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ZINC-INDUCED BIPHASIC MODULATION OF CHLOROPLAST PIGMENTS AND ANTIOXIDATIVE ENZYME ACTIVITIES IN ACID LIME (*Citrus aurantiifolia* Swingle): IMPLICATIONS ON PHOTOSYNTHETIC EFFICIENCY AND STRESS MANAGEMENT

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ABSTRACT

Zinc is an essential micronutrient vital for various physiological processes in plants; however, its narrow gap between deficiency and toxicity demands careful nutrient management. This study assessed the impact of zinc (zinc sulphate heptahydrate @ 0.0-15.0 mM L^{-1}) on the composition of chloroplast pigments and anti-oxidative enzyme activities within the chloroplasts of acid lime (*Citrus aurantiifolia* Swingle) leaves over two consecutive years. Moderate zinc concentrations (7.5-10.0 mM L^{-1}) significantly improved chlorophyll (a, b, total), carotenoids, xanthophylls and plastoquinone contents, signifying enhanced chloroplast development and better photosynthetic ability. Rubisco activity, a crucial enzyme for carbon fixation, reached its maximum at 10.0 mM L^{-1}. In contrast, both zinc deficiency (0.0 mM L^{-1}) and toxicity (≥ 12.5 mM L^{-1}) led to significant decline in pigments and Rubisco activity, associated with increased oxidative stress. Superoxide dismutase, glutathione, and ascorbate peroxidase activities within chloroplast were elevated under stress conditions, indicating a compensatory activation of antioxidant defence system. This biphasic response revealed the dual role of zinc in maintaining the physiological balance at optimal levels while triggering the oxidative stress and metabolic disturbances under deficiency or excess. The study emphasizes the critical zinc threshold required to sustain photosynthetic function and redox balance in acid lime.

Keywords: Acid lime, antioxidant enzymes, chloroplast pigments, nutrient management, Rubisco, oxidative stress, zinc

INTRODUCTION

Acid lime (*Citrus aurantiifolia* Swingle), a member of Rutaceae family, is a widely cultivated citrus species grown for its nutritional and medicinal benefits. Being a good source of vitamin C, flavonoids, and organic acids, acid lime serves as a vital dietary component and represents a commercially valuable fruit crop in tropical and subtropical regions (Kumar *et al*., 2020). India is one of the leading producers of acid lime and its cultivation plays a crucial role in supporting small holder livelihoods contributing to the rural economy (Bairwa *et al*., 2018). However, the productivity and fruit quality of acid lime is frequently hindered by inadequate nutrient management, particularly due to the imbalanced management of micronutrients like zinc.

As an indispensable micronutrient, zinc participates in numerous physiological and biochemical functions in plants, such as protein synthesis, auxin regulation, enzymatic activation, and preservation of membrane stability (Broadley *et al*., 2012; Hafeez *et al*., 2013). Zinc deficiency is common in calcareous and alkaline soils where citrus is predominantly cultivated, and the Zn deficiency leads to symptomatic development like interveinal chlorosis, stunted growth, poor fruit development, and reduced yield (Alloway, 2009; Saleem *et al*., 2022). Contrarily, Zn over-dose induces phytotoxic effects, disrupts cellular metabolism, damages chloroplast ultrastructure, and disturbs oxidative balance (Yang *et al*., 2019). Only appropriate zinc levels enhance pigment biosynthesis, improves anti-oxidative enzyme performance (Ahmad *et al*., 2018; Shahid *et al*., 2021), and the precise threshold distinguishing the beneficial and harmful concentrations is still undefined. Responses of zinc application on citrus species under diverse agroclimatic conditions have further complicated the formulation of consistent fertilization strategies (Pérez-Sanz *et al*., 2021; Ali *et al*., 2022). Thus, understanding the multifaceted interaction between zinc nutrition and redox control (Zhou *et al*., 2023). These pigments are sensitive to nutritional imbalances as well as oxidative stress, and thus considered as vital indicators of zinc deficiency or toxicity (Dimkpa and Bindraban, 2016; Ghasemi *et al*., 2020). Similarly, antioxidant enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione play key role in plant defence mechanism and have active role in redox state under zinc-induced stress conditions (Gill and Tuteja, 2010; Hasanuzzaman *et al*., 2020).

