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Response of Citrus Germplasm Seedlings to *Candidatus* Liberibacter Asiaticus Infection under Controlled Greenhouse Conditions

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Abstract. **Huanglongbing (HLB) is a major disease of citrus associated with phloem-limited bacteria in the genus *Candidatus* Liberibacter that affects all known citrus species and relatives, with many commercial cultivars being greatly damaged. Testing cultivar tolerance to HLB in field conditions is difficult because of the erratic spread of the bacteria, scion and rootstock interactions, and influence of many biotic and abiotic factors on the tree response to the disease. This study aimed to validate the effect of *C*Las infection on different citrus species and hybrids thought to have different levels of tolerance to the disease using *C*Las graft inoculation under controlled greenhouse conditions. Young potted seedlings from 12 different citrus germplasm selections were graft-inoculated with *C*Las or mock-inoculated. Plants were monitored periodically during 18 months for canopy growth, HLB and nutritional leaf symptoms, and leaf *C*Las titers. The leaf nutrient content was measured at the end of the experiment. Roots were also assessed at 18 months after inoculation (mai) for *C*Las titers and biomass distribution. There were significant differences in most analyzed variables of healthy and infected plants. Some plants of all cultivars were successfully infected; however, overall, the *C*Las transmission rate was low and inconsistent. Ct values of roots were generally higher than those in leaves at 18 mai. HLB symptoms were not observed on seedlings until 1 year after inoculation; at 18 mai, infected trees of all cultivars were HLB symptomatic. Significant shoot and root biomass reductions (44%–75%) in infected 'Cleopatra', 'Duncan', 'Olinda Valencia', 'Sunburst', and 'Valencia 1-14-19', considered susceptible to HLB, were measured. These cultivars also showed more severe HLB symptoms than the presumed tolerant cultivars such as *Microcitrus inodora*, Rich 16-6 trifoliate orange, and US-897. This study provides new knowledge of the efficacy and value of greenhouse screening of citrus germplasm for response to HLB to support the development of new cultivars with improved HLB tolerance or resistance.**

Worldwide citriculture has been impacted by the bacterial disease Huanglongbing (HLB) associated with phloem-limited *Candidatus* Liberibacter species. *Candidatus* Liberibacter asiaticus (*C*Las) is the most destructive and widespread of the three HLB causal agents (Song et al. 2017) and is naturally transmitted by the Asian citrus psyllid (ACP) (*Diaphorina citri* Kuwayaba-Hemiptera: Liviidae) (Capoor et al. 1967; Halbert and Manjunath 2004). The typical symptoms of HLB-affected trees are leaf yellowing, foliar blotchy mottle, canopy thinning, reduced cropping, premature fruit drop, reduced fruit quality, and tree decline (Bové 2006; Gottwald et al. 2007; Wang and Trivedi 2013).

Profitable cultivation of most commercial citrus cultivars in an HLB-endemic environment is challenging. Multiple strategies are being used to reduce the impact of HLB, including the suppression of the ACP using pesticides (Boina and Bloomquist 2015; De Carli et al. 2018; Qureshi et al. 2014), tree protective covers (Gaire et al. 2022), nutrient management (Hallman et al. 2022), and use of hormones (Singh et al. 2022) to improve the tree health and profitability and antibiotics to reduce bacterial titer (Archer et al. 2022a, 2022b, 2023). The cost of these strategies is high. Even with careful implementation, trees of all citrus fruit cultivars become or remain infected, and most continue to have significantly reduced growth, crop yield, and fruit quality. Although oxytetracycline injections were shown to effectively restore tree health, fruit quality, and yield (Archer and Albrecht 2023; Archer et al. 2022a, 2022b, 2023), it does not prevent re-infection, and the methodology is costly and logistically challenging. Although there are some citrus species and hybrids that are highly tolerant of *C*Las infection, most of the cultivars with good fruit quality and wide commercial acceptance are severely affected by the disease. Consequently, there is major interest in identifying and creating new citrus cultivars with good fruit quality that are tolerant and/or resistant to *C*Las.

HLB tolerance differs greatly among the sexually compatible citrus species, with *Poncirus trifoliata* (L.) Raf. more tolerant to HLB (Bowman and Albrecht 2020; Folimonova et al. 2009; Ramadugu et al. 2016), and sweet orange (*Citrus sinensis* L. Osbeck), grapefruit (*Citrus paradisi* Macf.), and mandarin (*Citrus reticulata* Blanco) more susceptible to HLB. Most information about HLB tolerance in citrus is from observations in the field, where irregular and recurring transmission occurs by ACP, and the disease spreads irregularly and sometimes slowly in the tree. It typically takes many years to affirm whether the reduced or lack of HLB symptoms on individual plants is caused by delayed transmission, slow disease movement through the tree, or the actual genetic tolerance or resistance to the bacteria. This complexity of interpreting the results from field observations is further complicated by the rather large influences of environment, nutrition, and other biotic and abiotic factors on the infection rate and severity of disease symptoms and tree decline. The tree response to HLB is also known to be influenced by the combination of scion and rootstock (Bowman and Albrecht 2020; Bowman and McCollum 2015; Bowman et al. 2016; Kunwar et al. 2023; Rodrigues et al. 2020; Viteri et al. 2021). For example, during a greenhouse study, 'Valencia' trees grafted on US-942 and US-812 rootstocks (both hybrids of *C. reticulata* 'Sunki' × *P. trifoliata*) were less damaged by *C*Las infection than Valencia trees grafted on Ridge (*C. sinensis*), Swingle (*C. paradisi* × *P. trifoliata*), and Cleopatra (*C. reticulata*) rootstock (Bowman and Albrecht 2020). During a field study in Brazil, the HLB incidence of the 'Valencia' scion was lower on Flying Dragon (*P. trifoliata*) than on Rangpur lime [*C.* ×*limon* (L.)

