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Rootstock Influence on Growth and Mineral Content of *Citrus limon* and *Citrus sinensis* cv. Valencia Inoculated with *Candidatus* Liberibacter Asiaticus

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Abstract: Huanglongbing (HLB) reduces the growth and development of citrus and induces changes in secondary metabolites such as flavonoids, limonoids, and polyamines. Likewise, infected plants have a deficient absorption of nutrients such as zinc, potassium, manganese, and copper. Therefore, the objective of this study was to evaluate the influence of different rootstocks on morphology and mineral changes of *Citrus limon* and *Citrus sinensis* cv. Valencia plants inoculated with *Candidatus* Liberibacter asiaticus. In a greenhouse of the Experimental Station-Autonomous University of Tamaulipas, the *Candidatus* Liberibacter asiaticus bacteria were inoculated to *Citrus limon* plants (growing on *Citrus volkameriana*, *Citrus macrophylla*, and *Citrus aurantium* rootstocks) and *Citrus sinensis* cv. Valencia (growing on *Citrus volkameriana* and *Citrus aurantium* rootstocks). The experiment was established under a completely randomized design with 45 graft/rootstock repetitions. In each graft/rootstock combination, the plant height and stem diameter were determined using a tape measurer and a Vernier, respectively. In addition, the nutrient content of foliar samples was determined by an X-ray fluorescence spectrometer. In both citrus species, the *C. aurantium* rootstock promoted a higher concentration of the bacteria. On the other hand, the rootstock that showed the best agronomical results after inoculation with the bacteria was *C. volkameriana*, presenting the least variation in mineral content and conferring greater plant height (15%) and stem diameter (23%). In contrast, the presence of *Ca.* Liberibacter asiaticus decreased S content and increased Cu concentration in *C. lemon* plants. Similarly, plants infected with *C. sinensis* presented higher Fe content. Finally, in both species, no significant differences were observed for Mn, P, and Zn concentration.

Keywords: Huanglongbing (HLB); height; scion; *Citrus volkameriana*; X-ray fluorescence spectrometry

1. Introduction

One of the factors that reduce citrus production is the presence of different diseases, among them, Huanglongbing disease (HLB), which is one of the biggest problems in citrus worldwide, reduces production up to 50%, and in advanced stages of infection, may cause death of trees [1,2]. Globally, every year, the HLB causes losses of billions of dollars in the citrus industry [3]. In different citrus areas around the world, HLB is caused by different strains of *Candidatus* Liberibacter; however, in Mexico and the Caribbean, the asiaticus strain is responsible for this disease. In Mexico, the decrease in production has reached 25%, that is, 1.84 million tons of citrus fruits [4]. The *Candidatus* Liberibacter asiaticus bacterium is spread by the Asian citrus psyllid (*Diaphorina citri*) [5]. The dissemination of the bacteria begins in the phloem; this plant tissue is affected by secretions of the bacteria that consist of dependent effectors and virulence factors. Likewise, *Candidatus* Liberibacter asiaticus interferes with protein mobilization and causes cell death and phloem necrosis [1]. In addition, within the primary infection process, the bacterium reaches the roots through the phloem [6]. Later, a dark material appears in the roots between the plasma membrane and cell walls. As this material accumulates, the roots show a decline and a low starch content [7]. Therefore, the growth of vertical roots decreases by 20% compared to horizontal roots, and finally, the root system shows a decline and death [8]. However, root death could also be a secondary symptom of infection in the absence of carbohydrates [6].

The pathogen raises starch concentration in the vascular bundles, induces a nutritional deficiency that is expressed in the leaves through irregular spots, and causes thickening of midribs [9]. In addition, it affects plant growth and development [10]; more specifically, in leaves and fruits it induces changes in secondary metabolites content, affecting flavonoids, limonoids, hydroxycinnamic acids, and polyamines [11,12]. Besides, the pathogen uses the nutrients present in the vascular bundle for its growth and reproduction; therefore, the plant has a deficient absorption of nutrients such as zinc, potassium, manganese, and copper [13,14]. Thus, diseased plants show a decrease of 16% to 30% in canopy height and volume [15]. In addition, the concentrations of these minerals vary depending on the cultivar and rootstock used [16]. The scion-rootstock connection is essential for optimal growth, absorption, and transport of water and nutrients [17]. In addition, the transmissibility of scion impacts plant shape and physiology. This is due to long-distance communication pathways between cells and may involve hormones, metabolites, water, and nutrient availability, as well as other molecules (proteins, transcripts, and sRNA) that provide a direct link to the underlying genetic mechanisms [18].

