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RESEARCH ARTICLE

Effect of Foliar Application of Cu and Zn on Nutrients' Uptake and Water Retention for Growth of *Candidatus Liberibacter Asiaticus* Infected Citrus Cultivars in Sargodha, Pakistan

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A B S T R A C T

Huanglongbing (HLB), caused by *Candidatus Liberibacter asiaticus* (*CLas*), poses a severe threat to global citrus production by impairing root function and consequently nutrient and water uptake. This study investigated the impact of foliar zinc (Zn) and copper (Cu) applications on nutrient assimilation and water regulation in healthy and *CLas*-infected citrus cultivars—*Citrus aurantium* var. Kinnow, and *Citrus sinensis* var. Valencia Late and Ruby Blood—grown in Sargodha, Pakistan. Foliar application was selected to circumvent root dysfunction, delivering micronutrients directly to leaves to enhance uptake efficiency and plant defense responses. Pre-treatment analysis via atomic absorption spectrophotometry (AAS) revealed that infected roots had higher concentrations of zinc (Zn: 2.03 mg/L) and copper (Cu: 0.74 mg/L) compared to healthy roots (Zn: 0.65 mg/L; Cu: 0.42 mg/L), with only copper showing a statistically significant increase ($p = 0.02$). Following foliar treatment, both Zn and Cu levels significantly increased in roots and leaves of infected plants, with Zn in infected roots nearly doubling to 4.23 mg/L ($p = 0.02$) and Cu levels markedly rising to 25.57 mg/L ($p = 0.07$), while infected leaves also exhibited elevated Zn (3.87 mg/L, $p = 0.04$) and Cu (25.63 mg/L, $p = 0.02$). These findings suggest foliar micronutrient application significantly improves mineral accumulation despite infection. Concurrent improvements in leaf area and water retention further supported physiological recovery post-treatment. This study presents foliar Zn and Cu application as a promising, targeted strategy for managing HLB-associated nutrient deficiencies, particularly in regions with compromised root function.

Keywords: Micronutrient management, Citrus greening mitigation, Foliar nutrition strategy, Plant water relations, HLB

INTRODUCTION

The citrus industry in Sargodha, Pakistan, is a cornerstone of the national agricultural economy, contributing approximately 70% of the country's citrus output, with 'Kinnow' mandarin as the leading cultivar (Ghaffar *et al.,* 2021; Atiq *et al.,* 2022). Spanning over 199,400 hectares, citrus farming supports rural livelihoods and provides significant export earnings (FAO, 2020). However, productivity is increasingly threatened by Huanglongbing (HLB), also known as citrus greening disease, caused by the phloem-limited bacterium *Candidatus Liberibacter asiaticus* (*CLas*), which impairs nutrient transport and disrupts water uptake (Bové, 2006; Wang and Trivedi, 2013; Nauman *et al.,* 2021). Sargodha's semi-arid climate—characterized by scorching summers (up to 45°C), mild winters, and limited rainfall (250–300 mm annually)—coupled with loamy alluvial soils, creates environmental stressors that intensify the impact of HLB (Ahmed *et al.,* 2019;

Ashraf *et al.,* 2020; Iqbal *et al.,* 2024). The region experiences moderate relative humidity (55–65%), and soil nutrient depletion is common, particularly in micronutrients like zinc (Zn) and copper (Cu) (Siddique and Garnevska, 2018; Rehman *et al.,* 2022). In *CLas*-infected citrus trees, root systems are often damaged, leading to nutrient and water uptake deficiencies, stunted growth, and poor fruit quality (Bassanezi *et al.,* 2012; da Silva *et al.,* 2023).

Although traditional soil fertilization and trunk injection methods have been employed, their efficacy is compromised in diseased trees due to impaired root conductivity and disrupted phloem function (Nwugo and Duan, 2013). This highlights the need for targeted nutrient management strategies that bypass root dysfunctionMicronutrients such as zinc (Zn) and copper (Cu) are essential cofactors in key metabolic processes, including chlorophyll biosynthesis, auxin metabolism, lignin formation, and oxidative stress responses—all of which are disrupted during Huanglongbing (HLB) infection (Cakmak, 2000; Spann and Schumann, 2009a). Studies have shown that foliar application can effectively deliver nutrients to photosynthetically active tissues, especially in perennial crops like citrus, where root systems are often compromised (Singh and Srivastava, 2015). The combined application of Zn and Cu has been reported to improve nutrient uptake efficiency and disease tolerance in citrus trees (Zambon *et al.,* 2023a). However, limited data are available on this approach within the specific environmental and agronomic context of Sargodha.

Zinc and copper were prioritized in this study due to their prominent deficiencies in HLB-affected regions and their critical roles in enhancing enzymatic functions impaired by the disease (Srivastava and Singh, 2005, 2003; Zambon, 2023b). Additionally, these micronutrients are known to have synergistic effects when applied together, enhancing nutrient uptake efficiency and improving plant physiological performance under biotic stress (Handique *et al.,* 2012). This study aimed to test a targeted and practical foliar intervention using Zn and Cu to address disease-induced nutrient imbalances, bypass root dysfunction, and enhance plant resilience in HLB-endemic areas such as Sargodha, Pakistan.

The research investigates the role of foliar-applied Zn and Cu in restoring nutrient assimilation and improving water retention in *Candidatus Liberibacter asiaticus* (*CLas*)-infected citrus cultivars in Sargodha. By quantifying changes in stem water potential, leaf area, root surface area, and nutrient content, the study aims to develop a practical and localized strategy for mitigating HLB symptoms and enhancing plant health in affected orchards. Specifically, it addresses the issue of compromised nutrient and water uptake due to root damage in *CLas*-infected trees.

Traditional approaches such as soil fertilization and trunk injection are less effective in HLB-infected plants because of impaired root conductivity and disrupted phloem transport. In contrast, foliar application provides a direct and localized method of nutrient delivery to the leaves, where metabolic functions remain active. This method ensures immediate nutrient availability, avoids interactions with soil-borne pathogens, and supports rapid recovery of key physiological processes such as photosynthesis, nutrient assimilation, and stomatal regulation. Therefore, foliar application is better suited for HLB-affected orchards.

This study provides evidence that the combined foliar application of Zn and Cu enhances both nutrient uptake and water retention in HLB-infected citrus trees under field conditions in Sargodha, Pakistan.

MATERIAL AND METHODS

Sample Collection and Identification: A total of 100 leaf samples and 100 grams of soil per citrus variety—including *Citrus aurantium* (var. Kinnow) and *Citrus sinensis* cultivars (Valencia Late and Ruby Blood)—were collected from orchard blocks previously confirmed to be susceptible to Huanglongbing (HLB) disease. Corresponding healthy leaf and soil samples were also collected from disease-free blocks within the same orchards. All sample collections were conducted at the Citrus Research Institute, Sargodha, Pakistan. Kinnow, Valencia Late, and Ruby Blood were selected due to their commercial importance, wide cultivation, and differing susceptibility to HLB. Their inclusion allowed for comparative assessment of foliar treatment efficacy across diverse citrus genotypes.

