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

by Dr. Khadija Gilani

Submission date: 29-May-2025 08:46AM (UTC+0500)
Submission ID: 2687299298
File name: 1241_similarity_indexed_report.docx (268.4K)
Word count: 5380
Character count: 31653

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

Khadija Gilani[1], Rehana Badar[2], Tehreem Salik[1], Fizza Ijaz[2], Rimsha Akbar[2], Aiza Ashfaq[1]

Khadija Gilani (11952)

Abstract

19

Citrus greening/Huanglongbing (HLB), caused by *Candidatus Liberibacter asiaticus* (*C*Las), persista a critical challenge to citrus agriculture production by impairing root function and consequently nutrient and water uptake. This study explored the impact of foliar zinc (Zn) and copper (Cu) applications on nutrient assimilation and water regulation in healthy and *C*Las-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). Spanning over 199,400 hectares, citrus farming supports rural livelihoods and provides significant export earnings (FAO, 2020). However, productivity is increasingly threatened by citrus greening/Huanglongbing (HLB), caused by the phloem-limited bacterium *Candidatus Liberibacter asiaticus* (*C*Las), which impairs nutrient transport and disrupts water uptake (Bové, 2006; Wang & Trivedi, 2013). 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 (Ashraf et al., 2020; Ahmed et al., 2019). The region experiences moderate relative humidity (55–65%), and soil nutrient depletion is common, particularly in micronutrients like zinc (Zn) and copper (Cu) (Siddique, & Garnevska 2018;

Rehman et al., 2022). In *C*Las-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 & 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 & 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 & 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 & Singh, 2005, 2003; Zambon et al., 2023b). Additionally, the combined effect of zinc and copper synergistically improve plant physiology under biotic stresses (Handique et al., 2012). This research aimed to target the particular nutrients foliar spray effects to identify nutrient imbalances due to abrupt bacterial disease, bypass of root dysfunction and enhance resistant in citrus plants against HLB disease in endemic areas i.e. Sargodha, Pakistan.

This study investigates the role of foliar application of Zn and Cu in restoring mineral deficiencies and correlate with the water retention in *C*Las-infected citrus cultivars in Sargodha. It also analyzed the stem water potential, leaf area and nutrient imbalances occurred due to this disease. This study developed particular strategy for overcoming HLB symptoms and enhancing plant's health in HLB-affected orchard areas. Particularly it addresses the issues regarding nutrient imbalances and water update by roots damages in HLB-affected tress.

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 criteria allowed for differential analysis of foliar applications efficacy against diverse citrus genotypes.

Extraction protocol using CTAB Method

Extraction of Genomic DNA was performed using citrus leaf midrib tissues using the cetyl-trimethyl-ammonium bromide (CTAB) protocol. The midribs were cutted using a sterile blade and crushed it into a fine paste using a pestle and mortar. Homogenization was performed using a CTAB buffer (composition of 100% w/v CTAB, Tris-HCl (pH 8.0), 1 M NaCl, 20 mM EDTA, and β-mercaptoethanol) by Doyle and Doyle (1987). Purification was performed after following incubation and chemical treatment steps.

Analysis of *C*Las-infection using qPCR

The extracted DNA was quantified using qPCR reaction analysis to determine the copy number of *C*Las. The protocol involved the cycle threshold (Ct) values, which correspond to the amplification cycles required for the fluorescence signal to exceed background levels. A gel-purified amplicons subjected to serial dilution resulting a standard calibration curve. The known concentrations plotted against their respective Ct values. This standard curve quantified the estimation of *Candidatus* liberibactar asiaticus DNA concentration in samples based on their threshold values. The protocol was followed by Chen et al., 2022.

