *Vicia faba* L. cv Nubaria at different Cd and Zn concentrations[45].

Zn supplementation reduced Cd damage by stimulating the cellular defense machinery, the effect being most noticeable at Zn 300 mg/L. The SOD, considered to be the first line of defense against abiotic challenge[46] increased the most among all antioxidant enzymes in Zn-supplemented Cd-treated plants. A high level of oxidative exposure tends to inhibit SOD activity[47]. The present result of Cd-treated plants complies with other studies, wherein a non-significant effect on SOD was recorded along

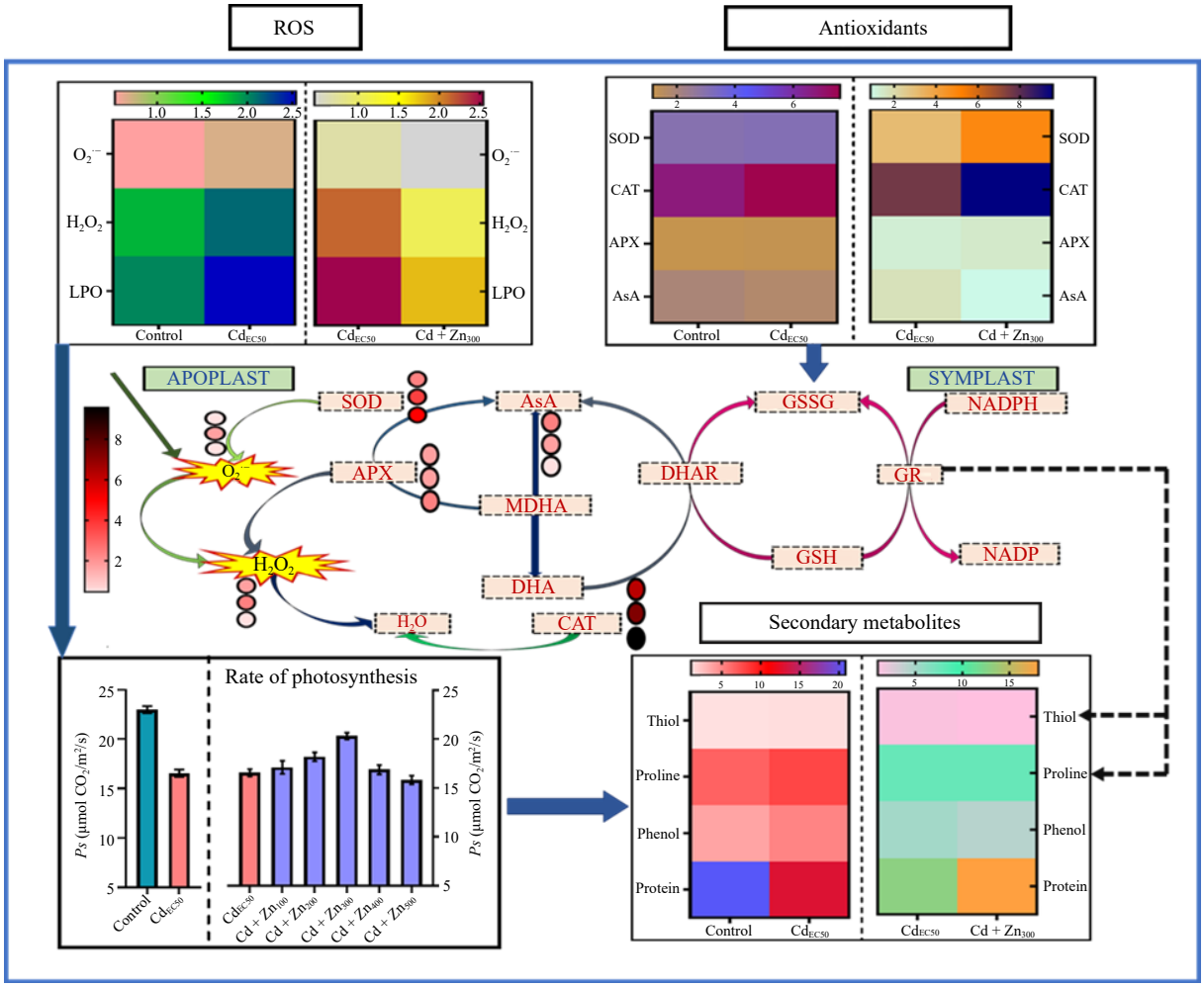

Fig. 5 Comparative analysis of the effect of treatments (Control-Cd_{EC50} and Cd_{EC50}-Cd_{EC50} combined with doses of Zn) on ROS (H_2O_2 and $O_2^{\cdot-}$) generation, lipid peroxidation (LPO), antioxidative response, rate of photosynthesis, secondary metabolite and protein contents of *Beta vulgaris* L. plants. The color gradient of the heatmap shows the variation in ROS, antioxidants and secondary metabolites. Lighter colors are showing lower concentration and dark colors are showing higher concentration. The pathway is showing conversion of antioxidant machinery with the color