Recently, the University of Florida and USDA/ARS in the USA have conducted joint research for the development of rootstocks resistant to HLB [19]. Evaluations have been made with different graft/rootstock combinations, for example: 'Hamlin/Kinkoji', 'Hamlin/Cleopatra', 'Temple/Cleopatra', 'Fallglo/Kinkoji', 'Sugar Belle/Sour Orange', 'Tango/Kuharske', and 'Ruby Red/Kinkoji', to name a few [20].

The most used technology for this bacterium detection is based on polymerase chain reaction (PCR), while techniques to determine changes in chemical compounds and mineral contents include gas chromatography, spectroscopy, and chemical analysis of volatile organic compounds [21,22]. However, these detection techniques are expensive or require a long time [23]. Another methodology for assessing mineral concentration is X-ray fluorescence spectrometry [24], which has the advantage that can generate spectra of a wide range of samples (powders, liquids, among others) with minimum sample preparation, and its analysis is performed in a short time. In addition, it has quantitative applications based on the relationship between spectral, and reference data obtained by conventional methods to establish prediction models and qualitative applications, to illustrate the studied diversity and classifying the samples according to their spectral characteristics [25]. Therefore, the objective of this study was to evaluate the changes in minerals and morphology of *Citrus limon* and *Citrus sinensis* plants inoculated with *Candidatus* Liberibacter asiaticus grown on different rootstocks.

2. Materials and Methods

2.1. Plant Material and Experimental Design

The experiment was established in a greenhouse of the "Ingeniero Herminio García González", Experimental Station-Autonomous University of Tamaulipas (23°56′26.5″ North Latitude and 99°05′59.9″ West Longitude, and 193 masl) at Güémez, Tamaulipas, México on January 2018. Italian lemon (*Citrus limon*) (which were grafted on *Citrus aurantium*, *Citrus macrophylla*, and *Citrus volkameriana*) and Valencia orange (*Citrus sinensis*) (grafted on *Citrus aurantium* and

Citrus volkameriana) rootstocks were grown under a completely randomized design. In each scion/rootstock combination, 40 plants were inoculated, and 5 plants were used as a control. After 6 months of inoculation, the data were collected monthly for two years.

2.2. Bacterial Inoculation of Plant Materials

One-and-a-half-year-old *Citrus limon* and *Citrus sinensis* plants grown under greenhouse conditions were grafted on January 2018 with symptomatic HLB buds (the presence of *Candidatus* Liberibacter asiaticus was previously confirmed by PCR). In addition, different plants were grafted with pathogen-free buds, which were used as a control.

2.3. Bacteria Identification

DNA extraction was performed from the leaf tissue of infected and control plants. Briefly, 0.05 g of leaf tissues was homogenized and 1 mL of the genomic DNA isolation reagent (Bio Basic Canda Inc., Markham ON, Canada) was added; then samples were centrifuged at 12,000× *g* for 10 min at 4 °C. The precipitate (pastille) was washed with 75% ethanol and then centrifuged and re-suspended in 30 µL of molecular biology grade water. The confirmation of the bacteria was carried out using the methodology reported by Li [26].

2.4. Test Parameters and Data Analysis

2.4.1. Test Parameters

Response variables included plant height, which was measured from the base of the bag to the apex of the highest leaf using a measuring tape. Likewise, the stem diameter was measured at 15 cm below the graft union by employing an electronic vernier Steren HER-411 (Steren®, Mexico City, Mexico). Both variables were measured monthly over a period of two years.

2.4.2. Mineral Content

To determine the mineral content from each specie/rootstock combination, a foliar sample was taken from the 40 inoculated plants to obtain a combined sample. In the same way, a combined sample was obtained from the 5 repetitions of healthy plants in each specie/rootstock combination. Then, 1 g of sampled graft leaves (previously dried and crushed) was placed on the energy dispersion X-ray fluorescence spectrometer (ED-XRF) (Epsilon 1, Malvern Panalytical, Almelo, The Netherlands). The underside of the tube was completely covered and, between each reading, the reader was cleaned with methanol. Mineral content analysis (Mg, Al, Si, P, S, Cl, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, and Zn) was performed using the software Omnian® (Malvern Panalytical, Almelo, The Netherlands) in a period of 20 min per sample.