Measurement of Soil Moisture and Water Uptake

Soil moisture content was examined by using HS-10 and EC-5 capacitance sensors installed at a depth of 10–15 cm of soil. The measurements were noted hourly, following gravimetric calibration as per previous conventional procedure. Ecological parameters involved; average temperature, solar radiation and relative humidity (RT). These parameters were recorded from an automated weather station to estimate the evapotranspiration within the controlled microclimate greenhouse conditions. Citrus cultivars evapotranspiration (ETc) for *C*Las-infected trees was calculated using the formula:

$$ETcHLB = KcHLB \times EToGH$$

$$ETcHLY = KcHLY \times EToGH$$

Where *ETcHLB* and *ETcHLY* represent evapotranspiration (water loss) for *C*Las-infected and healthy trees (Control), respectively, *Kc* repressents the crop coefficient, and *EToGH* denoted as the reference evapotranspiration obtained within the micro greenhouse parameters (Allen et al., 1998).

Measurement of Stem Water Potential

In the calculation of stem water potential, two leaves per tree was used. The symptoms of the leaves were analysed by sealing the samples in the aluminium foil and placed in the plastic bags for 24h to ensure water potential of stem and leaves before measurement. The leaves were cutted at the petiole immediately at the day of measurement and subjected to the pressure chamber (Model 1000, PMS Instrument Co., Corvallis, OR) for analysis. Compressed nitrogen, was progressively applied (1 MPa increments every 30 seconds) until xylem sap was released. This protocol yielded reliable and consistent measurements of stem water potential under pathogenic stresses (Scholander et al., 1965).

Assessment of Leaf Area

To elucidate the dynamics of plant transpiration, canopy leaf area was recorded on the biweekly assessment, differentiating effective transpiring surface area (Tsa) from total foliage with focus on stomatal conductance. The movement of water uptake (Up) was recorded by potential differential between soils to xylem water uptake movements influenced by total hydraulic resistance from roots to soil properties.

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

However, *Ψxylem* represent xylem and *Ψsoil* represents the soil water potential (MPa), Due to the root dysfunction and potential degradation with Cu toxicity in *C*Las-infected citrus plants, Tsa/Usa balance may be altered requiring recalibration of estimated framework.

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

Reconfigured to compute estimate resistance:

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

This protocols insights into evapotrapirational efficiency with the physiological influence of *C*Las on the root-soil dynamics (Pérez-Harguindeguy et al., 2013).

Analysis of Mineral by Atomic Absorption Spectroscopy (AAS)

The mineral analysis of roots and leaves were quantified in pre and post foliar applications using Atomic Absorption Spectroscopy (AAS). Ten leaves per tree were collected, surface sterilized by avoiding contaminant and preheated in oven (Memmert LDO-080F) at 75°C until equilibrium weight was obtained. Dried tissues were pulverized and 0.5g of the dried powder was heated in muffle furnace at 500°C for 16 h to obtain ash. 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).

Statistical Analysis

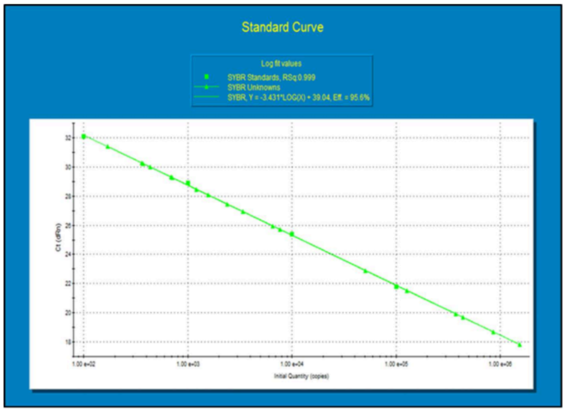
Statistical analysis was conducted using one-way ANOVA using SPSS software to compare physiological and nutrient uptake parameters between healthy and HLB-affected citrus cultivars. Although differences in zinc and copper uptake, leaf area, and soil moisture were not statistically significant ($p > 0.05$), 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