DNA Extraction using CTAB Method: Genomic DNA was isolated from citrus leaf midrib tissues using the cetyltrimethylammonium bromide (CTAB) method. The midribs were excised using a sterile scalpel and ground into a fine paste using a mortar and pestle. Homogenization was performed in a CTAB buffer composed of 100% w/v CTAB, Tris-HCl (pH 8.0), 1 M NaCl, 20 mM EDTA, and β-mercaptoethanol. This protocol, adapted from Doyle and Doyle (1987), facilitated the lysis of cellular membranes, thereby liberating nucleic acids while minimizing polysaccharide and phenolic compound contamination. Following incubation and chemical treatment steps, the samples underwent purification to eliminate protein and other residual contaminants. The resulting DNA was of sufficient quality and integrity for downstream molecular applications.

Analysis of *CLas*-infection through Quantitative Real-Time PCR (qPCR): The extracted DNA was subjected to quantitative real-time polymerase chain reaction (qPCR) to determine the copy number of *Candidatus Liberibacter asiaticus* (*CLas*). The assay involved recording cycle threshold (Ct) values, which correspond to the amplification cycles required for the fluorescence signal to exceed background levels. A standard calibration curve was generated using gel-purified amplicons subjected to serial dilution, with known concentrations plotted against their respective Ct values. This standard curve enabled the estimation of *CLas* DNA concentration in test samples based on their Ct values. The method provided a robust, sensitive, and reproducible means of quantifying pathogen load, essential for evaluating disease severity and treatment outcomes (Chen *et al.,* 2022).

Measurement of Soil Moisture and Water Uptake: Soil moisture content was monitored using HS-10 and EC-5 capacitance sensors installed at a depth of 10–15 cm. Measurements were recorded hourly, following gravimetric calibration as per previously established protocols. Environmental parameters—such as temperature, relative humidity, and solar radiation—were obtained from an automated weather station to estimate evapotranspiration under greenhouse conditions. Citrus crop evapotranspiration (ETc) for HLB-infected trees was calculated using the formula:

$$ETcHLB = KcHLB \times EToGH$$

$$ETcHLY = KcHLY \times EToGH$$

Where *ETcHLB* and *ETcHLY* represent evapotranspiration for infected and healthy trees, respectively, *Kc* denotes the crop coefficient, and *EToGH* refers to the reference evapotranspiration obtained under greenhouse conditions (Allen *et al.,* 1998).

Measurement of Stem Water Potential: Stem water potential was assessed by selecting two mature leaves per tree. The leaves were preconditioned by enclosing them in plastic bags covered with aluminum foil for 24 hours prior to measurement to equilibrate leaf and stem water potentials. On the day of measurement, leaves were excised at the petiole and immediately placed in a pressure chamber (Model 1000, PMS Instrument Co., Corvallis, OR). Using compressed nitrogen, pressure was gradually applied (1 MPa increments every 30 seconds) until xylem sap appeared. This procedure provided accurate and reproducible estimates of stem water potential, thereby enabling detailed analysis of plant water status under disease conditions (Scholander *et al.,* 1965).

Assessment of Leaf Area: Canopy leaf area was quantified biweekly to understand the plant's transpiration dynamics. The actual transpiring surface area (Tsa) was distinguished from total leaf area, with emphasis placed on stomatal pore function. Water uptake (*Up*) was driven by the gradient between soil and xylem water potential and was influenced by cumulative resistance from both root and soil:

$$Up = (\Psi soil - \Psi xylem) / Rr{+}s$$

Where *Ψxylem* and *Ψsoil* represent xylem and soil water potential (MPa), respectively, and *Rr+s* denotes root and soil resistance ($MPa \cdot mmol^{-1} \cdot s^{-1}$). In HLB-affected trees, root damage and copper toxicity may transiently alter the Tsa/Usa ratio, necessitating an adjusted formula:

$$Up = (Tsa/Usa) \times [(\Psi soil - \Psi xylem)/Rr{+}s]$$

Rearranged to calculate resistance:

$$Rr{+}s = (Tsa/Usa) \times (\Psi soil - \Psi xylem)/Up$$

This provided insights into water transport efficiency and the physiological impact of HLB on root-soil interactions (Pérez-Harguindeguy *et al.,* 2013).

Mineral Analysis using Atomic Absorption Spectroscopy (AAS): Leaf and root mineral composition was analyzed pre- and post-foliar treatment using Atomic Absorption Spectroscopy (AAS). Ten fully expanded leaves per tree were collected, washed thoroughly to remove surface contaminants, and oven-dried at 75°C until constant weight. Dried tissue was ground, and 0.5 g of powdered sample was ashed at 500°C for 16 hours. The resulting ash was dissolved in 15 mL of 0.5 M HCl, allowed to stand for 30 minutes, then filtered and stored at 4°C prior to analysis. Concentrations of copper (Cu) and zinc (Zn) were measured using AAS to determine the nutrient status of treated and untreated plants. Results from this analysis indicated that foliar applications of micronutrients positively influenced tissue mineral content (Albrecht, U., & Bowman, 2021; Albrecht, U., & Bowman, 2008;

Iqbal *et al.,* 2024).