gradient of the heatmap showing lower (light color) to higher concentrations (dark color) of antioxidants. Legend numbers represent the concentration of antioxidant machinery. LPO: lipid peroxidation, H_2O_2: hydrogen peroxides, $O_2^{\cdot-}$: superoxide, SOD: superoxide dismutase, CAT: catalase, AsA: ascorbic acid, DHA: dehydroascorbate, MDHA: monodehydroascorbate, GSH: glutathione, GSSG: glutathione, APX: ascorbate peroxidase, DHAR: dehydroascorbate reductase, MDHAR: monodehydroascorbate reductase, GR: glutathione reductase, NADP reductase; NADP: nicotinamide adenine dinucleotide phosphate; NADPH: reduced nicotinamide adenine dinucleotide phosphate, ROS: reactive oxygen species, *Ps*: net photosynthetic rate, *gs*: stomatal conductance.

with a steep increase in $O_2^{\cdot-}$ concentration. Zn supplementation triggered the inactive SOD of Cd-stressed plants, which was evident through the significant increments in the SOD activity, which was as high as 59.60% in Cd_{EC50} + Zn 300 mg/L dose as compared to the Cd_{EC50} dose. Zn is an essential metal prosthetic group up-regulated the Cu-Zn SOD expression, enhancing SOD activity in Cd-Zn treated plants, compared to Cd-treated plants[48].