2.4.3. Data Analysis

A discriminant canonical analysis was performed to identify if the nutrients, plant height, and stem diameter were associated with the phytosanitary state of Italian lemon and Valencia orange scions, which were inoculated with *Ca.* Liberibacter asiaticus. In addition, a cluster analysis was carried out with each citrus species in their respective rootstock and the concentration of minerals in order to determine whether the scion/rootstock combination and phytosanitary status influence nutrient absorption. Finally, the data were analyzed using ANOVA, and treatment means of plant height and rootstock stem diameter were compared using the Tukey multiple range tests ($p < 0.05$), and standard deviations were also determined. Data were analyzed using the SAS software Version 9.0 (SAS Institute Inc., Cary, NC, USA) [27].

3. Results

3.1. Bacteria Identification

Table 1 shows the Cq values for each graft/rootstock combination. Likewise, during means comparison of Cq values (Table 2), significant differences were observed among the rootstocks in the two cultivars. When *C. limon* was developed on *C. aurantium*, the concentration of the bacteria rose 5% compared to the rest of the slides. Similarly, in the combination *C. sinensis*/*C. aurantium*, the concentration of *Ca*. Liberibacter asiaticus increased by 5%. Likewise, both lemon and orange plants did not present symptoms of HLB.

Table 1. Values of the quantification cycle (Cq) of the bacterium *Candidatus* Liberibacter asiaticus in foliar samples of *Citrus limon* and *Citrus sinensis* developed in different patterns.

	C. limon			***C. sinensis***	
Plant	***C. aurantium***	***C. macrophylla***	***C. volkameriana***	***C. aurantium***	***C. volkameriana***
	Cq Value				
1	26.28	32.62	35.34	34.42	30.26
2	35.43	35.11	36.58	33.12	30.23
3	33.12	34.67	34.85	32.56	29.71
4	34.32	34.39	36.36	34.34	28.50
5	32.18	34.08	35.29	34.27	30.64
6	33.57	37.25	32.92	32.03	30.15
7	33.75	33.30	34.02	24.05	29.75
8	35.26	34.81	32.56	32.96	29.25
9	35.36	36.20	34.74	33.28	31.57
10	34.02	35.48	33.75	31.9	30.12
11	32.32	37.23	38.11	29.05	29.44
12	34.04	35.29	35.02	34.64	31.14
13	31.59	36.37	34.53	31.23	31.09
14	36.34	37.27	36.28	32.93	21.11
15	34.72	37.64	37.47	35.88	27.65
16	34.66	36.68	36.15	26.30	31.02
17	34.18	36.04	36.11	26.02	30.18
18	32.95	36.82	36.41	25.50	30.12
19	32.55	36.39	34.54	27.07	30.76
20	35.12	36.28	35.06	24.06	31.43
21	32.19	36.95	34.22	24.60	29.74
22	33.96	31.04	34.65	27.11	32.84
23	32.08	37.12	34.63	28.12	29.52
24	35.07	36.07	34.32	28.17	32.02
25	35.47	35.61	34.46	28.91	29.45
26	34.42	37.06	36.46	19.44	18.46
27	34.16	36.06	36.21	23.79	31.26
28	36.4	36.93	36.22	25.46	31.44
29	34.75	36.86	34.42	24.39	31.60
30	34.81	36.91	34.24	26.53	28.39
31	34.90	37.06	36.37	26.97	29.38
32	36.08	36.97	35.87	28.78	30.82
33	35.25	36.95	35.02	27.02	33.52
34	34.81	36.31	34.52	27.01	31.67
35	36.12	36.89	34.44	26.95	32.78
36	36.13	37.55	34.41	26.83	33.12
37	35.32	37.15	34.62	29.58	31.51
38	34.31	36.39	34.67	27.41	33.40
39	36.40	36.57	34.63	23.46	32.63
40	35.28	36.10	34.77	28.34	31.84

Table 2. Means comparison of quantification cycle (Cq) values of *C. limon* and *C. sinensis* developed on different rootstocks.