Considering the limited understanding of physiological thresholds of zinc in acid lime and lack of comprehensive studies on its effects on chloroplast pigments and antioxidant systems, there remains a notable research gap. It was hypothesized that zinc application induces a biphasic regulatory effect in acid lime, wherein optimal concentrations augment pigment biosynthesis and stress resilience, while both deficiency and excess levels disrupt pigment synthesis and exacerbate oxidative stress responses. Hence, the present study was aimed to assess the optimal zinc level that may maximize chloroplast pigment synthesis and sustain redox homeostasis, while delineating the critical thresholds beyond which zinc shifts from beneficial to phytotoxic in acid lime physiology.

MATERIALS AND METHODS

Seedling raising and other experimental setup

A pot experiment was conducted at the Horticultural Farm, Institute of Agriculture, Visva-Bharati, Sriniketan, West Bengal (India) during the years 2022 and 2023 to evaluate the impact of zinc on acid lime seedlings under greenhouse conditions (28-35ºC, 80-90% RH). Uniform plastic pots of 36 cm dia. were used, filled with acid-washed river sand to minimize the background nutrient contamination. The sand was thoroughly treated with 0.01 N HCl, rinsed many times with distilled water, and then air-dried before use. Healthy, uniform acid lime seedlings of 60 day's age were raised from the seeds extracted from freshly harvested ripe fruits of cv. 'Pramalinini'. For the first three months, seedlings were irrigated at regular intervals with a modified Hoagland's nutrient solution devoid of zinc. Zinc treatments were applied in the form of zinc sulphate in varying concentrations as per the experimental design. The pH of nutrient solution was maintained at 6.0. Zinc was applied weekly to the plants in nutrient solution form in various concentrations (0.0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mM L^{-1}) through sand substrate in a completely randomized design with each treatment replicated thrice. Standard plant protection practices were followed to maintain plant health (Bose *et al*., 2001). Observations related to chloroplast pigment profiles and stress-related enzyme activities were recorded at various time intervals throughout the study duration.

Determination of chloroplast pigments

Chloroplast pigments were quantified as per Lichtenthaler and Wellburn (1983) by extracting leaf tissue in 80% acetone and measuring the absorbance at 663, 645 and 470 nm using a spectrophotometer (Labman, India; model: LMSPUV 1000B). The following equations were used for quantification of chlorophyll:

Chlorophyll *a* = $12.7(A_{663}) - 2.69(A_{645})$
Chlorophyll *b* = $22.9(A_{645}) - 4.68(A_{663})$
Total chlorophyll = $20.2(A_{645}) + 8.02(A_{663})$
Carotenoids = $(1000 \times A_{470} - 1.82 \times \text{Chl } a - 85.02 \times \text{Chl } b) / 198$

Xanthophyll content was determined by using the following formula:

$$\text{Xanthophylls} = (A_{474} \times V \times 10) / (E \times W),$$

where *V* is the final extract volume (mL), *W* is the fresh weight of sample (g), and *E* is the extinction coefficient (≈198.3 for xanthophylls in 100% acetone).

Plastoquinone was extracted using cold 100% acetone, followed by partitioning with hexane. The hexane phase was scanned using a UV spectrophotometer across the 255-290 nm range as per Yamamoto and Kamite (1987). Quantification was performed by using the following equation:

$$\text{Plastoquinone } (\mu\text{mol g}^{-1}\text{ FW}) = (A_x \times V) / (\varepsilon \times d \times W)$$

where A_x is the absorbance at ~ 255-265 nm (oxidized plastoquinone), V is the extract volume (mL), ε is the molar extinction coefficient (≈ 12,000 M^{-1} cm^{-1} at 255 nm), d is the path length of cuvette (1 cm), and W is the fresh weight of sample (g).

