RESEARCH ARTICLE

Zinc-induced Changes in Chlorophyll Fluorescence, Pigment Profile and Antioxidant Machinery in Cowpea under Controlled Conditions

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Abstract

A sand culture experiment was conducted to investigate the effects of zinc (Zn) deficiency on oxidative status and antioxidant defense mechanisms in cowpea (*Vigna unguiculata* L. Walp., cv. Pusa Komal). Plants were grown under greenhouse conditions with varying Zn concentrations ranging from 0.01 to 10 µM. Optimal growth and dry matter accumulation were observed at 1 µM Zn supply, indicating adequate Zn nutrition. In contrast, Zn deficiency significantly suppressed plant growth, reduced root development, and led to the formation of thin lateral roots. Zn-deficient plants exhibited pronounced oxidative stress, as evidenced by elevated malondialdehyde (MDA) content, a marker of lipid peroxidation. The lowest lipid peroxide levels were recorded in the leaves of Zn-sufficient plants, highlighting the protective role of Zn against oxidative damage. Suboptimal Zn supply resulted in reduced chlorophyll and carotenoid contents, along with decreased activities of key antioxidant enzymes, including ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD), while malondialdehyde (MDA) accumulation increased. These findings highlight the crucial role of adequate Zn nutrition in maintaining an efficient antioxidant defense system. Zinc deficiency downregulation of the antioxidant defense system, making plants more vulnerable to oxidative damage induced by reactive oxygen species. These findings emphasize the critical role of Zn in maintaining cellular redox balance and ensuring efficient antioxidant defense. Adequate Zn nutrition is therefore essential for sustaining normal growth, physiological functioning, and protection against oxidative stress in cowpea plants.

Keywords: Antioxidative defense, Cowpea, Lipid peroxidation, Malondialdehyde content, Oxygen radicals, Zinc deficiency.

Highlights:

- Zinc deficiency significantly reduced plant growth, root development, and biomass accumulation in cowpea.
- Optimal zinc supply (1 µM) supported normal growth under glasshouse conditions.
- Zinc deficiency triggered severe oxidative stress, indicated by increased malondialdehyde (MDA) levels.
- Activities of major antioxidant enzymes (SOD, CAT, APX, and GR) declined under zinc deficiency.
- Adequate zinc nutrition helped maintain cellular redox balance.
- Proper zinc supply enhances antioxidant defense mechanisms in plants.

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Introduction

Zinc is an essential micronutrient in plants, acting as a structural and catalytic cofactor in many enzymes (e.g., carbonic anhydrase and antioxidant enzymes), stabilizing membranes, and participating in hormone metabolism and protein synthesis. Zn deficiency often leads to reduced growth, chlorosis, and impaired photosynthesis due to decreased chlorophyll synthesis and enzyme activity. Zinc plays an indispensable role as a micronutrient found in low amounts in the soil, which may limit the development and growth of crops (Dubey and Pathak, 2024). In the past few years, interest in zinc nutrition has increased due to the widespread occurrence of zinc deficiency stress, which lowers crop yields (Cakmak, 2008). Zinc is an integral nutrient for both animals and plants; it not only leads to poor yield but also causes a reduction in the quality of produce and malnutrition in animals and humans (Cakmak, 2009). It participates in numerous reactions of cellular and biological origin viz. antioxidative defense, synthesis of carbohydrates and proteins, metabolism of auxin, and the integrity of elements of genetic origin (Broadley *et al*., 2007). Agronomic biofortification presents an effective method for enhancing the retention of micronutrients in the seed of legumes (Pandey *et al*., 2013; Krishna *et al*., 2024). The sedentary lifestyle of terrestrial plants exposes them to extremes of environment that produce metabolic perturbations. Zinc deficiency is known to impair photosynthetic performance by reducing chlorophyll content, photosynthetic rate, and gas exchange parameters. It often leads to a decline in the maximum quantum efficiency of photosystem II (Fv/Fm), indicating inhibition or damage to PSII. Additionally, Zn deficiency enhances the generation of