Specie	Rootstock	Cq Values
C. limon	*C. aurantium*	34.24 b
	C. macrophylla	36.06 a
	C. volkameriana	35.13 ab
C. sinensis	*C. aurantium*	28.61 d
	C. volkameriana	30.23 c

Values with the same letter within a column are not statistically different as determined by the Tukey test ($p \leq 0.05$). MSD: Minimum significant difference.

3.2. Plant Height and Stem Diameter

No significant differences ($p \leq 0.05$) for plant height and stem diameter were observed between healthy and infected Italian lemon and Valencia orange plants in a general way (Table 3). However, when the variables were evaluated taking into account different rootstocks in each of the species, it was observed that uninfected Italian lemon and orange plants did not show significant differences in plant height and stem diameter between each of the rootstocks (Table 4).

Table 3. Plant height and stem diameter of uninfected and infected *C. limon* and *C. sinensis* plants.

Species	Presence	Height (cm)	Stem (mm)
C. limon	Not infected	167.66 a	16.44 a
	Infected	179.93 a	16.81 a
	MSD	13.14	1.25
C. sinensis	Not infected	149.40 a	15.26 a
	Infected	154.43 a	15.74 a
	MSD	19.83	2.08

Values with the same letter within a column are not statistically different as determined by the Tukey test ($p \leq 0.05$). MSD: Minimum significant difference.

Table 4. Plant height and rootstock stem diameter of uninfected and infected *C. limon* and *C. sinensis* plants.

C. limon				
Rootstock	**Not Infected**		**Infected**	
	Height (cm)	**Stem (mm)**	**Height (cm)**	**Stem (mm)**
C. volkameriana	170.60 a	17.82 a	196.88 a	19.35 a
C. macrophylla	162.40 a	16.86 a	176.27 b	16.32 b
C. aurantium	167.0 a	15.18 a	166.63 b	14.76 c
MSD	51.95	4.31	10.70	0.67
C. sinensis				
Rootstock	**Not Infected**		**Infected**	
	Height (cm)	**Stem (mm)**	**Height (cm)**	**Stem (mm)**
C. volkameriana	149.20 a	18.02 a	169.45 a	17.48 a
C. aurantium	149.60 a	13.46 b	131.61 b	13.04 b
MSD	22.05	2.73	12.17	0.99

Values with the same letter within a column are not statistically different as determined by the Tukey test ($p \leq 0.05$). MSD: Minimum significant difference.

In contrast, infected plants that were developed on rootstocks of *C. volkameriana* were significantly larger in height (15%) and stem diameter (23%) compared to *C. aurantium* and *C. macrophylla*. Like Italian lemon, other lemon species were affected by HLB. With *C. sinensis*, none of the rootstocks showed significant differences in plant height. However, when plants were infected, *C. volkameriana* showed a higher height (17%) compared to *C. aurantium*. In addition, for uninfected and infected plants, the stem diameter in *C. volkameriana* was 25% higher than *C. aurantium* (Table 4).

3.3. Mineral Concentration of Citrus limon and Citrus sinensis in Uninfected and Infected Plants

When a comparison of means was performed (Table 5), it was observed that most of the mineral contents in both citrus plants did not show significant differences ($p \leq 0.05$). However, in plants infected with *C. limon*, Cu significantly increased (77%) but sulfur decreased by 18%. In *C. sinensis* infected with HLB, the Fe content increased significantly (20%).

Table 5. Means comparison of mineral concentration (ppm) in *Citrus limon* and *Citrus sinensis*.

	***C. limon* (ppm)**							
	Mg	**Al**	**Si**	**P**	**S**	**Cl**	**K**	**Ca**
NI	7.40 a	1.02 a	0.32 a	1.38 a	3.97 a	6.15 a	27.89 a	50.33 a
I	8.47 a	1.04 a	0.56 a	1.40 a	3.27 b	7.63 a	24.56 a	51.47 a
MSD	1.23	0.34	0.96	0.49	0.32	2.01	4.98	6.7
	Sc	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Cu**	**Zn**
NI	0.11 a	0.0009 a	0.00002 a	0.00001 a	0.08 a	0.23 a	0.08 b	0.03 a
I	0.11 a	0.00009 a	0.00002 a	0.00001 a	0.08 a	0.24 a	0.01 a	0.03 a
MSD	0.03	0.01	0.0000005	0.0000002	0.01	0.12	0.02	0.02
	***C. sinensis* (ppm)**							
	Mg	**Al**	**Si**	**P**	**S**	**Cl**	**K**	**Ca**
NI	10.18 a	0.98 a	0.56 a	1.51 a	2.99 a	9.88 a	20.52 a	51.79 a
I	9.8 a	0.99 a	0.46 a	1.48 a	3.63 a	8.38 a	17.42 a	56.22 a
MSD	8.75	1.10	0.60	0.38	6.51	10.30	54.12	27.70
	Sc	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Cu**	**Zn**
NI	0.11 a	0.0050 a	0.00003 a	0.00002 a	0.08 a	0.20 b	0.10 a	0.03 a
I	0.07 a	0.00008 a	0.00003 a	0.000009 a	0.07 a	0.25 a	0.08 a	0.03 a
MSD	0.98	0.06	0.0001	0.0000006	0.29	0.03	0.04	0.06