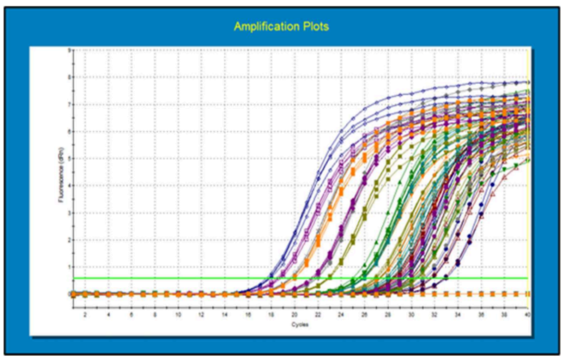
Quantitative Real-Time PCR Analysis for *Candidatus* Liberibacter asiaticus (*C*Las) Detection

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 (*C*Las). A standard calibration curve was developed from serial dilutions of gel-purified CLas-specific amplicons, enabling accurate quantification of target DNA copy numbers (Fig. 2a). The Ct values for healthy samples ranged between 15.5 and 36.3, corresponding to *C*Las 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 *C*Las presence.

Among *C*Las-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 *C*Las-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).



a



b

Figure 1 (a-b): Huanglongbing-infection status in *C*Las-infected citrus cultivars through qPCR

Legend: a) Standard Curve; calculated from real-time qPCR. A gel-purified amplicon was quantified by optical density (OD) and diluted serially to represent from 1 to 1x10^6 copies in PCR reaction cycles to construct the standard curve. 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 *C*Las 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.

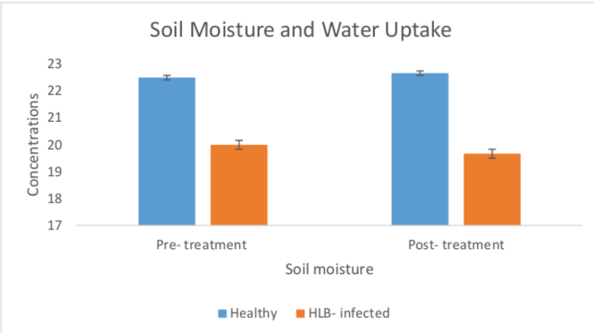


Figure 2: Analysis of soil moisture and water uptake in pre and post treatment of Cu and Zn foliar spray

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: $p \geq 0.05$

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 ($p = 0.005$)(Fig 2), validates the effectiveness of micronutrient supplementation in alleviating HLB-induced water deficit stress in citrus trees.

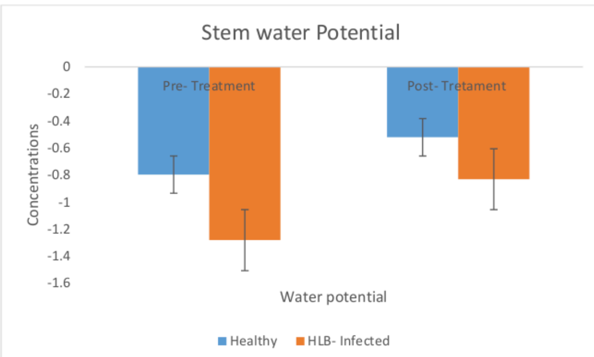


Fig 3. Analysis of stem water potential in healthy and *C*Las-infected citrus orchards of Kinnow, Valencia Late and Ruby Blood in Pre- and Post-Treatment of Cu and Zn Foliar Spray

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) (Fig 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.

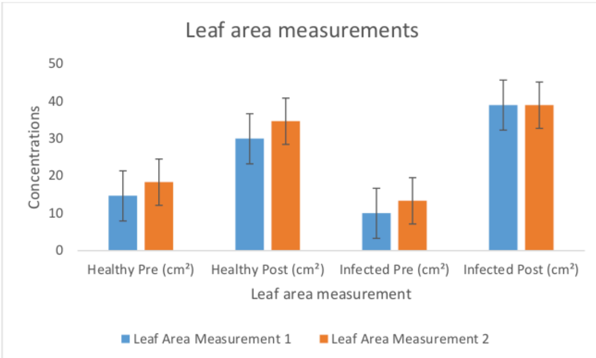


Figure 4: Visual representation of leaf area measurements of Healthy and *C*Las-infected citrus cultivars in pre- and post-treatment of Cu and Zn foliar spray