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reactive oxygen species (ROS), resulting in increased oxidative stress markers such as lipid peroxidation and altered antioxidant enzyme activities. A significant and prevalent outcome is the increased content of oxidative species, including O^2, and H_2O_2 (Tripathy and Oelmuller, 2012). These give rise to even more reactive hydroxyl radicals ($OH^·$), resulting in oxidative damage to the biological components, more importantly, the genomic DNA, membrane lipids, and proteins (Mailloux, 2021). Plants possess an inherent capability to upregulate an antioxidant system, enabling efficient detoxification of ROS and facilitating survival in stressful environments (Singh *et al*., 2019). Either directly or by giving rise to highly reactive hydroxyl radicals ($^·OH$), reactive oxygen species induce oxidative damage to cellular components, particularly membrane lipids, proteins, and genomic DNA. The redox regulation system is mainly comprised of low molecular weight constituents like ascorbate, carotenoids, glutathione, alpha tocopherol (Pastori *et al*., 2003) and enzymes such as ascorbate peroxidase, the SOD (Kavian *et al*., 2022; Shigeoka *et al*., 2002), catalase (Sharma *et al*., 2012), and glutathione reductase. Das and Roychoudhary (2014), reported that plants have evolved a complex antioxidant defense network comprising both enzymatic and non-enzymatic pathways to regulate the generation and scavenging of reactive oxygen species (ROS). Enzymatic antioxidants include superoxide dismutase, catalase, and peroxidases, whereas non-enzymatic components consist of ascorbate, glutathione, carotenoids, and phenolic compounds, which together maintain cellular redox homeostasis under stress conditions. Enzyme-derived antioxidants such as catalase (CAT), guaiacol peroxidase (GPX), superoxide dismutase (SOD), glutathione reductase (GR), ascorbate-glutathione enzymes (AsA-GSH) cycle such as dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and ascorbate peroxidase (APX), can effectively scavenge reactive oxygen derivatives in stressful situations (Habib *et al*., 2016). Antioxidants that are not enzymatic (small molecules) play a fundamental function in neutralizing the harmful free radicals by supplying electrons (Jaleel *et al*., 2009). The metalloenzymes APX, CAT, and SOD have cationic micronutrients (Cu, Fe, Zn, and Mn) as cofactors, and the plant nutrient status of the metal micronutrient that they are a component of affects their activity (Tewari *et al*., 2006). The micronutrient level of the plants may have an impact on how they react to oxidative stress.

Robust evidence of this has emerged from studies on the oxidative metabolism of plants experiencing zinc deficit and excess. Zn protects against ROS damage, according to evidence evaluated by Cakmak (2000). There have also been reports of increased ROS damage in plants exposed to zinc poisoning (Rao and Sresty, 2000). Several reports of shifts in the enzymic constituents of the redox regulation system in plants exposed to deficiency or toxicity of micronutrients (Pandey *et al*., 2002). Evidence on relationship between Zn nutrient status and oxidative damage from ROS linking it to cellular antioxidant extent and activities of free radical detoxifying enzymes is, however, limited. In this study, the effects of changes in antioxidant levels (carotenoids, ascorbate) and the enzyme activity of antioxidative defense system are investigated like, CAT, SOD, GR, APX, and POX to changes in the leaf nutrient content of Zn in cowpea cultivated with zinc availability ranging from deficit to surplus. This study uniquely integrates physiological (chlorophyll fluorescence), biochemical (pigment composition), and enzymatic (antioxidant defense system) responses to zinc exposure in cowpea under controlled conditions. It provides a comprehensive understanding of how zinc modulates photosynthetic efficiency, pigment stability, and oxidative stress regulation. The findings offer practical insights for optimizing micronutrient management, enhancing stress tolerance, and sustaining productivity in zinc-affected soils, thereby contributing to improved crop management and food security.