Values with the same letter within a column are not statistically different as determined by the Tukey test ($p \leq 0.05$). MSD: Minimum significant difference. Status: NI (*not infected*), I (Infected).

3.4. Standardized Canonical (Can) Nutrient Coefficients, Plant Height, and Rootstock Stem Diameter

The discriminant canonical analysis showed that the first canonical correlation was significant (0.0049 < 0.05) and explains 99.97% of the variability that was recorded on nutrient concentrations, plant height, and stem diameter (Table 6). Therefore, the first principal component is associated with the variables of nutrient concentration, plant height, and rootstock stem diameter and helped to discriminate the phytosanitary state of the Italian lemon and Valencia orange scions. The second canonical correlation was not significant (0.88 > 0.05). That is, the second principal component has no association with the variables and, therefore, does not help to determine the phytosanitary status of the evaluated citrus.

Table 6. Standardized canonical (Can) nutrient coefficients, plant height, and rootstock stem diameter.

Variable	Can1	Can2	Can3
Cl	89.39737621	2.96360649	−0.78333747
K	57.79235462	3.53450965	−1.92557449
Ca	90.20094892	2.60397644	−2.05580445
Height	28.91402537	1.13598256	−0.16235885
Stem	−15.01864614	−0.50627275	0.78861065

In order of importance, the variable that shapes the component and achieves a separation between the groups is Ca (90.20), then Cl (89.39), and in a smaller proportion K (57.59) and height (28.91). On the contrary, stem diameter (−15.01) does not contribute anything to the model (Table 1).

3.5. Significance of the Mahalanobis Distances between Each of the Groups and Canonical Dispersions of Uninfected and Infected Species

Table 7 shows the significance of the Mahalanobis distances between each of the groups. That is, it shows the separation between uninfected and infected plants of Italian lemon and Valencia orange cv. Valencia, respectively. The distances of uninfected lemon with the remaining groups, infected lemon (5695), uninfected orange (7398), and infected orange (15914), were significant ($p < 0.05$). However, there was no significant distance between infected lemon (group 1) and uninfected orange (group 2); therefore, the distance between these two groups is equal to 0. On the other hand, there is a significant distance of 2573 between the uninfected lemon and infected oranges. Finally, the distance of 1614 between the groups of uninfected and infected orange (2 and 3, respectively) is significant, that is, the distance is non-zero. Therefore, Figure 1 shows the separation between each of the evaluated groups (uninfected lemon, infected lemon, uninfected orange, and infected orange) plants.

Table 7. The significance of Mahalanobis distances between uninfected and diseased Italian lemon and Valencia orange plants.

Presence	0	1	2	3
0	0	5695	7398	15914
1	5695 *	0	114.04	2573
2	7398 *	114.04 §	0	1614
3	15914 *	2573 *	1614 *	0

*: $p \leq 0.05$, §: $p > 0.05$. 0 = not infected Italian lemon, 1 = infected Italian lemon, 2 = not infected Valencia orange and 3 = infected Valencia orange.