Isolation of chloroplasts and determination of antioxidative enzyme complex

Chloroplasts were isolated by homogenizing fresh green leaves in ice-cold extraction buffer containing 50 mM tris-HCl (pH, 7.5), 0.35 M sucrose and 1 mM EDTA. The homogenate was filtered through four layers of cheesecloth (100-120 µm mesh) and centrifuged at 1,000 rpm for 10 min at 4°C to eliminate debris. The supernatant was re-centrifuged at 5000 rpm for 10 min to pellet chloroplasts. For enhanced purity, the pellet was loaded onto a 40%/80% Percoll gradient and centrifuged at 7000 rpm for 15 min. The final chloroplast pellet was resuspended in cold buffer and kept on ice for immediate analysis (Aronsson and Jarvis, 2002). Rubisco activity (µmol CO_2 mg protein^{-1} min^{-1}) was assayed by extracting soluble proteins from fresh leaf tissue using ice-cold buffer, followed by incubation with ribulose-1,5-bisphosphate (RuBP). The rate of NADH oxidation was monitored at 340 nm spectrophotometerically (Parry *et al.*, 1997). The decline in absorbance was used to compute Rubisco activity, normalized to the protein concentration.

Superoxide dismutase (SOD) activity was measured from chloroplast extracts based on the enzyme's ability to inhibit photo-reduction of nitro blue tetrazolium (NBT) at 560 nm. The reaction mixture contained methionine, riboflavin, NBT, and EDTA under illumination. One unit of SOD activity is defined as the amount causing 50% inhibition of NBT reduction (Giannopolitis and Ries, 1977). The glutathione content in chloroplasts was assessed by extraction in cold phosphate buffer and reacting it with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and glutathione reductase in the presence of NADPH. The formation of yellow 5-thionitrobenzoic acid (TNB) product was measured at 412 nm and its concentration calculated using a standard curve (Noctor and Foyer, 1998). Ascorbate peroxidase (APX) activity was assessed by measuring the decrease in absorbance at 290 nm due to ascorbate oxidation in a reaction mixture containing ascorbate, hydrogen peroxide, and chloroplast extract. The activity was calculated using the extinction coefficient of ascorbate (Nakano and Asada, 1981).

Leaf zinc determination

Leaf zinc content was determined using diacid digestion (HNO_3: $HClO_4$, 3:1) of dried leaf, followed by analysis with atomic absorption spectrophotometry (Lindsay and Norvell, 1978). Dry leaf samples were digested, filtered, and aliquots fed to AAS (Perkin Elmer Analyst 800, Perkin Elmer Inc., USA) to determine zinc content against standards zinc solution (Perkin Elmer, USA).

Statistical analysis

Data collected over two successive years (2023, 2024 and pooled) were subjected to statistical analysis by using a completely randomized design. Treatment effects were evaluated by analysis of variance (ANOVA). When significant differences emerged, treatment means were separated using least significant difference test at 5% level (Gomez and Gomez, 1984). Statistical analyses were carried out using IBM SPSS Statistics (version 26.0). Prior to conducting ANOVA, assumptions such as normal distribution and homogeneity of variance were verified.

RESULTS AND DISCUSSION

Impact of zinc on chlorophyll a, chlorophyll b and total chlorophyll

Chlorophyll a (Chl a), the dominant pigment involved in photosynthesis, exhibited significant variation in response to foliar zinc application. The lowest Chl a concentration was recorded in untreated control plants (0.84 mg g^{-1} FW pooled) in both the years (2023 and 2024) datasets (Table 1), reflecting symptoms of Zn deficiency. With increasing Zn level, a progressive rise in Chl a was observed which reached its peak at 10.0 mM L^{-1} (1.75 mg g^{-1} FW pooled), beyond this a marked reduction occurred at 15.0 mM L^{-1} (0.97 mg g^{-1} FW). A comparable trend was observed for chlorophyll b (Chl b), which also increased with moderate Zn supply. The minimum pooled Chl b value was observed under control conditions (0.47 mg g^{-1} FW), whereas maximum occurred at 10.0 mM L^{-1} Zn (0.83 mg g^{-1} FW). Chl b is closely associated with light-harvesting complex protein elements that are notably responsive to nutrient status and oxidative stress. It declined at 15.0 mM L^{-1} Zn (0.44 mg g^{-1} FW) suggesting possible impairment of these structures under toxic Zn conditions. Total chlorophyll followed a cumulative trend, showing highest pooled content at 10.0 mM L^{-1} Zn (2.52 mg g^{-1} FW) and lowest in control treatment (1.30 mg g^{-1} FW).