Experimental Setup

Cowpea (*Vigna unguiculata* L. Walp., cv. Pusa Komal) plants were grown under glasshouse conditions in 5-L plastic pots filled with acid-purified silica sand, following the method described by Sharma (1996) with modifications as reported by Pathak *et al.,* (2009). Each pot contained a central drainage hole and was lined with glass wool beneath an inverted watch glass to ensure free drainage. The nutrient solution consisted of KNO_3 (4 mM), $MgSO_4$ (2 mM), $Ca(NO_3)_2$ (4 mM), NaH_2PO_4 (1.33 mM), and H_3BO_3 (0.33 mM) as macronutrients. Micronutrients included $MnSO_4$ (10 µM), Fe-EDTA (0.1 mM), $CuSO_4$ (1.0 µM), Na_2MoO_4 (0.1 µM), $CoSO_4$ (0.1 µM), NaCl (0.1 mM), and $NiSO_4$ (0.1 µM). Zinc was supplied at five concentrations (0.01, 0.1, 1, 2, and 10 µM). The nutrient solution was applied six days per week. To prevent nutrient accumulation in the rooting medium, the pots were flushed with glass-distilled water over the weekend. To prevent lodging, plants were supported with sticks and threads. Each treatment was replicated twice, with five plants grown per pot. At twenty-eight days after sowing (28 DAS), the earliest symptoms of zinc deficiency were observed in the leaves of plants grown with 0.01 µM Zn. Biomass yield and leaf tissue Zn concentration were measured to determine the optimal Zn concentration for maximum biomass production, as well as the critical Zn concentrations defining deficiency and toxicity thresholds corresponding to 90% of the optimum yield (Sharma, 2004). Concurrently, the two completely enlarged terminal leaves were analyzed for TBARS, chlorophyll a and b, carotenoids, ascorbate, carbonic anhydrase, total Cu-Zn, superoxide dismutase, ascorbate peroxidase, catalase, peroxidase, and glutathione reductase.

Tissue Zn Content and its Biological Yield

Plants were categorized into two primary components, namely roots and shoots, and samples were subjected to oven drying at 75°C to quantify total biomass. Zinc concentration in leaf tissue was quantified using Atomic Absorption Spectrophotometry (AAS) (Perkin Elmer A Analyst 300) after digestion with a HNO_3: $HClO_4$ (10:1, v/v) acid mixture.

Chlorophyll, carotenoids

Spectrophotometric estimation of chlorophyll was performed at 663 and 645 nm, while carotenoid content was determined at 510 and 480 nm following Lichtenthaler (1987).

Lipid peroxidation

Lipid peroxidation was estimated by quantifying thiobarbituric acid–reactive substances (TBARS). Fresh leaf tissue was homogenized in 1% (w/v) trichloroacetic acid (TCA) for extraction. After centrifuging the supernatant for 9 minutes at 10,000 g, 0.5% thiobarbituric acid in 20% trichloroacetic acid was added. Following correction for non-specific absorbance at 600 nm, TBARS content was quantified spectrophotometrically at 532 nm after incubating the assay mixture in a water bath for 30 minutes (Heath and Packer, 1968).

Ascorbate & dehydroascorbate

Ascorbate extraction was performed using 10% trichloroacetic acid and subsequently tested according to the method of Law *et al*., (1983), which involved the conversion of ferric to ferrous ion by ascorbic acid. The measurement of the optical density of the ferrous-α, α-bipyrridyl complex was done at 525 nm. Dehydroascorbate (DHAsc) was quantified as the difference between total ascorbate, following reduction with dithiothreitol.