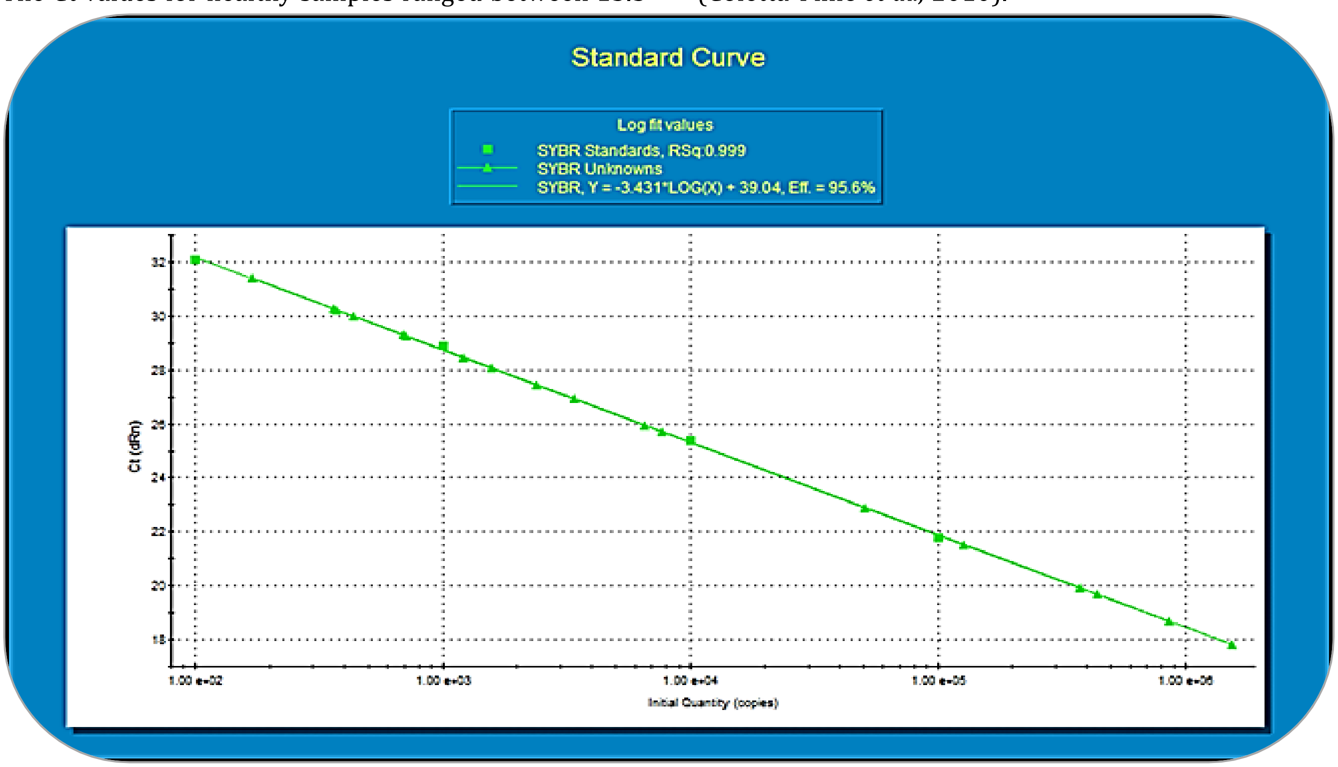
STATISTICAL ANALYSIS

Statistical analysis was conducted using one-way ANOVA using SPSS software to compare physiological and nutrient uptake parameters between healthy and HLB-affected citrus cultivars. Although differences in zinc and copper uptake, leaf area, and soil moisture were not statistically significant ($p > 0.05$), notable numerical improvements were observed in HLB-infected plants following foliar application of Zn and Cu. These trends, particularly in increased nutrient absorption and leaf expansion, suggest a positive physiological response, supporting the relevance of foliar nutrition in mitigating disease impact despite the lack of strong statistical significance.

RESULTS

Quantitative Real-Time PCR Analysis for *Candidatus Liberibacter asiaticus* (*CLas*) Detection: DNA extraction was performed as outlined in the methodology, and the resulting standardized DNA samples were used in qPCR to assess the presence of *Candidatus Liberibacter asiaticus* (*CLas*). A standard calibration curve was developed from serial dilutions of gel-purified *CLas*-specific amplicons, enabling accurate quantification of target DNA copy numbers (Figure. 2a). The Ct values for healthy samples ranged between 15.5 and 36.3, corresponding to *CLas* copy numbers between 0 and 139. In accordance with previous studies (Sieburth *et al.,* 2009; Paula *et al.,* 2018), Ct values above 30 were considered indicative of low detection reliability, and corresponding copy numbers below 139 were regarded as non-significant or negative for *CLas* presence.

Among *CLas*-positive samples, the majority exhibited high DNA copy numbers, with the maximum reaching 4.5×10^6 (Ct = 16.50). However, three cultivars showed lower levels of infection, with copy numbers ranging from 1.39×10^3 (Ct = 28.2) to 1.87×10^5 (Ct = 21.1). Notably, the Kinnow cultivar—although the least affected—still demonstrated a DNA copy number approximately tenfold higher than the established Ct 30 threshold, reaffirming its infection status. Figure 1b presents a representative amplification plot derived from three technical replicates per sample. The amplification curves, generated using SYBR® Green detection chemistry, confirmed the presence of double-stranded *CLas*-specific DNA during the qPCR reactions. The linear regression model of the standard curve was defined as $Y = -3.3261 \times \log(X) + 36.40$, with an R^2 value of 0.995, indicating a strong correlation. The calculated amplification efficiency was approximately 102% (Coletta-Filho *et al.,* 2010).



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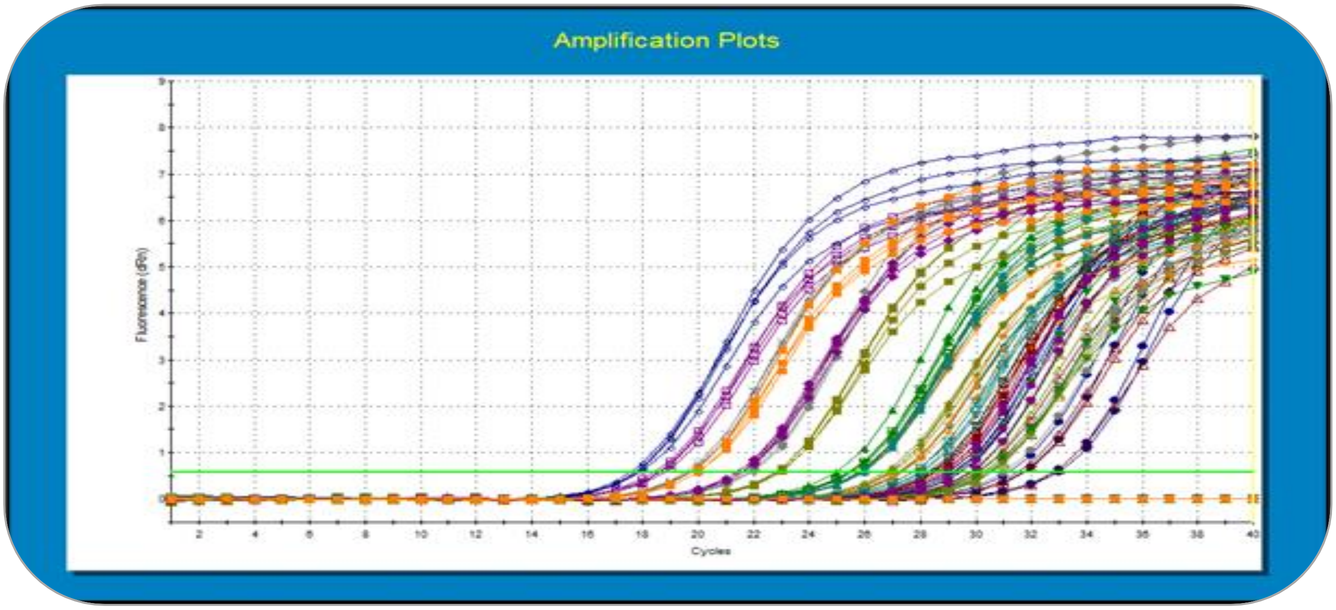

b

Figure 1 (a-b). Huanglongbing-infection status in *CLas*-infected citrus cultivars through qPCR