Table 1: Influence of zinc application on chlorophyll content in the leaves of acid lime seedlings

Treatments	Chl a (mg 100 g^{-1} FW)			Chl b (mg 100 g^{-1} FW)			Total Chl (mg 100 g^{-1} FW)		
(mM Zn L^{-1})	2023	2024	Pooled	2023	2024	Pooled	2023	2024	Pooled
0.0	82.5^{fg}	86.3^{fg}	84.4^{fg}	45.7^{f}	48.2^{f}	47.4^{f}	127.5^{fg}	134.1^{fg}	130.3^{fg}
2.5	124.7^{cde}	129.2^{de}	126.5^{e}	62.0^{d}	65.7^{d}	64.3^{d}	186.3^{cde}	193.2^{de}	189.2^{de}
5.0	137.1^{c}	145.6^{c}	141.3^{c}	69.1^{c}	72.8^{c}	71.5^{c}	206.4^{c}	217.7^{c}	211.5^{c}
7.5	168.5^{ab}	173.2^{ab}	170.3^{ab}	78.3^{ab}	82.5^{ab}	80.4^{ab}	246.6^{ab}	254.2^{ab}	250.4^{ab}
10.0	172.8^{a}	178.1^{a}	175.4^{a}	81.2^{a}	84.2^{a}	83.2^{a}	250.9^{a}	254.2^{ab}	252.6^{a}
12.5	131.3^{cd}	135.7^{cd}	133.5^{cd}	59.5^{de}	62.6^{de}	61.5^{de}	190.2^{cd}	196.5^{d}	193.3^{d}
15.0	94.2^{f}	99.6^{f}	97.4^{f}	42.4^{fg}	46.8^{fg}	44.6^{fg}	136.5^{f}	143.9^{f}	140.7^{f}
SE±m	5.3	3.7	3.8	1.7	2.0	2.1	7.1	6.9	8.7
CD$_{0.05}$	16.1	14.2	14.6	5.2	6.2	6.5	22.4	20.7	26.1

The values in a column superscripted with the same letter(s) do not significantly differ from each other.

The upward shift in total chlorophyll from 0.0 - 10.0 mM L^{-1} indicated improvement in pigment biosynthesis and photosynthetic capacity under moderate Zn nutrition. This biphasic pattern revealed that moderate Zn enhances pigment production, while excessive levels become phytotoxic, likely due to oxidative stress and ultrastructural damage in chloroplasts (Dimkpa and Bindraban, 2016; Fukao *et al*., 2021). Zn plays vital role in the maintenance of membrane integrity and serves as a cofactor in enzymes linked to chlorophyll biosynthetic pathways (Broadley *et al*., 2007). The pigment reduction at higher Zn levels may be attributed to lipid peroxidation, resulting in chloroplast dysfunction and pigment loss (Hussain *et al*., 2020). The sharp drop in Chl b at 15.0 mM L^{-1} supports this interpretation, highlighting photo-damage and compromised light-harvesting efficiency under Zn toxicity (Zhang *et al*., 2023). The improvement in both Chl a and b under optimal Zn conditions signifies enhanced chloroplast activity and photosynthetic efficiency. In contrast, when Zn exceeds physiological thresholds, pigment biosynthesis gets suppressed due to gene-level disruptions and elevated ROS accumulation (Ghasemi *et al*., 2020). The observed variation in total chlorophyll aligns with Pérez-Sanz *et al*. (2021) wherein Zn application promoted chlorophyll accumulation in citrus species. However, the pigment decline under 12.5 and 15.0 mM L^{-1} treatments signals a toxicity threshold, beyond which Zn exerts detrimental effects. Such responses may stem from oxidative damage to thylakoid membranes, ultimately inhibiting pigment formation and accelerating breakdown processes (Ali *et al*., 2022).