In the present study, the positive effect of Zn on SOD activity can further be verified by a significant reduction in concentrations of $O_2^{\cdot-}$ in Cd_{EC50} + Zn treated plants, compared to Cd_{EC50} treated plants, which experienced a substantial accumulation of $O_2^{\cdot-}$. Activation of SOD due to Zn treatment stopped dismutation of $O_2^{\cdot-}$, thereby reducing its contents in Cd_{EC50} + Zn plants, compared to Cd-treated plants (Fig. 5). The ROS behavior reduced $O_2^{\cdot-}$ content was of higher magnitude than that of H_2O_2 content in Cd-Zn treated plants (Fig. 5). This response of ROS can be due to the disparate stimulation of enzymatic antioxidants with Cd-Zn treatment. In the present study Zn supplementation, although an increment in the activities of all the studied enzymatic antioxidants was recorded, the enhancement was less in H_2O_2 scavenging enzymes like APX and CAT as compared to SOD (0.82), (Fig. 5), which are responsible for the dismutation of $O_2^{\cdot-}$ to H_2O_2. Lesser reduction of H_2O_2 to $O_2^{\cdot-}$ in Cd_{EC50} + Zn treated plants, compared to Cd_{EC50} treated plants, can be accounted for through less efficiency of APX and CAT and higher feedback of SOD upon Zn treatment. An extrapolation of responses of CAT and APX in the present study indicated CAT (0.81) to be a more efficient scavenger of H_2O_2 in Cd + Zn treated plants. This is further evident through correlation values (R) and regression value (R^2), which was higher for CAT (R = 0.97, R^2= 0.94), compared to APX (R = 0.90, R^2 = 0.82) (Fig. 6), and in PCA evident, higher synchronization of CAT (0.81) and SOD (0.82) (Fig. 7). This observation contradicts earlier literature as APX has a higher affinity toward H_2O_2 compared to CAT[49]. In the present study, Zn treatment was not able to revive the APX activity of Cd-treated plants, it is also unable to sustain AsA regeneration, which is crucial in scavenging H_2O_2. The antagonistic effect of Zn treatment towards antioxidant response in Cd_{EC50} + Zn treated plants was only up to 300 mg/L Zn, after which Zn and Cd act synergistically leading to enhanced negative effects on plants. Significant reductions in thiol contents in Cd_{EC50} + Zn treated plants indicate a decline in the GSH pool which can be correlated with a disturbed continuity of the AsA-GSH cycle. This explains the reduction in AsA in Cd_{EC50} + Zn treated plants compared to Cd_{EC50} treated plants. The present results suggest that the AsA-GSH cycle may not play a significant role in imparting Cd tolerance to spinach plants upon Zn supplementation, due to inefficiency of sustenance of the GSH pool. Antioxidants response of Cd $_{EC50}$ + Zn treated spinach plants suggests the role of SOD and CAT activity is more decisive in providing tolerance towards Cd, upon 300 mg/L dose of Zn. Higher Zn concentration acted synergistically with Cd, denigrating the positive response of antioxidative enzymes. Stomatal regulation is an important biophysical aspect of plants that determines their sensitivity toward abiotic challenges[42]. In the present study, although Cd application reduced stomatal conductance, Zn application in Cd_{EC50} + Zn plans favor stomatal opening. Zn promotes stomatal regulation owing to its positive effect on membrane permeability which sustains the K^+ transportation across the guard cell membrane, a feature that was disrupted due to Cd[50]. In addition, Zn treatment favors the accumulation of osmolytes in Cd-treated plants which assist in stomatal opening[50]. The positive effect of Zn extends to carbon fixation, as evident by increased rate of photosynthesis in Cd-treated plants.

The positive effects of Zn in Cd $_{EC50}$ + Zn treated plants can be understood in light and dark reactions but also through increased concentration of chlorophyll contents. In the present study, increased chlorophyll contents in Cd $_{EC50}$ + Zn treated plants were recorded compared to plants treated with Cd_{EC50}. Ma et al.,[51] also reported a considerable increase in chlorophyll content in wheat plants treated with Zn. Zn application not only insulates PSII from the detrimental effects of abiotic stress[52]; it also ensures the regular functioning of the different enzymes of dark reactions[53,54]. The present study indicated an

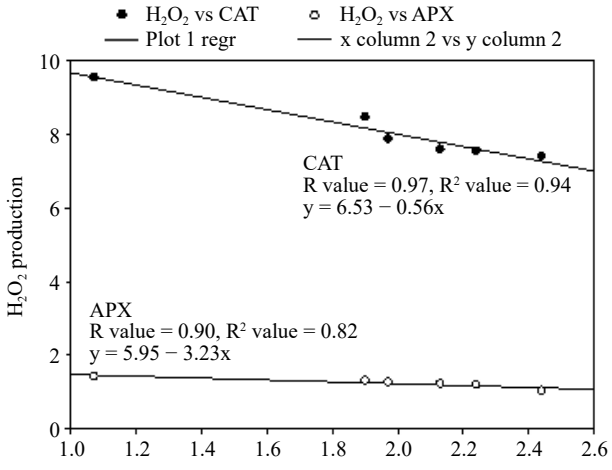

Fig. 6 Regression analysis to estimate the correlation of H_2O_2 with ascorbate peroxidase (APX) and catalase (CAT) in *Beta vulgaris* L. plants treated with combined EC_{50} Cd dose and different doses of Zn at 45 DAE. X-axis shows enzymatic antioxidants.