Mineral Analysis of Leaves and Roots Using Atomic Absorption Spectrophotometry

In total, three representative samples from each cultivar were analysed 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 *C*Las-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

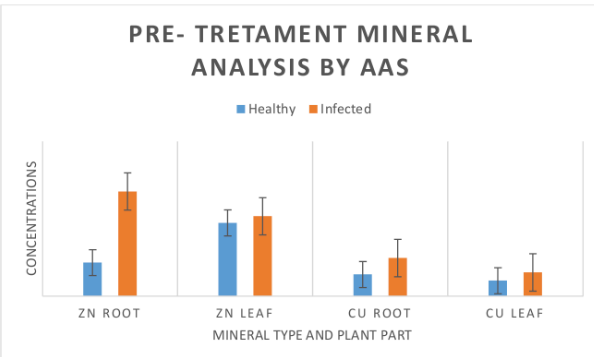


Figure 5a: Pre-treatment Mineral Analysis of Roots and Leaves Using AAS in Healthy (H) and HLB-infected (I) Citrus Cultivars

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 (Fig 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.

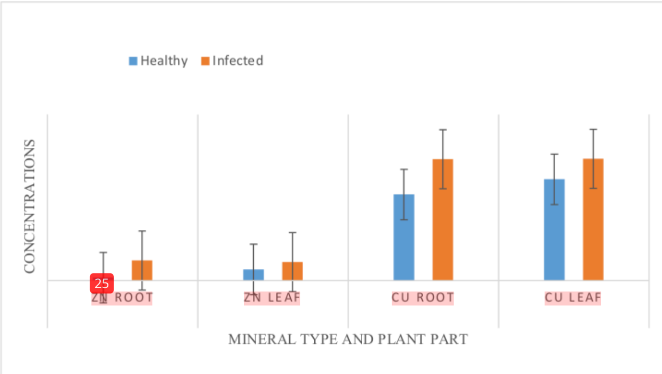


Figure 5b: Post-treatment Mineral Analysis of Roots and Leaves Using AAS in Healthy and HLB-infected Citrus Cultivars

Discussion

This study aimed to analysed the effect of foliar application of zinc and copper on mineral disruption and physiological imbalances in *C*Las infected citrus cultivars vs. healthy control. The selection of zinc and copper were chose due to the direct impact of these mineral on physiological responses and their characteristic depletion in citrus trees under biotic and abiotic stresses. 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 (Atta et al., 2021; Cakmak, 2000; Dučić & Polle, 2005). 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 (da Silva et al., 2023 a &b; Nwugo & Duan, 2013). 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. This study showed the elevation of zinc levels from 2.03-4.23 mg/L in roots, whereas, 1.55-3.87 mg/L were observed in leaves. For instance Cu concentration was elevated from 0.74-25.57 mg/L in roots and in leaves it was increased from 0.46 to 25.63 mg/L. These results supported the notion that foliar application of Zn and Cu counterparts the mineral deficiencies caused by *C*Las-infection related stresses in citrus cultivars (Srivastava & Singh, 2003, Srivastava & Singh, 2005; Bose et al., 2014; Tian et al., 2014).

In addition to restoring micro and macronutrients deficiencies through foliar application led to promotes the physiological responses such as increased leaf area in treated citrus plants. Controlling mineral deficiencies promotes the regulation of hormonal balances with enhanced enzymatic activities with stress tolerance and water uptake efficiencies (Spann & Schumann, 2009; Vert et al., 2009). However, this study did not directly analyzed the water regulation and stomatal conductance, the explained results reflects the citrus plant responses with well-documented pathways associated with mineral uptakes associated with biotic and abiotic stress tolerance (Handique et al., 2012; Marschner, 1995).