Influence of Zn application on carotenoids, xanthophylls and plastoquinone

The concentrations of carotenoids, xanthophylls, and plastoquinone in acid lime seedlings were significantly affected by varying levels of Zn foliar application during 2023 and 2024 growing seasons (Table 2). Carotenoid content showed a steady upward trend with increasing Zn concentrations, achieving maximum levels at 10.0 mM L^{-1}, where measurements reached 37.0 mg 100 g^{-1} FW in 2023, 38.4 mg 100 g^{-1} FW in 2024, and a pooled mean of 37.7 mg 100 g^{-1} FW. Slightly lower yet still elevated values were observed at 7.5 mM L^{-1}. In contrast, control group (0.0 mM L^{-1}) and highest Zn treatment (15.0 mM L^{-1}) exhibited significantly lower carotenoid concentrations (21.4 and 20.1 mg 100 g^{-1} FW pooled, respectively), implying that moderate Zn enhances carotenoid synthesis, and excessive application disrupt the biosynthetic pathway. A parallel trend was found in xanthophyll accumulation. Peak levels were observed at 10.0 mM L^{-1} in both the years. The 7.5 mM L^{-1} treatment also supported relatively high xanthophyll levels. The lowest values were associated with control and 15.0 mM L^{-1} Zn treatments. These findings suggest a positive role of Zn in promoting xanthophyll production at optimal concentrations, whereas excessive Zn appears to impede its accumulation. Plastoquinone levels also followed biphasic pattern with maximum concentrations observed at 10.0 mM L^{-1}. The 7.5 mM L^{-1} Zn treatment maintained relatively high plastoquinone levels, while lowest content was seen in control and 15.0 mM L^{-1} Zn treatments. This support Zn's regulatory function in plastoquinone biosynthesis, acting beneficially at moderate doses and detrimentally at toxic levels.

Table 2: Influence of zinc application on other associated chloroplast pigments in the leaves of acid lime seedlings

Treatments	Carotenoids (mg 100 g^{-1} FW)			Xanthophylls (mg 100 g^{-1} FW)			Plastoquinone (µg g^{-1} FW)		
(mM Zn L^{-1})	2023	2024	Pooled	2023	2024	Pooled	2023	2024	Pooled
0.0	20.1^{f}	22.5^{f}	21.4^{ef}	7.4^{fg}	8.0^{fg}	7.7^{fg}	5.2^{g}	5.5^{fg}	5.4^{g}
2.5	28.0^{cd}	31.4^{cd}	30.2^{cd}	9.8^{cd}	10.5^{de}	10.3^{d}	7.8^{cde}	8.1^{cde}	8.0^{cde}
5.0	29.7^{c}	32.2^{c}	30.4^{c}	11.4^{c}	13.6^{c}	12.5^{c}	8.2^{cd}	8.5^{cd}	8.4^{cd}
7.5	34.2^{ab}	37.8^{ab}	36.5^{ab}	14.7^{ab}	16.8^{ab}	15.7^{ab}	10.5^{ab}	10.9^{ab}	10.7^{ab}
10.0	37.0^{a}	38.4^{a}	37.7^{a}	15.4^{a}	17.3^{a}	16.4^{a}	10.7^{a}	11.0^{a}	10.9^{a}
12.5	25.6^{cde}	28.1^{cde}	26.3^{cde}	8.7^{def}	10.6^{d}	9.6^{de}	8.2^{cd}	8.5^{cd}	8.4^{cd}
15.0	18.2^{fg}	22.0^{fg}	20.1^{fg}	9.0^{de}	9.4^{def}	9.2^{def}	6.0^{f}	6.3^{f}	6.2^{f}
SE±m	1.4	1.4	1.7	0.7	0.6	0.6	0.24	0.26	0.26
CD $_{(0.05)}$	4.1	4.3	5.0	2.1	2.0	2.0	0.74	0.80	0.77

The values in a column superscripted with the same letter(s) do not significantly differ from each other.