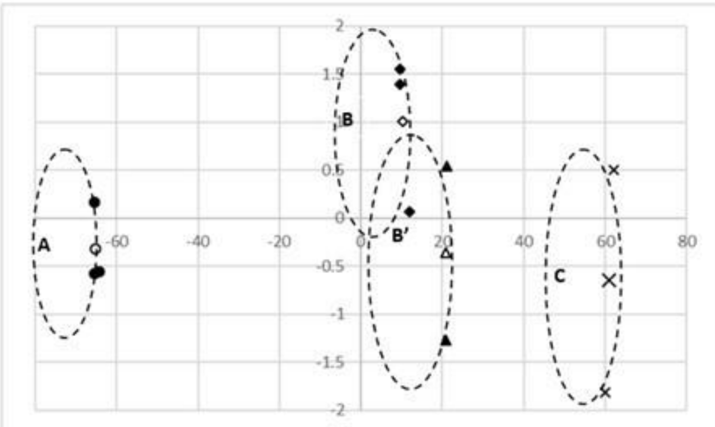

Figure 1. Canonical dispersions of uninfected Italian lemon (**A**), infected Italian lemon (**B**), uninfected Valencia orange (**B′**), and infected Valencia orange (**C**). The ovals represent each of the aforementioned groups. Likewise, figures represent the values of the canonical dispersions, the white figures are the centroids and in the case of group C, the centroid is located in the larger X.

3.6. Dendrogram of Mineral Content in Citrus limon and Citrus sinensis (Graft/Rootstock) Treatments

When a division of the dendrogram (Figure 2) was performed at 70% of the total distance, four different groups were observed: group 1 formed by treatment 8, group 2 composed of treatments 2, 9, and 10, group 3 with treatments 4 and 7, and group 4 formed by treatments 1, 3, 5, and 6. In the different combinations of scion/rootstock, it was observed that inoculation with *Ca*. Liberibacter asiaticus affected the mineral content since two different combinations of scion/rootstock did not have the same content. Branches of different lengths were observed, the smallest branches corresponded to *C. limon/C. volkameriana* (infected and uninfected), which suggests that there were fewer changes in mineral content due to inoculation with the bacteria.

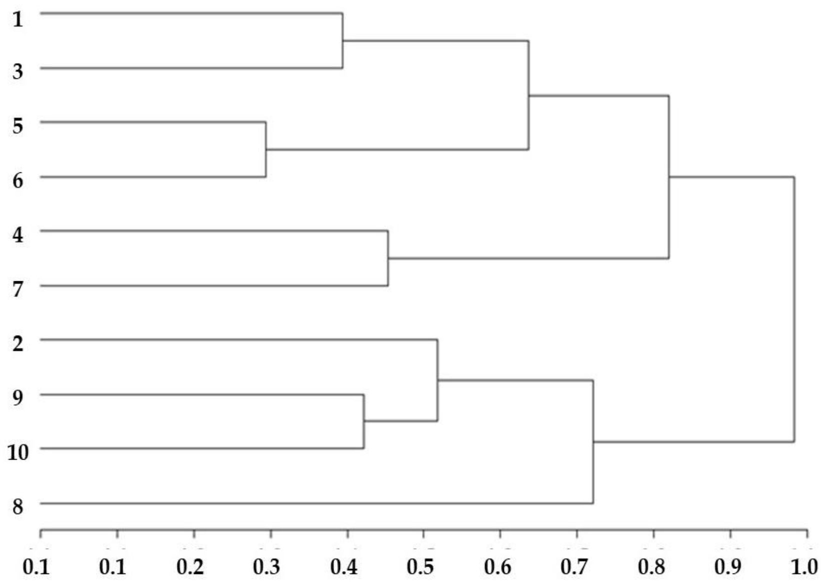

Figure 2. Dendrogram of treatments in *Citrus limon* and *Citrus sinensis* (graft/rootstock) plants. Uninfected (−) and infected (+) graft/rootstock, 1: −*C. limon/C. aurantium*), 2: +*C. limon/C. aurantium* 3: −*C. limon/C. macrophylla*, 4: +*C. limon/C. macrophylla*, 5: −*C. limon/C. volkameriana*, 6: +*C. limon/C. volkameriana*, 7: −*C. sinensis/C. aurantium*, 8: +*C. sinensis/C. aurantium*, 9: −*C. sinensis/C. volkameriana* and 10: +*C. sinensis/C. volkameriana*.