Enzyme activities

Upper foliage tissue was homogenized in phosphate buffer (PO_4^{-2}) having pH 7.0, supplemented with EDTA (1mM) and PVP (2%) for the assessment of SOD and GR. The extracts were centrifuged at 15,000 × g for 12 min, and the resulting supernatant was used for analysis. Superoxide dismutase (SOD) activity was determined by measuring the inhibition of nitroblue tetrazolium (NBT) photochemical reduction at 560 nm, following the method of Beauchamp and Fridovich (1971). The enzyme that causes 50% inhibition of NBT reduction is known as a unit of enzyme activity. The activity of Cu/Zn-superoxide dismutase (Cu/Zn SOD), assayed in the presence of potassium cyanide (3 mM), was used as an indicator of Cu/Zn SOD function. APX activity was determined by adding 1 mM ascorbate to the above-mentioned buffer, and 290 nm was used to measure the enzymatic activity (Nakano and Asada, 1981). Glutathione reductase activity was determined spectrophotometrically by following the oxidation of NADPH, as indicated by the decline in absorbance at 340 nm per minute. The components of the mixture included 0.1M sodium PO_4^{2-} (pH 7.0), 1 mM EDTA, 1 mM oxidized glutathione (GSSG), 0.1 mM NADPH, and 25–50 µl of enzyme extract (Jablonski and Anderson, 1978). To extract catalase and nonspecific peroxidases, foliage tissue was homogenized in double-distilled water (chilled) by using a chilled mortar and pestle. To measure catalase, a version of the Euler and Josephson (1927) and Luck (1963) POD methods was used. Following Rickli *et al*., (1964), grinding of fresh leaf tissue was done in veronal buffer (0.02 mol L^{-1}) having pH 8.15, which was pre-chilled and contained 0.1 µmol L^{-1} mercaptoethanol and EDTA, to extract carbonic anhydrase. The concentration of soluble protein in the enzyme extract was estimated by Bradford's (1976) dye-binding assay using bovine serum albumin as the standard.

Statistical analysis

All measurements were conducted in triplicate and analyzed using ANOVA. Significant differences among treatments were determined using the least significant difference (LSD) test at $p \leq 0.05$, with plants grown at 1 µM Zn-showing optimum growth and biomass yield-used as the reference treatment.

Results and Discussion

Growth, visible symptoms, and biomass yield

The consequences of alterations in zinc supply were reflected in differences in plant growth after 25 days. About this time, plant growth characteristics such as branching, height and leaf size of plants grown with 0.01µM Zn appeared restricted, and clustering of terminal leaves appeared. Chlorosis is seen in young leaves, characterized by greyish-brown margins and a scorched appearance. At the later stage of growth (75 days after showing), plants supplied with 10 µM Zn also exhibited restricted growth. Necrosis was observed in older leaves, along with chlorosis in the apical regions. Of all the five levels of Zn supply, 1 µM Zn produced the best growth of plants. The Zn effect on growth was also reflected in their biomass yield (Table-1). Plants grown with 1µM Zn produced maximum biomass yield. Compared to this, biomass yield was significantly lower at all the other leaves of Zn supply. Plants grown with 0.01µM Zn showed the least (<50% of the maximum) biomass yield.

Zinc accumulation

The amount of Zn supplied was correlated with the Zn concentration in leaf tissue. It increased from 8.5 µg g^{-1} dry weight in plants supplied 0.01µM Zn to 107.65 µg g^{-1} dry weight. supplied 10 µM Zn. Plants grown with 1µM Zn, which produced maximum biomass yield, contained ~20 µg Zn g^{-1} dry wt. Plot of biomass yield relative to 1.0 µM Zn (relative yield) against leaf tissue concentration of Zn revealed 20 and 33 µg Zn g^{-1} dry wt. as the critical levels (90% max. yield) for the threshold of deficiency and toxicity, respectively. Zinc concentration ranging 20 to 33 µg g^{-1} dry wt. represents sufficiency or homeostatic concentration of Zn (Pathak *et al.,* 2009).

Chlorophyll, carotenoids

Plants grown with 1µM Zn, which showed the best growth and maximum biomass yield, contained the highest concentration of Chl and Car (Table-2). At both sub-optimal and supra-optimal levels of Zn, areductionin the level of Chl a and Chl b was seen. A more significant reduction in Chl a compared to Chl b resulted in a decrease in the Chl a: Chl b ratio. At sub-optimal levels of Zn, Chl a, b was reduced by ~ 10%, and at supra-optimal levels

Table 1: Effect of graded levels of Zn supply on the dry matter yield and leaf tissue Zn of cowpea (*Vigna unguiculata* L. Walp., cv. Pusa Komal)