An elevated carotenoid content under moderate Zn supply suggests enhanced photo-protective capacity and structural integrity of photosynthetic apparatus (Pereira *et al*., 2020). However, pronounced reduction in carotenoids at 15.0 mM Zn L^{-1} (0.20 mg g^{-1} FW pooled) indicates oxidative damage, photo-inhibition. Excess Zn interferes the enzyme systems involved in carotenoid synthesis, leading to pigment degradation and impaired light-harvesting efficiency (Yang *et al*., 2021). Xanthophylls, critical components of xanthophyll cycle, are integral to non-photochemical quenching (NPQ) and protection against photo-oxidative damage (Demmig-Adams and Adams, 2006). Enhanced accumulation at optimal Zn reflects effective ROS detoxification and photo-protection. Conversely, toxic Zn content may compromise the xanthophyll cycle enzymes, resulting in reduced pigment levels and diminished NPQ potential due to excessive ROS generation (Zhou *et al*., 2019). The peak in plastoquinone concentration at 10.0 mM Zn L^{-1}, followed a steep decline at higher Zn levels and in control. PQ reduction under Zn stress is consistent with impaired PSII activity and over-accumulation of ROS (Tikkanen and Aro, 2014; Zhou *et al*., 2023). The decline in

PQ at high Zn levels may also result from disrupted PQ biosynthesis or accelerated degradation due to ROS-mediated oxidation, which negatively affects cyclic electron flow and ATP production (Yamori and Shikanai, 2016). Zn deficiency in control leads to suppressed pigment synthesis due to impaired enzymatic activity, disrupted chloroplast ultrastructure, and increased oxidative stress (Alloway, 2009). The optimal Zn levels (7.5 to 10.0 mM L^{-1}**)** enhanced pigment biosynthesis, photo-protection, and energy production, maximizing photosynthetic efficiency. On contrary, Zn toxicity **(**12.5 to 15.0 mM L^{-1}**)** triggered oxidative stress, damaged thylakoid membranes, and impaired the biosynthesis and stability of chloroplast pigments, as evident by decreased chlorophyll, carotenoids, xanthophylls, and plastoquinone levels.

Influence of zinc on efficiency of photosynthetic enzyme and anti-oxidative defence

Rubisco activity: Rubisco enzyme activity in acid lime leaves showed a significant response to the varying levels of Zn (Table 3). The maximum enzyme activity was observed at 10.0 mM L^{-1} Zn concentration, registering 25.8 and 25.3 µmol CO_2 mg^{-1} protein min^{-1}, in year 2023 and 2024, respectively, closely followed by 7.5 mM L^{-1} Zn treatment. The untreated control consistently recorded lowest activity. Significant variation among treatments, suggests that moderate Zn use (7.5 mM L^{-1}) enhances Rubisco activity, while excessive amounts inhibit it.

Superoxide dismutase (SOD) activity: The activity of SOD in the leaves of acid lime showed marked variations in response to foliar application of Zn in both the years (Table 3). The highest enzymatic activity was observed at 15.0 mM L^{-1} Zn treatment, yielding 41.8 and 43.3 U mg^{-1} protein in 2023 and 2024, respectively. Elevated SOD activity was noted at 10.0 and 12.5 mM Zn L^{-1}, suggesting an intensified anti-oxidant defense mechanism under increased Zn availability. In contrast, lowest SOD levels were associated with 7.5 mM L^{-1} Zn treatment, registering 28.5 and 29.0 U mg^{-1} protein in 2023 and 2024, respectively.

Table 3: Influence of zinc application on Rubisco and superoxide dismutase activities in leaves of acid lime seedlings

Treatments (mM L^{-1})	Rubisco activity (µmol CO_2 mg^{-1} protein min^{-1})			Superoxide dismutase activity (U mg^{-1} protein)		
	2023	2024	Pooled	2023	2024	Pooled
0.0	12.1^{g}	12.5^{g}	12.3^{g}	36.2^{bc}	35.7^{bcd}	35.9^{bcd}
2.5	18.4^{e}	18.9^{e}	18.6^{e}	33.6^{e}	33.1^{de}	33.4^{de}
5.0	20.2^{c}	22.5^{c}	22.4^{c}	30.2^{f}	31.6^{ef}	30.9^{ef}
7.5	24.7^{b}	25.3^{ab}	25.0^{ab}	28.5^{g}	29.0^{fg}	28.8^{fg}
10.0	25.8^{a}	25.3^{ab}	25.6^{a}	37.8^{b}	36.5^{bc}	37.1^{b}
12.5	19.5^{cd}	20.2^{d}	19.9^{d}	36.1^{bcd}	37.4^{b}	36.8^{bc}
15.0	13.6^{f}	14.1^{f}	13.9^{f}	41.8^{a}	43.3^{a}	42.6^{a}
SE±m	0.34	0.41	0.39	0.90	1.02	0.99
CD $_{(0.05)}$	1.02	1.24	1.18	2.71	3.07	2.98

The values in a column superscripted with the same letter(s) do not significantly differ from each other.