The next least affected treatments were infected and uninfected *C. sinensis/C. volkameriana* due to being grouped, although they had larger branches than those of *C. limon/C. volkameriana*. The mineral concentration of the other combinations was greatly affected since the infected and uninfected versions were not grouped. For example, according to its mineral content, the uninfected *C. limon/C. macrophylla* forms a group with treatment 1 (uninfected *C. limon/C. aurantium*) and then is grouped with treatment 7 (uninfected *C. sinensis/C. aurantium*). On the other hand, the combination *C. limon/C. aurantium* (in its two versions) had a great disparity in concentration and different minerals.

4. Discussion

4.1. Detection of Ca. *Liberibacter Asiaticus*

The number of PCR cycles ranged from 18 to 38, indicating abundant to moderate target nucleic acid in the sample, respectively, and samples are accurately identified as infected or uninfected, as they are considered positive for *Ca*. Liberibacter asiaticus when the cycle quantification values (Cq) <40 [28]. Likewise, there is a high correlation between bacterial concentration and the severity of HLB. That is, as the inoculum of the bacteria increases, is greater the expression of severity [29]. Therefore, *C. aurantium* rootstock is not suitable for *C. limon* and *C. sinensis* since it had the highest level of bacteria inoculum. On the contrary, *C. macropylla* and *C. volkameriana* present lower Cq values. Furthermore, the symptoms of HLB did not occur in either species; this is because at times the expression of symptoms can be sporadic, or the plant can be asymptomatic [30].

4.2. Plant Height and Stem Diameter

In previous research, the susceptibility of 18 citrus species to HLB was evaluated 6 months after inoculating the plants with bacteria. They mentioned that plant height and stem diameter were 2%

to 28% significantly lower in infected plants in comparison to uninfected plants [31]. In the same way, another study [32] evaluated the effects of HLB on seven-year-old Valencia orange trees and observed a significant reduction (33% and 21%) in fruit yield and height of plants that tested positive for the pathogen. Probably, there were no significant differences due to a scarce or zero overproduction of starch that would obstruct the vascular bundles and prevent the absorption of water and nutrients [13]. Furthermore, other authors mentioned that other lemon varieties, such as rough lemon, have less sensitivity to HLB attack [33]. Moreover, *C. aurantifolia* showed lower height (17.7%) and stem diameter (20%) when they were positive to *Ca.* Liberibacter asiaticus [31].

In previous works, the development of Valencia orange plants on 15 different rootstocks was evaluated to determine if rootstocks have an effect on HLB development. They observed an increase in plant height (16.3%) and stem diameter (19.1%) when orange was grown on *C. volkameriana* in comparison when it was grown on *C. aurantium*. It should be noted that these authors mentioned that rootstocks conferred vigor to the trees on *C. volkameriana* and showed higher tolerance to HLB and growth and less canopy damage. Therefore, they suggested that this rootstock can allow trees in younger stages to overcome the adverse effects of the disease [34]. The tolerance mechanism of this rootstock against HLB occurs through the expression of proteins not susceptible to diseases (lectin-related proteins, hitinase, and miraculin-like protein), the photosynthesis protein, and redox homeostasis [35].

Citrus sinensis is less sensitive to HLB when it grows on *C. volkameriana*; species such as Siem Pontianak (*C. nobilis* Lour) and Keprok Tejakula (*C. reticulata* Blanco) show lower bacterial concentration, less intensity of canopy damage, and greater plant height and stem diameter when grafted on *C. volkameriana* [36]. In addition, *C. aurantium*, *C. macrophylla*, and *C. volkameriana* are varieties moderately tolerant to HLB [37].

Previous studies indicate that the susceptibility of citrus fruits is closely related to the content of secondary metabolites; the most susceptible citrus have high levels of proline, serine, aspartic acid, galactose, among others. In contrast, less susceptible citrus species have high levels of glycine and mannose [38]. In addition to metabolites, rootstocks also have a direct effect on the stem in the presence of hormones, availability of water and nutrients, as well as other molecules (proteins, transcripts, and RNAs) that can act as a defense mechanism in the presence of pathogens [18].

4.3. Mineral Content of Citrus limon and Citrus sinensis

Mineral deficiencies such as Zn, P, Mn, and Cu usually occur in HLB-infected plants [13,14,39]. However, in the specific case of Zn, this nutrient did not decrease in any of the infected combinations compared to healthy plants. However, high levels of Zn were reported in citrus with HLB located in Punjab, Pakistan; the plants presented up to 11% more concentration of this nutrient than healthy plants. Similar results were also observed in Florida, United States, in lemon plants (*C. limon* cv. 'Todo del Ano' grafted on *C. paradisi* cv. 'Duncan' rootstock) infected with HLB, where the Zn content tended to increase in lemon varieties and decrease in others citrus [40]. These results are contradictory with those presented in other investigations, and they may have their origin in the physiological changes caused by HLB or the coexistence of *Phytophthora* [41].