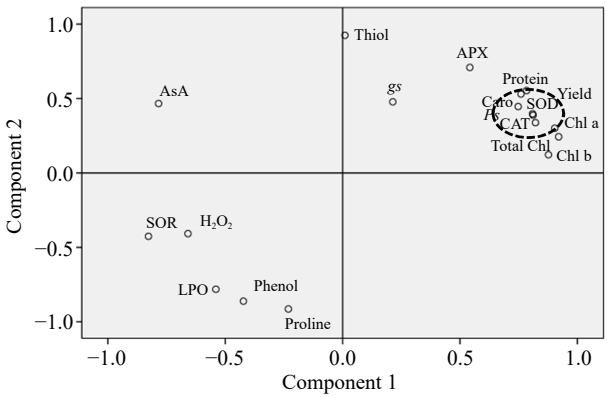

Fig. 7 Principal component analysis (PCA) showing the effect of cadmium (Cd) and zinc (Zn) on considered biochemical, physiological and yield parameters of spinach. The parameters studied are Total Chl (total chlorophyll), Chl a (chlorophyll a), Chl b (chlorophyll b), Caro (carotenoids), AsA (ascorbic acid), phenol, proline, APX (ascorbate peroxidase), SOD (superoxide dismutase), CAT (catalase), *gs* (stomatal conductance), *Ps* (rate of photosynthesis), Thiol, Protein, LPO (lipid peroxidation), H_2O_2 (hydrogen peroxide), and SOR (superoxide radical). Black dotted circles showed the synchronization of SOD and CAT.

ameliorated rate of photosynthesis in Cd $_{EC50}$ + Zn plants, due to Zn compared to Cd_{EC50} exposed plants, which further justifies the use of Zn in the management of Cd damage in plants.

Conclusions

The negative effects of Cd_{EC50}, as evident through the biomass and yield reduction, in the present study were improved by Zn application, with the maximum positive effect observed at 300 mg/L. The results suggest that Zn application is beneficial in improving the yield of spinach grown under Cd stress. Enhancement of yield upon Zn application can be attributed to the beneficial effect of Zn on the antioxidant activity which reduces the oxidative stress. Feedback of enzyme action showed that the SOD and CAT activity of plants treated with Cd_{EC50} was more responsive to Zn compared to APX. The reduction in the thiol pool and inefficiency of the AsA-GSH cycle in Cd_{EC50} + Zn treated plants depreciated their ascorbate regeneration potential, and enhanced the photosynthetic efficiency of plants. Biophysical characteristics responded positively to Zn amendment which led to improved carbon fixation efficiency of plants and resulted in increased yield of Cd $_{EC50}$ + Zn treated plants compared to Cd_{EC50} plants. Zn amendments improved membrane stability, enzymatic response, stomatal regulation, and photosynthetic yield of Cd treated plants, which resulted in enhancement of yield. The results of the present study justify the use of Zn as an efficient tool in the management of Cd toxicity in spinach plants. Zn (300 mg/L) diminished the Cd_{EC50}-induced oxidative activity by exhibiting antagonistic effects toward Cd toxicity. It is, however, necessary to establish a dose-response relationship to identify the appropriate functional dose of Zn, which does not show any synergistic effects with Cd toxicity.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Yadav G; data collection: Madheshiya P; analysis and interpretation of results: Madheshiya P; draft manuscript preparation: Tiwari S; All authors reviewed the results and approved the final version of the manuscript.