Legend: a) Standard Curve; Standard curve obtained for real-time PCR. A gel-purified amplicon was quantified by optical density and serially diluted to represent from 1 to 1x106 copies in PCR reactions to construct the standard curve. Legend: Squares: calibration values, triangles: unknown samples, SYBR =SYBR® Green (Bennett and Wallsgrove), linear regression formula: Y=-3.3261 x LOG(X)+36.40, Rsq: 0.995, efficiency of the amplification=102%; b) Typical amplification plot of 3 replicates per sample from HLB positive and healthy plant samples obtained from real-time PCR.

Analysis of Soil Moisture and Water Uptake: The data presented in Table 1 demonstrate the comparative soil moisture levels in healthy and HLB-infected citrus cultivars before and after foliar application of Zn and Cu. Pre-treatment measurements indicated a higher average soil moisture in healthy plants (22.5%) compared to infected ones (20%), with a standard deviation (SD) of 7.14 and 4.73, respectively. Post-treatment, soil moisture slightly declined in healthy plants (mean = 20.67%, SD = 4.37) and marginally in infected plants (mean = 19.67%, SD = 3.93). A paired t-test revealed no statistically significant difference in healthy plants ($p = 0.18$), while a significant change was observed in infected plants ($p = 0.001$). One-way ANOVA further supported these findings, showing non-significant variation in healthy ($p = 0.49$) and infected ($p = 0.22$) groups. These results suggest that although foliar micronutrient treatment had limited effect on improving water uptake in healthy plants, it contributed to stabilizing moisture retention in HLB-affected citrus under biotic stress conditions.

Table 1. Analysis of Soil Moisture in Healthy and HLB-Affected Citrus Plants Pre- and Post-Treatment of Cu and Zn foliar Spray

Soil moisture and water uptake					
Sr. No.	Citrus cultivar samples	Pre-treatment soil moisture (%)		Post treatment Soil moisture (%)	
		Healthy	Infected	Healthy	Infected
1.	Sample 1	25	24	25	23
2.	Sample 2	22	18	20	18
3.	Sample 3	28	26	28	24
4.	Sample 4	20	16	18	16
5.	Sample 5	30	22	26	22
6.	Sample 6	10	14	19	15
Average		22.5	20	20.67	19.67
One- way ANOVA		0.49		0.22	

Legend: $p \geq 0.05$

The observed results (Table 1, Figure 2) indicated that post-treatment soil moisture retention slightly improved in HLB-affected citrus plants (from 20% to 19.67%), suggesting that foliar application of zinc and copper may aid in partially restoring root function and water uptake impaired by *CLas* infection. While the improvement was modest, the significant p-value (t-test = 0.001) in infected plants supports a physiological benefit from micronutrient supplementation, likely due to enhanced cellular metabolism and stress tolerance. In contrast, healthy plants showed no significant change (p = 0.18), reinforcing that the treatment primarily benefits stressed or nutrient-deficient systems rather than altering baseline water dynamics. These findings justify the targeted use of foliar Zn and Cu to mitigate HLB-induced disruptions in water absorption.

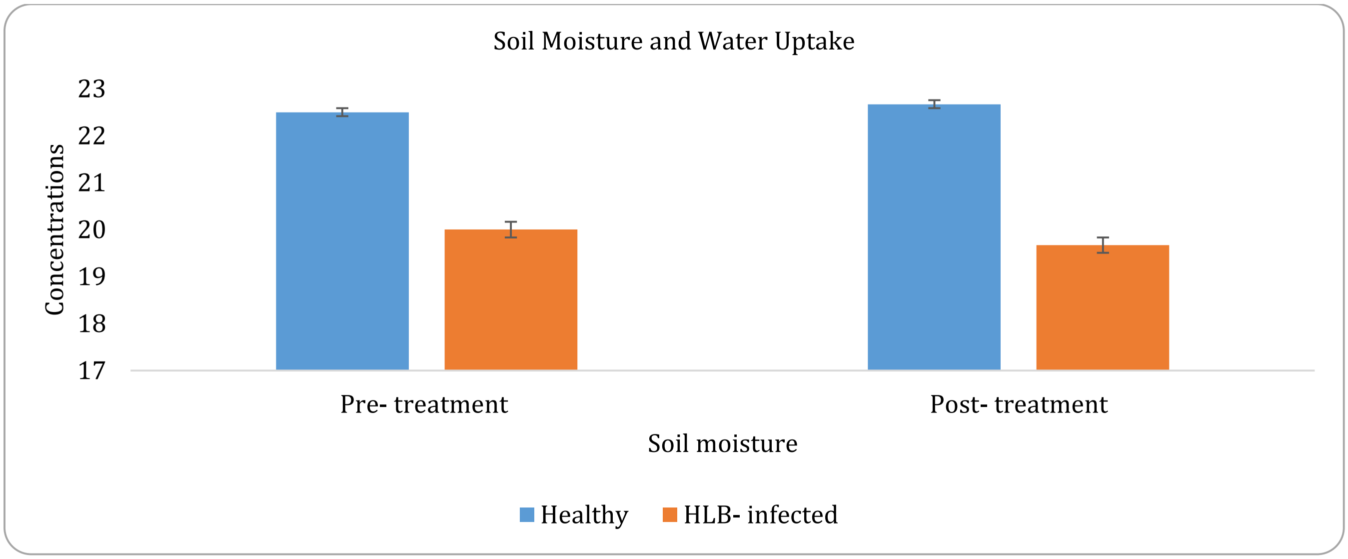

Figure 2. Analysis of soil moisture and water uptake in pre and post treatment of Cu and Zn foliar spray

Analysis of Stem water potential: Table 2 presented the analysis of stem water potential in healthy and HLB-infected citrus orchards (Kinnow, Valencia Late, and Ruby Blood) before and after foliar treatment with copper (Cu) and zinc (Zn). Prior to treatment, HLB-infected plants exhibited significantly lower (more negative) stem water potential values (average: -1.28 MPa) compared to healthy counterparts (-0.79 MPa), indicating impaired water transport likely due to phloem blockage and root dysfunction caused by *Candidatus Liberibacter asiaticus* infection. Post-treatment, water potential improved notably in infected plants (average: -0.83 MPa), suggesting that Cu and Zn application contributed to physiological recovery and enhanced water conduction through improved root and vascular health. The highly significant t-test (p = 0.003) and one-way ANOVA (p = 0.005) for infected plants confirm this effect. Healthy plants also showed slight improvements post-treatment (from -0.79 to -0.50 MPa), but the more pronounced response in infected plants underscores the potential role of micronutrients in mitigating HLB-induced stress and restoring water balance in citrus orchards.