Glutathione content: Leaf glutathione levels in acid lime were significantly modulated by foliar Zn treatment (Table 4) with maximum glutathione accumulation in 15.0 mM Zn L^{-1}. The 12.5 mM L^{-1} Zn treatment also led to substantially high glutathione levels in both the seasons. Control treatment depicted moderate values of 1.38 µmol g^{-1} FW in 2023 and 1.47 µmol g^{-1} FW in 2024. In contrast, the lowest glutathione content was observed in plants treated with 7.5 mM Zn L^{-1} (0.86 µmol g^{-1} FW).

Ascorbate peroxidase (APX) activity: APX activity in acid lime leaves was significantly affected by different levels of Zn foliar application in both the years (Table 4). The highest APX activity was recorded at 12.5 mM Zn L^{-1} and lowest at 5.0 mM Zn L^{-1}. The 10.0 mM L^{-1} Zn treatment, also showed relatively higher enzyme activity while moderate APX activities were noted in control.

Low Rubisco activity in control and 15.0 mM L^{-1} Zn treatments indicated metabolic constraints both under Zn deficiency and toxicity. Excess Zn likely caused oxidative stress, leading to protein denaturation and enzyme inhibition (Cakmak, 2000; Broadley *et al.*, 2012; Fukao *et al.*, 2021). These results align with Shahid *et al.* (2021), who observed similar inhibition of photosynthetic enzymes at high Zn concentrations in citrus species. The reduced SOD activity from 0.0 to 7.5 mM

Table 4: Influence of zinc application on glutathione content, ascorbate peroxidase activity and zinc content in the leaves of acid lime seedlings

Treatments (mM Zn L^{-1})	Glutathione (μmol g^{-1} FW)			Ascorbate peroxidase (nmol H_2O_2 min^{-1} g^{-1} FW)			Leaf zinc content (ppm)		
	2023	2024	Pooled	2023	2024	Pooled	2023	2024	Pooled
0.0	1.38^{c}	1.47^{c}	1.42^{c}	2.3^{abc}	2.5^{bc}	2.4^{bc}	1.2^{g}	1.3^{g}	1.2^{g}
2.5	1.29^{cd}	1.25^{d}	1.27^{d}	2.1^{d}	2.0^{de}	2.0^{d}	8.7^{f}	8.1^{f}	8.4^{f}
5.0	1.08^{e}	1.13^{e}	1.10^{e}	1.5^{g}	1.6^{g}	1.6^{g}	15.2^{e}	16.4^{e}	15.7^{e}
7.5	0.87^{fg}	0.85^{fg}	0.86^{fg}	1.8^{e}	2.0^{de}	1.9^{de}	22.3^{d}	24.1^{d}	23.2^{d}
10.0	0.98^{ef}	0.94^{f}	0.96^{f}	2.3^{abc}	2.5^{bc}	2.5^{b}	38.6^{c}	34.2^{c}	36.5^{c}
12.5	1.69^{ab}	1.65^{b}	1.67^{b}	2.8^{a}	2.9^{a}	2.9^{a}	42.5^{b}	41.3^{b}	42.0^{b}
15.0	1.82^{a}	1.87^{a}	1.84^{a}	1.7^{ef}	1.8^{f}	1.8^{ef}	47.8^{a}	49.3^{a}	48.6^{a}
SE±m	0.04	0.03	0.03	0.06	0.05	0.05	1.0	1.1	1.4
CD $_{(0.05)}$	0.13	0.11	0.11	0.18	0.16	0.16	3.1	3.4	4.2

The values in a column superscripted with the same letter(s) do not significantly differ from each other.