This research is relevant to those citrus cultivation areas where mineral deficiencies hindered in the growth of citrus with inadequate soil fertility which particularly enhanced haunglongbing disease infection (Ramadugu et al., 2016). The foliar application strategies not only improved the nutrient deficiencies but also improve the plant vigor and ultimately increase the growth and yield production (Obreza & Morgan, 2008; Gottwald et al., 2007). In contrast to previous researches of the assessment of mineral nutrients, this study demonstrated the combined application of Zn and Cu produced synergistic effects on the improvement of plant health with enhanced water potential and nutrient efficiencies. The notable movement in micronutrient uptake in post-treatment citrus samples illustrated the effectiveness of foliar application in rectifying mineral deficiencies and sustained improved physiological performances of the citrus plants (Smith & Specht, 1953; Koen & 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 & 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 & 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 results are consistent with other researches of micronutrient foliar application which supports the revival of growth with physiological parameters in *C*Las-infected citrus cultivars (Morgan et al., 2016; Shahzad et al., 2021). In summary, management of foliar applications of zzinc and copper enhaces the nutrient efficies with uptake of water potential in citrus plant infected with citrus greening. This methods provides the sustainable ecofriendly protocols which enhance the tree tolerance to biotic and abiotic stresses, boosting the growth and productivity by optimizing nutrient imbalances and diseases mitigation (Bassanezi et al., 2011; Aubert, 1979).

Conclusion

Citrus greenin/Huagnlongbing is the robust disease caused by *Candidatus Liberibacter asiaticus* (*C*Las), which poses a critical risk to citrus world industry. This study supporting the evidence of foliar application practices of Zinc (Zn) and Copper (Cu) as and effective approach to overcoming *C*Las-infection associated mineral deficiencies. The post-treatment application of foliar spray significantly affects the concentration of Zn and Cu in roots and leaves of infected citrus cultivars. The clear enhancement of micronutrients were seen in this study (Zinc increases from 2.03-4.23 mg/L in roots; however, Cu was alleviated from 0.74-25.57 mg/L). The combined synergistic effect of foliar application also enhanced leaves expansion areas by increasing water retention indicating overall strengthen the physiological health of the citrus plants. This strategy provided a feasible approach of replacement of traditional soil based nutrient delivery, specifically in *C*Las-infected citrus orchards where root dysfunction is prominently effect the plant growth. The foliar management practices directly targeting the nutrient assimilated foliar tissues. In summary, this research provides a valuable insight of understanding the Zn and Cu foliar feeding in mitigation nutrient deficiencies in citrus cultivars affected with HLB. The results reinforce the effectiveness of targeted foliar management practicesin improving plant growth and supports agriculture productivity against pathogenic affects. 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.

Acknowledgement

The authors acknowledge the Citrus Research Institute, Sargodha, for providing plant material and field support. We also thank the University of Lahore for access to laboratory facilities and analytical instrumentation. Their contributions significantly supported the completion of this research.

Authors' Contributions

Khadija Gilani conceptualized and supervised the study, designed the experiments, and critically revised the manuscript. Rehana Bader conducted the experimental work, performed data analysis, and contributed to manuscript drafting. Tehreem Salik assisted in sample collection, laboratory analyses, and preparation of figures and tables. All authors read and approved the final manuscript.

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

ORIGINALITY REPORT

7%	5%	4%	2%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to Higher Education Commission Pakistan Student Paper	2%
2	www.science.gov Internet Source	<1%
3	Ravendra Kumar Singh, Sugandha L. Chavan, Pravin H. Sapkale. "Heavy Metal Concentrations in Water, Sediments and Body Tissues of Red Worm (Tubifex spp.) Collected from Natural Habitats in Mumbai, India", Environmental Monitoring and Assessment, 2006 Publication	<1%
4	prr.hec.gov.pk Internet Source	<1%
5	www.ncbi.nlm.nih.gov Internet Source	<1%
6	Sabah Abdul-Wahab, Fouzul Marikar. "The environmental impact of gold mines: pollution by heavy metals", Open Engineering, 2012 Publication	<1%
7	Choi, Y.H.. "Transfer of ^9^0Sr to rice plants after its acute deposition onto flooded paddy soils", Journal of Environmental Radioactivity, 2007 Publication	<1%