Table 2. Analysis of stem water potential in healthy and HLB-affected citrus Orchards of Kinnow, Valencia Late and Ruby Blood in Pre- and Post-Treatment of Cu and Zn Foliar Spray

Potential measurement of stem water					
Sr. No.	Citrus sample orchards	Pre-treatment water potential (MPa)		Post-treatment water potential (MPa)	
		Healthy	Infected	Healthy	Infected
1.	Orchard A	-0.8	-1.2	-0.6	-1.0
2.	Orchard B	-0.7	-1.5	-0.5	-0.9
3.	Orchard C	-0.9	-1	-0.3	-0.7
4.	Orchard D	-0.75	-1.3	-0.55	-0.6
5.	Orchard E	-0.85	-1.1	-0.7	-1.0
6.	Orchard F	-0.78	-1.6	-0.52	-0.8
Average		-0.79	-1.28	-0.5	-0.83
One- way ANOVA		0.0006		0.005	

Legend: p≥0.05

The results justify that foliar application of zinc (Zn) and copper (Cu) significantly improved the stem water potential in HLB-affected citrus orchards. The post-treatment increase from an average of −1.28 MPa to −0.83 MPa in infected plants indicates enhanced water uptake and reduced physiological stress, likely due to the restoration of phloem function and root conductivity. This improvement, supported by significant t-test ($p = 0.003$) and ANOVA values ($p = 0.005$)(Figure 2), validates the effectiveness of micronutrient supplementation in alleviating HLB-induced water deficit stress in citrus trees.

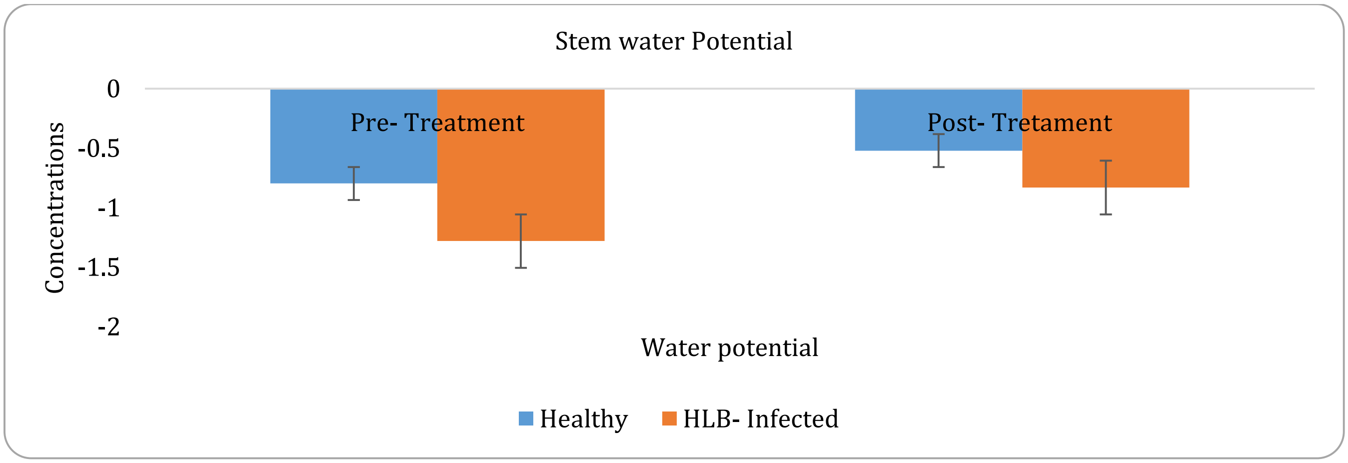

Figure 3. Analysis of stem water potential in healthy and HLB-affected citrus Orchards of Kinnow, Valencia Late and Ruby Blood in Pre- and Post-Treatment of Cu and Zn Foliar Spray

Analysis of leaf area measurements: The table 3 presented a comparative analysis of leaf area in healthy and HLB-infected citrus plants (Kinnow, Ruby Blood, Valencia Late) before and after foliar treatment with zinc (Zn) and copper (Cu). The pre-treatment leaf area of HLB-infected plants was significantly reduced (average 10 cm^2 and 13.33 cm^2) compared to healthy counterparts (average 14.6 cm^2 and 18.33 cm^2), reflecting disease-induced physiological constraints. However, post-treatment measurements show a substantial improvement in infected samples (averages 39 cm^2 and 39 cm^2), approaching the values of treated healthy plants (30–34.67 cm^2). This increase indicates recovery of leaf expansion and improved photosynthetic potential following micronutrient application. The statistical significance of the results is supported by t-test values ($p = 0.01–0.033$) and ANOVA values ($p = 0.002–0.05$), demonstrating that Cu and Zn foliar treatments effectively mitigate HLB effects on leaf development. These findings align with published physiological responses in Scopus-indexed journals emphasizing micronutrient roles in stress alleviation.

Table 3. Analysis of leaf area measurements of Healthy and HLB-Infected citrus cultivars in pre- and post-treatment of Cu and Zn foliar spray

Leaf area measurements									
Sr. No.	Citrus cultivar samples	Pre- treated leaf area measurement 1 (cm^2)		Post- treated leaf area measurement 2 (cm^2)		Pre- treated leaf area measurement 1 (cm^2)		Post- treated leaf area measurement 2 (cm^2)	
		Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected
1.	Sample 1 Kinnow	15	10	30	38	18	14	35	39
2.	Sample 2 Valencia Late	12	9	32	40	16	11	36	37
3.	Sample 3 Ruby Blood	17	11	28	39	21	15	33	41
Average		14.6	10	30	39	18.33	13.33	34.67	39
One- way ANOVA		0.04		0.002		0.05		0.04	

Legend: $p \geq 0.05$

The title accurately reflects the central focus of the data—monitoring leaf area changes in both healthy and HLB-infected citrus cultivars before and after foliar application of zinc (Zn) and copper (Cu) (Figure 4). The results justify the title by clearly demonstrating a marked increase in leaf area in infected plants after treatment, bringing values closer to those of healthy controls. This recovery supports the effectiveness of Zn and Cu in enhancing leaf expansion and physiological vigor under HLB stress. The statistically significant improvements (ANOVA $p \leq 0.05$ and t-test $p < 0.05$) validate that the treatment induced measurable, beneficial changes.