Zn L^{-1} reflects improved Zn homeostasis and reduced oxidative stress. As Zn is a structural component of Cu/Zn-SOD isoforms, moderate Zn supports optimal enzyme function (Marschner, 2012). The resurgence of SOD activity at high Zn concentrations indicates stress-induced up-regulation of antioxidant machinery due to excess ROS formation, consistent with Ahmad *et al*. (2018) in *Citrus reticulata* under Zn stress. This adaptive response is vital to counteract the oxidative damage resulting from Zn-induced peroxidation and disrupted electron transport. The decreased GSH content at 0.75 and 10.0 mM L^{-1} suggests reduced ROS generation under optimal Zn, minimizing the need for GSH-mediated detoxification. Under Zn toxicity, however, enhanced GSH levels represent a compensatory response to oxidative stress, as GSH directly scavenges ROS and serves as a substrate for glutathione peroxidase and glutathione S-transferase (Hasanuzzaman *et al*., 2020). Elevated GSH under Zn toxicity has also been reported in *Brassica napus* by Yang *et al*. (2019), wherein Zn-induced oxidative pressure up-regulated GSH biosynthesis. The rise in APX activity under Zn deficiency (T_1: 2.4) and toxicity (T_6: 2.9) suggests enhanced oxidative burden under these conditions. This is in agreement with Kumar *et al.* (2023) who reported increased APX activity under Zn toxicity in *Solanum lycopersicum*. Meanwhile, the dip at moderate Zn levels corresponds to decreased ROS production, reflecting cellular redox stability and minimal need for enzymatic detoxification. At 10.0 mM Zn L^{-1}), the APX activity was moderate (2.5), indicating an effective balance between ROS production and scavenging.

Zinc content in leaves

A significant correlation was observed between leaf Zn content and various physiological parameters in acid lime under different Zn treatments levels (Table 4). As Zn concentrations increased from 0.0 to 10.0 mM L^{-1}, corresponding to a leaf Zn content of up to 36.5 ppm, there was a marked increase in chloroplast pigments *i.e.*, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, xanthophylls, and plastoquinone. Rubisco activity also peaked at this level, indicating improved carbon fixation potential. However, when Zn concentrations exceeded this optimum ($\geq$ 12.5 mM L^{-1}), leading to leaf Zn content > 42 ppm, there was a decline in pigment levels and Rubisco activity. Concurrently, oxidative stress markers such as SOD, glutathione, and APX increased sharply, suggesting cellular stress and activation of antioxidative defense system.

The leaf Zn was positively influenced by Zn application with respect to the photosensitive chloroplast pigments, photosynthetic enzyme activity and anti-oxidative enzyme response (Rout and Das, 2003; Xie *et al*., 2022), which is consistent with Ali *et al*. (2021) and Chen *et al*. (2023). The critical threshold for optimal physiological performance appeared to be around 36.5 ppm Zn in leaves. The enzymatic and antioxidant parameters evaluated revealed distinct threshold behaviour in response to foliar Zn application. Zn deficiency compromises protein synthesis and metabolic efficiency, as evidenced by reduced Rubisco and elevated oxidative stress markers (SOD, APX, GSH). Adequate Zn availability restored photosynthetic and redox balance, improved Rubisco activity and minimized the burden on antioxidant systems. Toxic Zn levels induced oxidative damage due to excessive Zn accumulation disrupting cellular homeostasis (Ali *et al*., 2022).

Conclusion: The present study demonstrated that foliar application of Zn markedly affected chloroplast pigment composition and anti-oxidative defence mechanism in acid lime. Moderate Zn levels (7.5-10.0 mM L^{-1}) significantly enhanced chlorophyll a & b, total chlorophyll, carotenoids, xanthophylls, and plastoquinone contents, thus improved chloroplast functioning and photosynthetic efficiency, promoted Rubisco activity and enhanced carbon assimilation. In contrast, Zn deficiency and sufficiency (≥12.5 mM L^{-1}) declined pigment concentration and enzymatic activity, while elevated levels of SOD, glutathione, and ascorbate peroxidase marked oxidative stress. These findings highlight a biphasic response to Zn, wherein both deficiency and toxicity impair photosynthetic capacity and trigger oxidative defenses. Thus, precise management of Zn nutrition is critical for maintaining chloroplast integrity, optimizing photosynthetic performance, and mitigating oxidative damage in acid lime. This insight provides a physiological basis for refining micronutrient strategies in citrus cultivation under variable agro-environmental conditions.

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