8	bmcplantbiol.biomedcentral.com Internet Source	<1%
9	discovery.researcher.life Internet Source	<1%
10	nar.oxfordjournals.org Internet Source	<1%
11	www.tandfonline.com Internet Source	<1%
12	G.R. Smitha, Virendra S. Rana. " The Effect of Viral Infection on Essential Oil Content, Chemical Composition and Biomass Yield of cultivars ", Journal of Essential Oil Bearing Plants, 2015 Publication	<1%
13	jhe.cnu.edu.ph Internet Source	<1%
14	"Precision agriculture '21", Walter de Gruyter GmbH, 2021 Publication	<1%
15	www.microbiologyjournal.org Internet Source	<1%
16	"Abstracts Submitted for Presentation at the 2008 APS Centennial Meeting", Phytopathology, 2008 Publication	<1%
17	Christine A. Edmiston, William P. Cochlan, Christopher E. Ikeda, Andrew L. Chang. "Impacts of a temperate to tropical voyage on the microalgal hull fouling community of an atypically-operated vessel", Marine Pollution Bulletin, 2021 Publication	<1%
18	Maria Calabritto, Alba N. Mininni, Roberto Di Biase, Angela Pietrafesa, Bartolomeo Dichio.	<1%

"Spatio-temporal dynamics of root water uptake and identification of soil moisture thresholds for precision irrigation in a Mediterranean yellow-fleshed kiwifruit orchard", Frontiers in Plant Science, 2024
Publication

19	content.govdelivery.com Internet Source	<1 %
20	www.ogscience.org Internet Source	<1 %
21	"Abstracts Submitted for Presentation at the 2009 APS Annual Meeting", Phytopathology, 2009 Publication	<1 %
22	Hanan Sharif, Reza Arabi Belaghi, Kiran Kumar Jagarlamudi, Sara Saellström, Liya Wang, Henrik Rönnberg, Staffan Eriksson. "A novel cross-validated machine learning based Alertix-Cancer Risk Index for early detection of canine malignancies", Frontiers in Veterinary Science, 2025 Publication	<1 %
23	Said A. Hamido, Robert C. Ebel, Kelly T. Morgan. "Interaction of Huanglongbing and Foliar Applications of Copper on Water Relations of Citrus sinensis cv. Valencia", Plants, 2019 Publication	<1 %
24	Xiaocong Jiao, Xuemei Yu, Yajing Yuan, Jianming Li. "Effects of vapor pressure deficit combined with different N levels on tomato seedling anatomy, photosynthetic performance, and N uptake", Plant Science, 2022 Publication	<1 %

25	notulaebotanicae.ro Internet Source	<1 %
26	pubmed.ncbi.nlm.nih.gov Internet Source	<1 %
27	research.ou.nl Internet Source	<1 %
28	"Sustainable Solutions for Elemental Deficiency and Excess in Crop Plants", Springer Science and Business Media LLC, 2020 Publication	<1 %
29	Margie Lynn Stratton, George L. Good, Allen V. Barker. "THE EFFECTS OF NITROGEN SOURCE AND CONCENTRATION ON THE GROWTH AND MINERAL COMPOSITION OF PRIVET", Journal of Plant Nutrition, 2001 Publication	<1 %
30	Mustafa Dogan. "The Influence of Heavy Metals on the Urban Flora / UTICAJ TEŠKIH METALA NA URBANU FLORU", Biodiverzitet - Teorijski i praktični aspekti / Biodiversity - Theorectial and Practical Aspects, 2012 Publication	<1 %

Exclude quotes	Off	Exclude matches	Off
Exclude bibliography	Off		