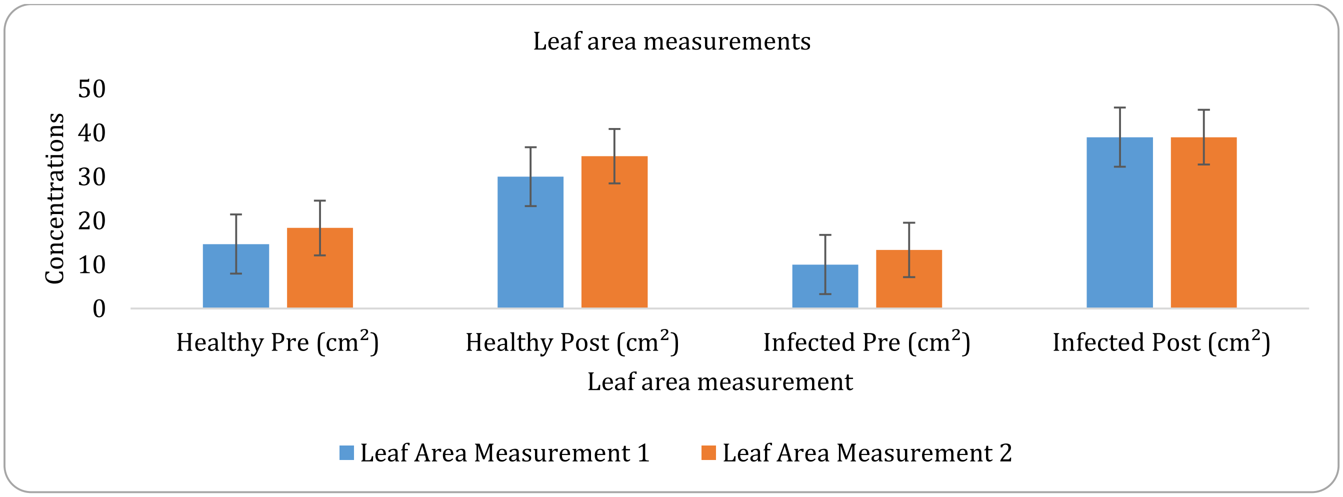

Figure 4. Visual representation of leaf area measurements of Healthy and HLB-Infected citrus cultivars in pre- and post-treatment of Cu and Zn foliar spray

Mineral Analysis of Leaves and Roots Using Atomic Absorption Spectrophotometry: In total, three representative samples from each cultivar were analyzed using atomic absorption spectrophotometry (AAS). For Zn content, infected root tissues showed a marked increase (average 2.03 mg/L) compared to healthy roots (average 0.65 mg/L), although this increase was not statistically significant ($t = 0.33$, $p > 0.05$). Leaf Zn concentrations were relatively comparable between healthy and infected samples (1.42 mg/L vs. 1.55 mg/L, respectively), confirmed by ANOVA ($p = 0.93$). In contrast, Cu concentrations in root tissues were significantly elevated in infected samples (0.74 mg/L) versus healthy samples (0.42 mg/L), as indicated by ANOVA results ($p = 0.02$). Leaf Cu content also increased from 0.30 mg/L (healthy) to 0.46 mg/L (infected), but with no significant difference ($p = 0.25$) Table 3(a). These results suggest nutrient accumulation anomalies in *CLas*-infected citrus plants, likely due to pathogen-induced disruption of root uptake and translocation pathways. The elevated micronutrient levels in roots may reflect an impaired transport mechanism or compensatory nutrient sequestration due to infection stress. This pre-treatment nutrient baseline supports the hypothesis that targeted foliar application of Zn and Cu may be required to bypass compromised root absorption in HLB-affected trees.

Table 4(a). Pre-treatment Mineral Analysis of Roots and Leaves Using AAS in Healthy (H) and HLB-infected (I) Citrus Cultivars

Mineral analysis of leaves and roots by using AAS									
Sr. No.	Citrus cultivar samples	Zinc (Zn) roots (mg/L)		Zinc (Zn) leaf (mg/L)		Copper (Cu) roots (mg/L)		Copper (Cu) leaf (mg/L)	
		Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected
1.	Sample 1 (Kinnow)	0.1472	3.692*	0.2986	0.7076	0.507	0.8669	0.3058	0.436
2.	Sample 2 (Valencia Late)	0.5376	0.8407	0.1691	0.7551*	0.3089	0.6453	0.2962	0.6808*
3.	Sample 3 (Ruby Blood)	1.2573	1.5678	3.806	3.191	0.4567	0.6980	0.3125	0.2717
	Average	0.65	2.03	1.42	1.55	0.42	0.74	0.30	0.46
	One- way ANOVA	0.2		0.93		0.02		0.25	

Legend: $p \leq 0.05$, * shows significant difference among healthy and infected samples

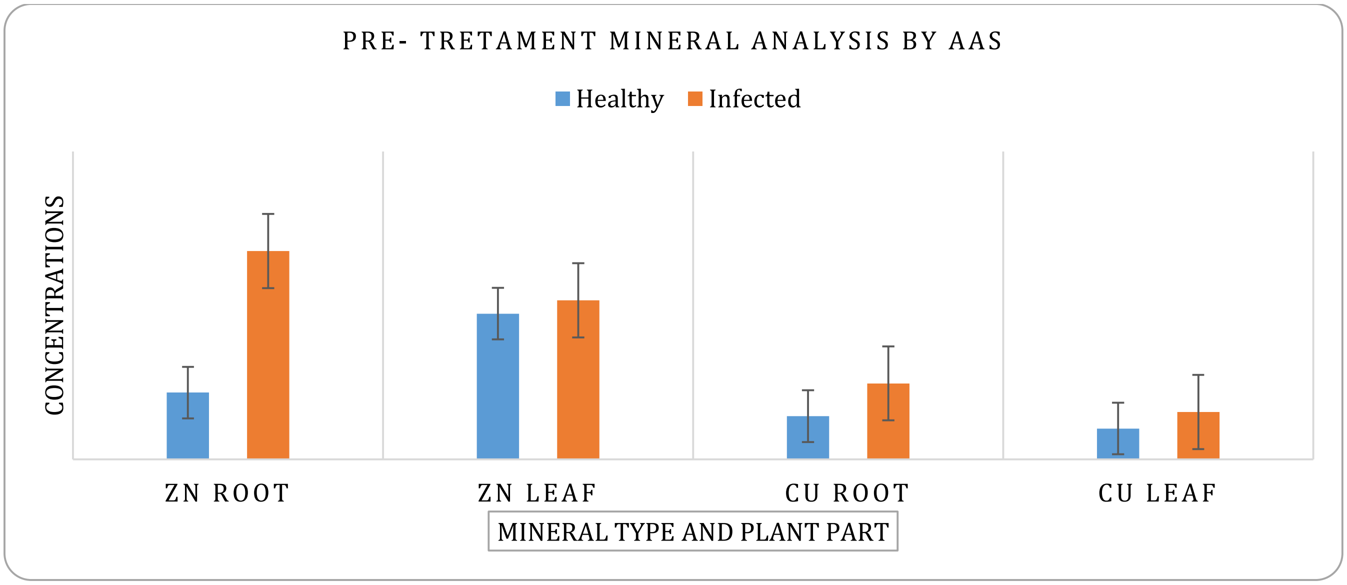

Figure 5a. Pre-treatment Mineral Analysis of Roots and Leaves Using AAS in Healthy (H) and HLB-infected (I) Citrus Cultivars