In previous studies, it has been observed that HLB-infected *C. sinensis* plants show significant decreases in Cu and Mn because *C. sinensis* is one of the most sensitive species to HLB compared to other citrus species [31]. Therefore, one of the measures to mitigate the HLB attack is the applications of Cu [42]. However, in the present investigation, no significant differences were observed in the concentrations of Cu and Mn. On the contrary, Cu increased in plants infected with *C. lemon*.

In the case of P, deficiencies of up to 26% are more likely in older infected trees [43]. Potassium has great mobility and is a promoter of flowering, increasing the number of fruits, and enhancing the transport of water, sugars, and other nutrients [44]. Consequently, when the plant lacks potassium, symptomatic fruits have a lower sugar concentration and high acidity [45].

Therefore, as in the present investigation there were no significant variations of P in either of the two species, this behavior may be correlated with the age of the plants due to the fact that *C. limon* and *C. sinensis* were too young [43].

Another mineral found in lower concentrations in plants infected with HLB is Mg [46]. This mineral participates in photosynthesis, affecting chloroplasts and functions related to sugar accumulation and metabolic activities [47]. Since there were no significant differences in the concentration of Mg in either of the two species, the absence of green and irregular spots on the leaf, characteristic symptoms of the disease, would be explained [46].

Ca concentration changed in none of the species. On the contrary, in other investigations, it has been observed that *Citrus sinensis* cv. Navelina has a higher Ca content when infected with HLB [48]. The literature mentions that citrus fruits infected with HLB can present Ca deficiencies because bacteria use this mineral for their growth [49]. Therefore, the plant presents an active abiotic or biotic stress through the signaling pathways of Ca kinase and calmodulin-like proteins as defense mechanisms; and Ca is another nutrient that improves the health status of the plant [50,51]. This mechanism occurs across the plasma membrane via permeable ion channels [39]. In addition, calcium strengthens cell walls and provides greater protection against pathogens, which is one of the reasons why symptomatic fruits with HLB are harder and have a thicker shell [48,52].

K also did not vary between healthy and infected plants of *C. lemon* and *C. sinensis*. In previous research, a 34% reduction in K was observed in seven citrus species infected with HLB [53]. This mineral can mitigate the adverse effects of *Ca.* Liberibacter asiaticus, as it performs an antibacterial function by being present in protein synthesis, carbohydrate metabolism, and enzyme activation [54,55].

Like the aforementioned nutrients, HLB also causes Fe deficiencies [42,56]. On the contrary, the concentration of this mineral was higher (20%) in infected plants of *C. sinensis* and there were no variations in the content of *C. limon.* In this same species, a sulfur deficiency was observed; it should be clarified that other works do not report deficiencies of this mineral but it is an essential nutrient in foliar nutrition programs to counteract the adverse effects of HLB for its antibacterial activity [56,57].

Some of the results of this study differ with those reported in the literature, this is because different species were evaluated. In addition, factors such as soil, climate, and stage of development of HLB-infected plants have a direct influence on the data analyzed [58]. Finally, in some cases, there are no differences in nutrient content between healthy and HLB-infected plants [59]. The way each nutrient contributes to a plant's response varies, and in general, the interactions between nutrients and pathogens are not well understood [46]. Nutrient deficiencies or excesses in citrus fruits negatively affect susceptibility to HLB through metabolic changes in plants, thus creating a more favorable environment for this disease development [60].

5. Conclusions

In both citrus species, the *C. aurantium* rootstock showed a higher concentration of the bacteria. On the other hand, the rootstock that showed the best results after inoculation with the bacteria was *C. volkameriana*, presenting the least variation in mineral content and conferring greater plant height (15%) and stem diameter (23%). In contrast, the presence of *Ca.* Liberibacter asiaticus decreased S content and increased Cu concentration in *C. lemon* plants. Similarly, infected plants with *C. sinensis* presented higher Fe content. Finally, in both species, no significant differences were observed in the concentration of Mn, P, and Zn.

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