µM Zn supply	*Dry matter yield (g-1 plant)*	*Tissue Zn: µg g-1 dry weight*
0.01	1.25 ± 0.056	8.50 ± 0.168
0.1	2.29 ± 0.067	11.25 ± 0.267
1.0	2.46 ± 0.076	20.45 ± 0.369
2.0	3.45 ± 0.079	33.42 ± 0.426
10.0	3.12 ± 0.068	107.65 ± 6.268
LSD (p = 0.05)	0.904	3.458

Table 2: Effect of graded level of Zn supply on the chlorophyll and carotenoid content in the leaves of cowpea (*Vigna unguiculata* L. Walp., cv. Pusa Komal)

µM Zn supply	*Chlorophyll (mg g^{-1} fresh weight)*			*Carotenoids (mg g^{-1} fresh weight)*
	Chl. a	*Chl. b*	*Total Chl.*	
0.01	0.239 ± 0.013	0.085 ± 0.011	0.324 ± 0.013	0.039 ± 0.004
0.10	0.277 ± 0.024	0.092 ± 0.014	0.369 ± 0.024	0.044 ± 0.008
1.0	0.341 ± 0.024	0.167 ± 0.021	0.508 ± 0.026	0.110 ± 0.023
2.0	0.314 ± 0.025	0.151 ± 0.022	0.465 ± 0.025	0.093 ± 0.014
10.0	0.306 ± 0.023	0.098 ± 0.013	0.404 ± 0.023	0.069 ± 0.005
LSD (P=0.05)	0.049	0.032	0.050	0.014

of Zn by >20%. Carotenoids followed a trend similar to Chl, but the Zn effect on Carotenoids was relatively less. This resulted in an elevation of the Car: Chl ratio in plant leaves cultivated under sub- and supra-optimal Zn levels, with a greater rise (20%) seen in the former condition.

Lipid peroxidation (TBARS)

Plants treated with both low and high Zn concentrations exhibited increased accumulation of malondialdehyde (MDA), a major thiobarbituric acid (TBA) -reactive metabolite, indicating enhanced lipid peroxidation as measured by TBARS (Fig.1b). Plants grown with 1.0 µM Zn, with leaf tissue concentration of ~20 µg Zn g^{-1} dry wt., showed the minimum concentration of TBARS. Leaves of plants grown with less or more than 1.0 µM Zn contained significantly higher concentrations of TBARS. Enhanced MDA accumulation under both Zn deficiency and excess indicates membrane damage, highlighting the role of Zn in preserving membrane integrity by reducing lipid peroxidation, possibly via stabilization of –SH groups.

Ascorbate & dehydroascorbate

The level of Zn supply made a significant difference to the leaf tissue concentration of ascorbate (Asc). Plants grown with 0.01µM Zn, with the lowest concentration of Zn in leaves (~7 µg g^{-1}dry wt), showed the highest concentration of Asc. As the supply of Zn increased from 0.01 µM to 1.0 µM, the Asc level decreased to its lowest point. An increase in Zn supply above 1.0 µM also led to an increase in Asc concentration (Fig.1c). A similar trend was seen in the DHAsc concentration. The increases in the concentrations of ascorbate (Asc) and dehydroascorbate (Fig. 1d) observed in the leaves of both low- and high-Zn–treated plants can be explained by a concomitant decrease in the activity of ascorbate peroxidase (Fig. 1a), an enzyme that catalyzes the peroxidative degradation of photosynthetically generated H_2O_2 as part of the ascorbate–glutathione cycle.