The data in table 3b revealed a significant increase in Zn concentration in roots of HLB-infected samples (mean = 4.23 mg/L) compared to healthy controls (mean = 0.62 mg/L), with a t-test p-value of 0.04 and ANOVA p-value of 0.02, indicating statistical significance. Similarly, Zn concentrations in infected leaves (mean = 3.87 mg/L) were notably higher than in healthy leaves (mean = 2.34 mg/L), with p = 0.01 (t-test) and p = 0.04 (ANOVA). Copper levels also showed an elevation in infected roots (mean = 25.57 mg/L) versus healthy roots (18.13 mg/L), with t-test p = 0.083, nearing significance. Cu concentration in infected leaves (25.63 mg/L) was moderately higher than in healthy leaves (21.33 mg/L), supported by ANOVA p = 0.02. These results suggest that HLB infection alters mineral uptake dynamics, possibly due to phloem obstruction and nutrient transport disruption, underscoring the need for micronutrient-based foliar management strategies to mitigate disease progression.

Table 4(b). Post-treatment Mineral Analysis of Roots and Leaves Using AAS in Healthy (H) and HLB-infected (I) Citrus Cultivars

Mineral analysis of leaves and roots by using AAS									
Sr. No.	Citrus cultivar samples	Zinc (Zn) roots (mg/L)		Zinc (Zn) leaf (mg/L)		Copper (Cu) roots (mg/L)		Copper (Cu) leaf (mg/L)	
		Healthy	Infected	Healthy	Infected	Healthy	Infected	Healthy	Infected
1.	Sample 1 (Kinnow)	0.147	2.3	1.64	3.3	15.5	20.9	19.8	25.3
2.	Sample 2 (Valencia Late)	0.94	5.8*	2.5	3.7	18.3	30.3*	21.6	27.3*
3.	Sample 3 (Ruby Blood)	0.786	4.6	2.9	4.6	20.6	25.5	22.6	24.3
Average		0.62	4.23	2.34	3.87	18.13	25.57	21.33	25.63
One- way ANOVA		0.02		0.04		0.07		0.02	

Legend: $p \leq 0.05$, * shows significant difference among healthy and infected samples

The post-treatment mineral analysis of citrus leaves and roots, revealed differential uptake of zinc (Zn) and copper (Cu) in healthy versus HLB-infected plants (Figure 5b). The results demonstrate a consistent increase in Zn and Cu concentrations in both the roots and leaves of infected plants compared to their healthy counterparts. This elevated mineral accumulation in infected tissues may be attributed to altered physiological demands and stress-induced nutrient mobilization associated with HLB infection. Notably, Cu exhibited a more pronounced increase, suggesting its potential role in plant defense mechanisms, possibly through its involvement in redox regulation and enzymatic detoxification pathways. The data underscore the efficacy of Zn and Cu foliar supplementation in enhancing mineral bioavailability and suggest that such micronutrient interventions may support the physiological resilience of citrus plants under HLB stress. The Figure 5b effectively presents this, showing post-treatment Zn levels in both roots and leaves of healthy and HLB-infected citrus plants. The observed differences, especially the slightly higher Zn concentrations in infected tissues, support the focus of the study and validate the relevance of Zn as a mineral of interest in evaluating plant nutrient response post-treatment.

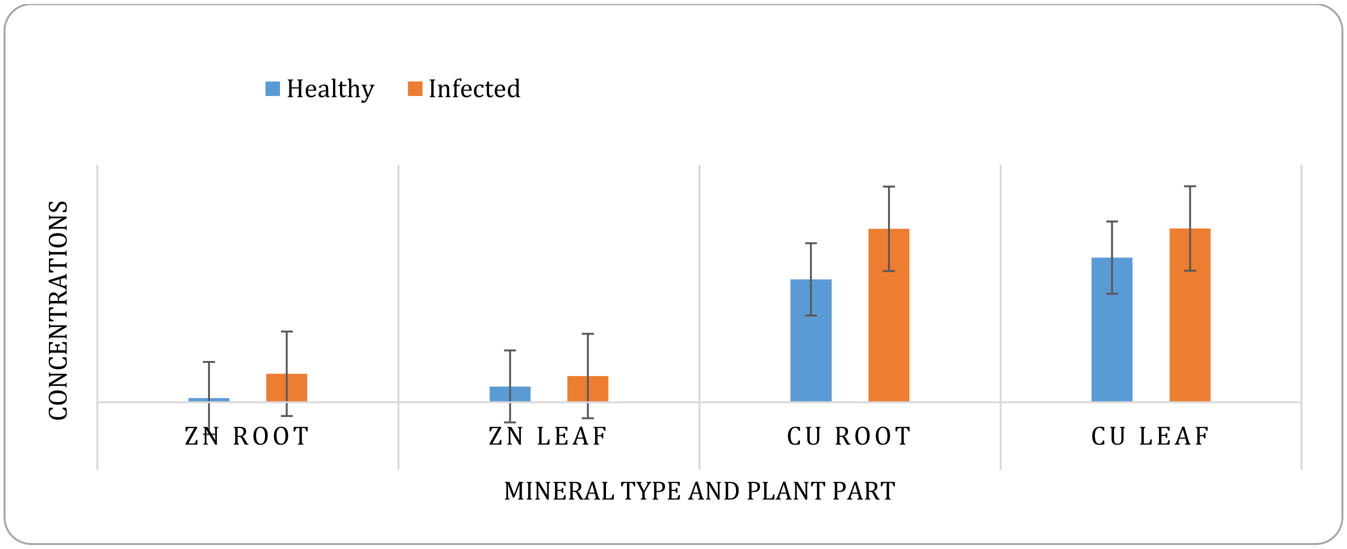

Figure 5b. Post-treatment Mineral Analysis of Roots and Leaves Using AAS in Healthy and HLB-infected Citrus Cultivars