Data avaidlability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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References

1. Rai PK. 2019. Heavy metals/metalloids remediation from wastewater using free floating macrophytes of a natural wetland. *Environmental Technology & Innovation* 15:100393
2. Haider FU, Cai L, Coulter JA, Cheema SA, Jun W, et al. 2021. Cadmium toxicity in plants: impacts and remediation strategies. *Ecotoxicology and Environmental Safety* 211:111887
3. Ghori NH, Ghori T, Hayat MQ, Imadi SR, Gul A, et al. 2019. Heavy metal stress and responses in plants. *International Journal of Environmental Science and Technology* 16(3):1807–28
4. Petrovic D, Krivokapic S. 2020. The effect of Cu, Zn, Cd, and Pb accumulation on biochemical parameters (proline, chlorophyll) in the water caltrop (*Trapa natans* L.), lake skadar, Montenegro. *Plants* 9(10):1287
5. Rizwan M, Ali S, Adrees M, Ibrahim M, Tsang DCW, et al. 2017. A critical review on effects, tolerance mechanisms and management of cadmium in vegetables. *Chemosphere* 182:90–105
6. Abbas T, Rizwan M, Ali S, Adrees M, Zia-ur-Rehman M, et al. 2018. Effect of biochar on alleviation of cadmium toxicity in wheat (*Triticum aestivum* L.) grown on Cd-contaminated saline soil. *Environmental Science and Pollution Research* 25:25668–80
7. Madheshiya P, Gupta GS, Sahoo A, Tiwari S. 2023. Role of elevated ozone on development and metabolite contents of lemongrass [*Cymbopogon flexuosus* (Steud.) (Wats.)]. *Metabolites* 13(5):597
8. Nazar R, Iqbal N, Masood A, Khan MIR, Syeed S, et al. 2012. Cadmium toxicity in plants and role of mineral nutrients in its alleviation. *American Journal of Plant Sciences* 3:1476–89
9. Bojórquez C, Frías Espericueta MG, Voltolina D. 2016. Removal of cadmium and lead by adapted strains of *Pseudomonas aeruginosa* and *Enterobacter cloacae*. *Revista Internacional De Contaminación Ambiental* 32(4):407–12
10. Samreen T, Humaira, Shah HU, Ullah S, Javid M. 2017. Zinc effect on growth rate, chlorophyll, protein and mineral contents of hydroponically grown mung beans plant (*Vigna radiata*). *Arabian Journal of Chemistry* 10:S1802–S1807
11. Noulas C, Tziouvalekas M, Karyotis T. 2018. Zinc in soils, water and food crops. *Journal of Trace Elements in Medicine and Biology* 49:252–60
12. Zaheer IE, Ali S, Saleem MH, Yousaf HS, Malik A, et al. 2022. Combined application of zinc and iron-lysine and its effects on morpho-physiological traits, antioxidant capacity and chromium uptake in rapeseed (*Brassica napus* L.). *PLoS One* 17:e0262140
13. Kaur H, Garg N. 2021. Zinc toxicity in plants: a review. *Planta* 253(6):129
14. Zare AA, Khoshgoftarmanesh AH, Malakouti MJ, Bahrami HA, Chaney RL. 2018. Root uptake and shoot accumulation of cadmium by lettuce at various Cd: Zn ratios in nutrient solution. *Ecotoxicology and Environmental Safety* 148:441–46
15. Hart JJ, Welch RM, Norvell WA, Kochian LV. 2002. Transport interactions between cadmium and zinc in roots of bread and durum wheat seedlings. *Physiologia Plantarum* 116:73–78
16. Cherif J, Mediouni C, Ben Ammar W, Jemal F. 2011. Interactions of zinc and cadmium toxicity in their effects on growth and in antioxidative systems in tomato plants (*Solanum lycopersicum*). *Journal of Environmental Sciences* 23(5):837–44
17. Küpper H, Kochian LV. 2010. Transcriptional regulation of metal transport genes and mineral nutrition during acclimatization to cadmium and zinc in the Cd/Zn hyperaccumulator, *Thlaspi caerulescens* (Ganges population). *New Phytologist* 185:114–29
18. Palusińska M, Barabasz A, Kozak K, Papierniak A, Maślińska K, et al. 2020. Zn/Cd status-dependent accumulation of Zn and Cd in root