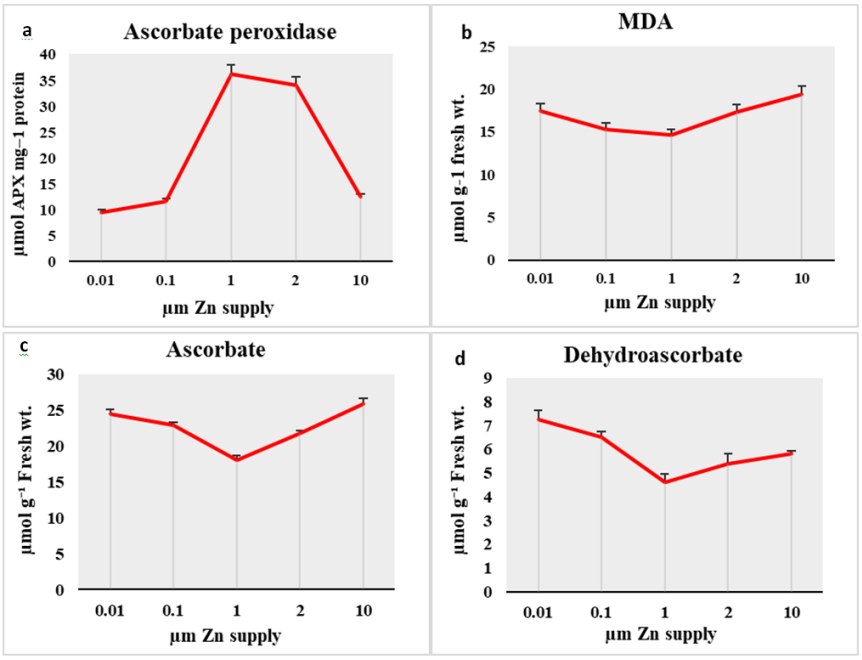

Fig. 1: Effect of graded zinc (Zn) concentrations on the activities of (a) ascorbate peroxidase (APX), (b) malondialdehyde (MDA), (c) ascorbate (Asc), and (d) dehydroascorbate (DHAsc) content in plants 28 days after treatments

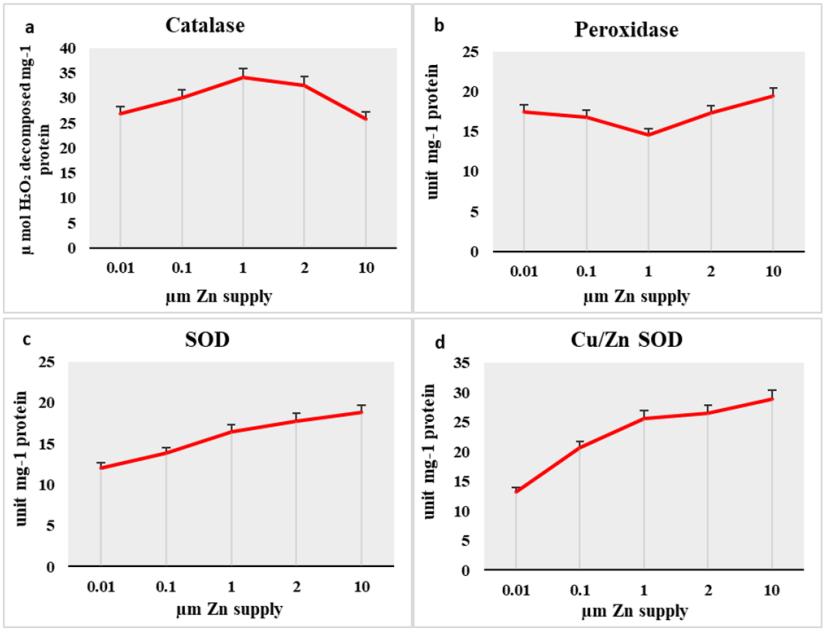

Fig. 2: Effect of graded zinc (Zn) concentrations on the activities of antioxidant enzymes- (a) catalase (CAT), (b) peroxidase (POD), (c) superoxide dismutase (SOD), and (d) Cu/Zn-superoxide dismutase (Cu/Zn-SOD), 28 days after treatment

Enzymatic Antioxidants

As the amount of Zn supplied increased from 0.01 to 10 µM, the activity of the enzyme SOD steadily increased. Total as well as Cu/Zn- SOD activities followed similar trend (Fig. 2c, 2d). As the Zn fertilization increased from 0.1 to 1.0 µM, the other enzymatic antioxidant enzymes performance either improved or declined, beyond which the trend was reversed. Thus, the reactions of APX and CAT are maximized in leaves which cultivated with 1.0 µM Zn (Fig. 2a). At both sub- and supra-optimal levels of Zn, both APX and the POX (Fig. 2b) activities were significantly enhanced at sub-optimal levels of Zn. Supra-optimal supply of Zn made little change in POX and CAT activities compared to Zn supply of 1.0 µM.