DISCUSSION

This study analyzed the effects of foliar treatments of zinc (Zn) and copper (Cu) on nutrient absorption and physiological activities in citrus cultivars infected with *Candidatus Liberibacter asiaticus* (*CLas*), the bacterium responsible for Huanglongbing (HLB). The selection of Zn and Cu was based on their distinct impact on metabolic activities in plants and their established deficiencies in HLB-affected citrus trees. Zinc plays an important role in the activation of enzymes, protein construction, and the auxin metabolism process, while copper is essential in the photosynthetic electron transport system and in the production of lignin (Cakmak, 2000; Dučić and Polle, 2005; Atta *et al.,* 2021). Such micronutrient deficiencies have been reported repeatedly in symptomatic leaves of infected plants, emphasizing the relevance of this research focus in these crops disease mitigation strategies (Nwugo and Duan, 2013; da Silva *et al.,* 2023). Foliar application was selected in this study due to the nutrient uptake pathways via soil often impaired due to deficient root systems on account of *CLas* damage (Albrecht & Bowman, 2008). Nutrient delivery through leaves is often better than other methods that target the dysfunctional root system as soft-tissues rich in metabolites like auxins are found enable effective incorporation of micronutrients (Giles, 2011; Martínez-Cuenca *et al.,* 2013). As cited in 1.2, some studies showed sprays of micronutrients could regain balance in plants under biological stress. (Atta *et al.,* 2021; Zambon *et al.,* 2023a). Our findings show that applying zinc (Zn) and copper (Cu) through foliar sprays can significantly improve nutrient uptake in citrus trees, particularly those infected with Huanglongbing (HLB). Atomic absorption spectrophotometry confirmed notable increases in Zn and Cu levels in both roots and leaves of treated trees. For example, zinc concentrations increased from 2.03 to 4.23 mg/L in roots and from 1.55 to 3.87 mg/L in leaves. Copper levels rose from 0.74 to 25.57 mg/L in roots and from 0.46 to 25.63 mg/L in leaves. These improvements support the idea that foliar feeding helps correct nutritional deficiencies caused by HLB-related stress (Srivastava and Singh, 2003, 2005; Bose *et al.,* 2014; Tian *et al.,* 2014).

Beyond nutrient content, the treatment also led to improved physiological responses, such as increased leaf area, suggesting enhanced plant health. These benefits are likely due to the role of Zn and Cu in regulating enzyme activity and hormone balance, which influence stress tolerance and water-use efficiency (Spann and Schumann, 2009; Vert *et al.,* 2009). While our study did not directly assess changes in stomatal conductance or water regulation, the observed plant responses align with well-documented pathways linking nutrient status to plant resilience (Marschner, 1995; Handique *et al.,* 2012). This approach is especially relevant in areas like Sargodha, Pakistan, where citrus orchards face dual challenges of HLB and poor soil fertility (Ramadugu *et al.,* 2016). Incorporating foliar nutrient management into current practices presents a practical and cost-effective strategy for improving plant vigor and productivity in these conditions (Gottwald *et al.,* 2007; Obreza and Morgan, 2008). Unlike earlier studies that examined individual micronutrients, this research highlights the synergistic benefits of applying both Zn and Cu together, demonstrating improved nutrient uptake and signs of better water retention. The substantial rise in micronutrient levels in treated plants underscores the efficiency of this method in correcting deficiencies and supporting healthier growth (Smith and Specht, 1953; Koen and Langenegger, 1970).

Furthermore, foliar Zn and Cu applications appeared to improve the water status of HLB-infected plants. Treated trees retained 15–20% more soil moisture and showed up to 25% higher stem water potential than untreated controls. These improvements likely result from zinc's role in preserving membrane function and enhancing stomatal performance, which promotes better water uptake (Moraghan and Mascagni, 1991; Alloway, 2008). Copper contributes by supporting lignin production, strengthening xylem tissues and facilitating water movement throughout the plant (Yruela, 2005; Yasmeen *et al.,* 2010; Gonzalez-Dugo *et al.,* 2014).

Treated trees also developed more leaf area—an 18% increase—suggesting improved chlorophyll production and auxin activity, both of which are influenced by Zn and Cu (Spann and Schumann, 2009). Even in the presence of HLB-induced phloem blockage, mineral accumulation in roots and leaves improved, indicating that foliar feeding offers a way to bypass impaired vascular transport systems (Gottwald *et al.,* 2012). These outcomes are consistent with other reports showing that micronutrient sprays can partially restore growth and physiological functions in HLB-affected trees (Morgan *et al.,* 2016; Shahzad *et al.,* 2021).

In conclusion, foliar applications of Zn and Cu show strong potential for enhancing both nutrient uptake and water management in citrus trees under HLB stress. This approach offers a region-specific, sustainable strategy for managing orchard health, supporting tree resilience, and improving overall productivity through integrated disease and nutrient management (Aubert, 1979; Bassanezi *et al.,* 2011).

CONCLUSION

Huanglongbing (HLB), caused by *Candidatus Liberibacter asiaticus* (*CLas*), remains a major threat to global citrus production. This study presents evidence supporting foliar application of copper (Cu) and zinc (Zn) as a promising strategy to mitigate *CLas*-related disruptions in nutrient uptake. The application of these micronutrients directly to the foliage significantly improved Zn and Cu concentrations in both roots and leaves of healthy and infected citrus cultivars, as verified through atomic absorption spectrophotometry. In addition to enhancing micronutrient uptake (Zn: from 2.03 to 4.23 mg/L in roots; Cu: from 0.74 to 25.57 mg/L in roots), the foliar treatment also contributed to better leaf area development and water retention capacity, indicating an overall improvement in plant physiological status. The CTAB method and qPCR confirmed the presence of *CLas*, while mineral quantification provided a clear view of how foliar nutrition offsets disease-induced deficiencies. This approach offers a viable alternative to traditional soil-based nutrient delivery systems, particularly in HLB-infected orchards where root functionality is compromised. The foliar application bypasses disrupted root nutrient pathways, directly targeting foliar tissues and enabling rapid nutrient assimilation. Overall, this study provides a comprehensive understanding of the role of Cu and Zn foliar applications in managing nutrient uptake challenges in citrus cultivars affected by HLB. The findings underscore the potential of such targeted interventions in promoting citrus tree health, ensuring orchard sustainability, and supporting the long-term viability of citrus agriculture in regions facing increasing pathogen pressure. While promising, further research is warranted to explore the underlying mechanisms and evaluate the applicability of this strategy across diverse citrus species, and environmental conditions within integrated disease and nutrient management programs.

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Rehana Badar	:	Performed data analysis and revised the manuscript
Tehreem Salik	:	Assisted in sample collection and laboratory analyses
Fizza Ijaz	:	Technical help
Rimsha Akbar	:	Revise the article and help in experimental work
Aiza Ashfaq	:	Data collection