parts in tobacco is accompanied by specific expression of *ZIP* genes. *BMC Plant Biology* 20:37
19. Harris NS, Taylor GJ. 2001. Remobilization of cadmium in maturing shoots of near isogenic lines of durum wheat that differ in grain cadmium accumulation. *Journal of Experimental Botany* 52(360):1473–81
20. Garg N, Singh S. 2018. Arbuscular mycorrhiza *Rhizophagus irregularis* and silicon modulate growth, proline biosynthesis and yield in *Cajanus cajan* L. Millsp. (pigeonpea) genotypes under cadmium and zinc stress. *Journal of Plant Growth Regulation* 37:46–63
21. Zhang XD, Meng JG, Zhao KX, Chen X, Yang ZM. 2018. Annotation and characterization of Cd-responsive metal transporter genes in rapeseed (*Brassica napus*). *BioMetals* 31:107–21
22. Yadav G, Tiwari S. 2021. Determination of EC_{50} of Cd and evaluation of growth and biochemical response of Palak plants (*Beta Vulgaris* L.) to different Cd treatments. *Journal of Scientific & Industrial Research* 80:491–98
23. MacLachlan S, Zalik S. 1963. Plastid structure, chlorophyll concentration, and free amino acid composition of a chlorophyll mutant of barley. *Canadian Journal of Botany* 41(7):1053–62
24. Duxbury AC, Yentsch CS. 1956. Plankton pigment monographs. *Journal of Marine Research* 15:19–101
25. Heath RL, Packer L. 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* 125:189–98
26. Elstner EF, Heupel A. 1976. Inhibition of nitrite formation from hydroxyl ammonium chloride: a simple assay for superoxide dismutase. *Analytical Biochemistry* 70:616–20
27. Alexieva V, Sergiev I, Mapelli S, Karanov E. 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant, Cell & Environment* 24(12):1337–44
28. Fridovich I. 1975. Superoxide dismutases. *Annual Review of Biochemistry* 44:147–59
29. Nakano Y, Asada K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology* 22(5):867–80
30. Aebi H. 1984. Catalase *in vitro*. *Methods in Enzymology* 105:121–26
31. Keller T, Schwager H. 1977. Air pollution and ascorbic acid. *Forest Pathology* 7(6):338–50
32. Bray HG, Thorpe WV. 1954. Estimation of phenols. *Methods of Biochemical Analysis* 1:27–52
33. Aravind P, Prasad MNV. 2005. Modulation of cadmium-induced oxidative stress in *Ceratophyllum demersum* by zinc involves ascorbate-glutathione cycle and glutathione metabolism. *Plant Physiology and Biochemistry* 43(2):107–16
34. Bates LS, Waldren RP, Teare ID. 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil* 39:205–7
35. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry* 193:265–75
36. Guo D, Ali A, Ren C, Du J, Li R, et al. 2019. EDTA and organic acids assisted phytoextraction of Cd and Zn from a smelter contaminated soil by potherb mustard (*Brassica juncea*, *Coss*) and evaluation of its bioindicators. *Ecotoxicology and Environmental Safety* 167:396–403
37. Manzoor M, Gul I, Kallerhoff J, Arshad M. 2019. Fungi-assisted phytoextraction of lead: tolerance, plant growth–promoting activities and phytoavailability. *Environmental Science and Pollution Research* 26(23):23788–97
38. El Rasafi T, Oukarroum A, Haddioui A, Song H, Kwon EE, et al. 2022. Cadmium stress in plants: a critical review of the effects, mechanisms, and tolerance strategies. *Critical Reviews in Environmental Science and Technology* 52(5):675–726