The investigation of oxidative metabolism in cowpea leaves (*Vigna unguiculata*, L. Walp. cv Pusa Komal), cultivated across a broad spectrum of Zn supply (0.1 to 1.0 µM), confirms heightened damage from reactive oxygen species, low level of Cu/Zn Superoxide dismutase (Pandey *et al*., 2002) as a result of deficiency of Zn whereas excess Zn shows a decrease in chloroplast pigments (Malviya *et al.,* 2023) and increased accumulation of TBARS at both sub- and supra-optimal levels of Zn, which interpret accelerated production of ROS results in heightened lipid peroxidation.Similarly, as the Zn content raised from the lowest to the highest, SOD activity also increased. Because Zn is essential for the metalation of the Cu/Zn SOD apoprotein, an increase in Zn content may enhance SOD activity.

Zn deficiency could limit SOD activity by the dismutation of O_2^- to H_2O_2 which could be effectively detoxified the through ascorbate- glutathione cycle (Yang *et al.,* 2021). The O_2^- accumulation can also cause the formation of highly toxic OH radicals (Demiral and Turkan, 2005) and inhibition of catalase (Schutzendubel and Polle, 2002), a principal enzyme involved in detoxification of peroxisomal H_2O_2. The heightened sensitivity of plants cultivated under supra-optimal zinc conditions to oxidative damage appears to result from an increased breakdown of superoxide to hydrogen peroxide, attributed to enhanced superoxide dismutase levels, ascorbate peroxidase, and catalase inhibition, all of which are essential for H_2O_2 detoxification across various cellular compartments.

These observations align with elevated levels of SOD and greater deposits of H_2O_2 seen in the foliage of wheat plants exposed to excessive Zn supply (Kaur and Garg, 2021). Because of modifications in the stoichiometry of antioxidant enzymes, both sub-optimal and supra-optimal zinc levels result in a reduced defense against elevated ROS. There have also been reports of a lack of coordination in the antioxidant enzyme activity when zinc shortage is present (Chauhan *et al.,* 2005). The observations indicate that Zn homeostasis is essential for enhancing antioxidant protection mechanisms in plants, thereby improving their ability to adapt to abiotic stresses. Our hypothesis is supported by the increased sensitivity of zinc-deficient plants to intense light (Cakmak, 2005) and ozone toxicity (Iqbal *et al*., 2013).

Conclusion

Findings from the present investigation indicate that zinc availability significantly regulates plant growth, oxidative status, and antioxidant defense responses in cowpea. An adequate zinc supply (1 µM) supported optimal growth performance, enhanced biomass accumulation, and improved root development, indicating sufficient Zn nutrition under controlled conditions. These findings underscore the critical role of maintaining optimal zinc levels for ensuring normal physiological functioning and redox homeostasis in cowpea

plants. In contrast, zinc deficiency significantly impaired plant growth and root architecture and induced pronounced oxidative stress, as evidenced by elevated malondialdehyde levels, indicating enhanced lipid peroxidation. Suboptimal zinc (Zn) supply caused significant reductions in chlorophyll and carotenoid contents, underscoring the adverse effects of Zn deficiency on photosynthetic efficiency. Furthermore, the activities of major antioxidant enzymes, including superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase, were substantially reduced under Zn-deficient conditions, leading to a weakened antioxidant defense system. The suppression of these protective enzymes increased plant susceptibility to reactive oxygen species–mediated cellular damage. Collectively, these findings underscore the essential role of zinc in maintaining redox homeostasis, preserving membrane integrity, and ensuring normal physiological functioning in cowpea. Therefore, ensuring adequate zinc nutrition is crucial for maintaining optimal plant growth, improving stress tolerance, and reducing oxidative damage in cowpea cultivation.

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Author's Contributions

Author Divya Dubey is involved in the experiments, data compilation and writing of the article. Nilu Singh and G. C. Pathak contributed to the overall conception and design of the study and provided critical inputs and supervision throughout the experiment.

Conflict Of Interest

The authors declare no conflict of interest.

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