39. Moradi R, Pourghasemian N, Naghizadeh M. 2019. Effect of beeswax waste biochar on growth, physiology and cadmium uptake in saffron. *Journal of Cleaner Production* 229:1251–61
40. Rizwan M, Ali S, Abbas T, Zia-Ur-Rehman M, Hannan F, et al. 2016. Cadmium minimization in wheat: a critical review. *Ecotoxicology and Environmental Safety* 130:43–53
41. Rizvi A, Zaidi A, Ameen F, Ahmed B, AlKahtani MDF, et al. 2020. Heavy metal induced stress on wheat: phytotoxicity and microbiological management. *RSC Advances* 10(63):38379–403
42. Sarwar N, Ishaq W, Farid G, Shaheen MR, Imran M, et al. 2015. Zinc-cadmium interactions: impact on wheat physiology and mineral acquisition. *Ecotoxicology and Environmental Safety* 122:528–36
43. Zulfiqar U, Ayub A, Hussain S, Ahmad Waraich E, El-Esawi MA, et al. 2022. Cadmium toxicity in plants: recent progress on *Morpho*-physiological effects and remediation strategies. *Journal of Soil Science and Plant Nutrition* 22:212–69
44. Bueno P, Piqueras A. 2002. Effect of transition metals on stress, lipid peroxidation and antioxidant enzyme activities in tobacco cell cultures. *Plant Growth Regulation* 36(2):161–67
45. Tammam A, Hatata M, Sadek O. 2016. Effect of Cd and Zn interaction on reactive oxygen species and antioxidant machinery of broad bean plants (*Vicia faba* L). *The Egyptian Journal of Experimental Biology (Botany)* 12:193–209
46. Noctor G, Reichheld JP, Foyer CH. 2018. ROS-related redox regulation and signaling in plants. *Seminars in Cell & Developmental Biology* 80:3–12
47. Zhu Q, Zhang J, Yu H, Li L, Chen X, et al. 2019. Maize Cd-tolerant ZmVTE4 encoding γ-tocopherol-methyl-transferase alleviated Cd-toxicity through its product α-tocopherol. *Environmental and Experimental Botany* 158:171–79
48. Yang F, Zhang H, Wang Y, He G, Wang J, et al. 2021. The role of antioxidant mechanism in photosynthesis under heavy metals Cd or Zn exposure in tobacco leaves. *Journal of Plant Interactions* 16:354–66
49. Smirnoff N. 2019. Engineering of metabolic pathways using synthetic enzyme complexes. *Plant Physiology* 179(3):918–28
50. Jawad Hassan M, Ali Raza M, Rehman SU, Ansar M, Gitari H, et al. 2020. Effect of cadmium toxicity on growth, oxidative damage, antioxidant defense system and cadmium accumulation in two *Sorghum* cultivars. *Plants* 9(11):1575
51. Ma D, Sun D, Wang C, Ding H, Qin H, et al. 2017. Physiological responses and yield of wheat plants in zinc-mediated alleviation of drought stress. *Frontiers in Plant Science* 8:860
52. Tsonev T, Cebola Lidon FJ. 2012. Zinc in plants - an overview. *Emirates Journal of Food and Agriculture* 24(4):322–33
53. Ahanger MA, Morad-Talab N, Abd-Allah EF, Ahmad P, Hajiboland R. 2016. Plant growth under drought stress: significance of mineral nutrients. In *Water Stress and Crop Plants: A Sustainable Approach*, ed. Ahmad P. US: John Wiley & Sons, Ltd. pp. 649–68. doi: 10.1002/9781119054450.ch37
54. Zhang H, Xu Z, Guo K, Huo Y, He G, et al. 2020. Toxic effects of heavy metal Cd and Zn on chlorophyll, carotenoid metabolism and photosynthetic function in tobacco leaves revealed by physiological and proteomics analysis. *Ecotoxicology and Environmental Safety* 202:110856