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Osbeck] rootstock (Rodrigues et al. 2020). Therefore, significant efforts are underway to develop new citrus scions and rootstocks with high levels of tolerance to HLB (Bowman and Joubert 2020; Bowman et al. 2021, 2023; McCollum et al. 2016).

The objective of this study was to evaluate the effect of *C*Las infection on different citrus species and hybrids with different levels of tolerance to the disease using graft inoculation under controlled greenhouse conditions. Precise knowledge of the HLB tolerance of different species and cultivars will enable growers to make informed decisions that reduce the economic loss caused by *C*Las infection and assist citrus breeding programs with the development of new cultivars with improved HLB tolerance or resistance.

Materials and Methods

Plant material

Twelve citrus scion and rootstock cultivars were included in this experiment, and their responses to *C*Las infection during previous studies are listed in Table 1. Plants were grown in soilless potting mix (Pro Mix BX; Premier Horticulture, Inc., Quakertown, PA, USA) by planting one seed each in 3.8-cm × 21-cm cone cells (Cone-tainers; Stuewe and Sons, Tangent, OR, USA). Off-type seedlings were visually identified by morphology and discarded (Bisi et al. 2020), and true-to-type seedlings were transplanted into 19.7-cm × 31.8-cm plastic tree pots (Treepots; Stuewe and Sons) using the same soilless potting mix (Pro Mix BX; Premier Horticulture, Inc.).

Eight months after planting, 24 seedlings per genotype were graft-inoculated with *C*Las-infected buds, except for 'Kinnow' and *Microcitrus inodora*, of which fewer seedlings (15 and 18, respectively) were available. For *C*Las graft inoculation, budwood was collected from greenhouse-grown *C*Las-infected [confirmed by polymerase chain reaction (PCR)] and HLB-symptomatic 'Cleopatra' seedlings. Three buds were used to inoculate each test seedling using inverted T-budding (Albrecht et al. 2021). Inoculations were performed during Oct 2019. Stem diameters of seedlings at budding ranged from 3 mm (*M. inodora*) to 9 mm ('Assad'). Depending on the stem diameter, buds were inserted between 5 and 17 cm above the soil. Seedlings from different cultivars were grouped during inoculation to ensure that each plant received buds from more than one inoculum tree, and buds from each inoculum tree were distributed among many cultivars. Six seedlings per genotype were mock-inoculated with buds from healthy greenhouse-grown 'Cleopatra' seedlings in the same manner. Immediately after inoculation, all side branches and ~25% of the apical portion of the seedling shoot were removed. Buds were unwrapped after 2 weeks and not allowed to grow out.

Plants were arranged in a completely randomized design on the greenhouse benches under natural daylight. Supplemental light using a 150-W LED lightbar (XC150 vegetative spectrum; Kind Grow Lights, Santa Rosa, CA, USA) was applied during the early morning to increase the daylength to 16 h and improve growth (Bowman and Albrecht 2021). Plants were irrigated as needed and fertilized every 3 weeks using a water-soluble fertilizer (20N–10P–20K; Peters Professional, The Scotts Company, Marysville, OH, USA) at a dose of 400 mg $N \cdot L^{-1}$, and insecticides were applied as needed. At 12 months after inoculation (mai), six mock-inoculated plants and six *C*Las-inoculated infected plants were selected from each cultivar for data analysis from all timepoints. To classify as infected, plants needed to exhibit a Ct value of ≤38 at some time point during 3 to 12 mai (Table 2). To perform a thorough data analysis, we focused on the six plants with the lowest Ct values within each cultivar, except for 'Duncan' and 'Kinnow', of which only five plants became infected, and 'Assad', Rich 16-6, and US-897, of which only four plants became infected.

Canopy measurements were performed by dividing the plant canopy into four quadrants for a better representation of the disease spread in the plant (Fig. 1). Plants were assessed at 3, 8, 12, and 18 mai for trunk diameter, pruned dry biomass, canopy/leaf *C*Las titer, leaf nutrients, and HLB symptoms. Leaf area, leaf nutrient concentration, root *C*Las titer, and root biomass were assessed at 18 mai.

CLas titer

We collected 10 to 12 fully expanded leaves from the most recent growth flush of each tree at 3, 8, 12, and 18 mai. Fibrous root samples were collected at 18 mai. Tissues were pulverized in liquid nitrogen using a mortar and pestle. One hundred milligrams from leaf midrib and fibrous root ground tissue were used for DNA extraction. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Real-time PCR assays were performed using primers HLBas (5′-TCGAGCGCGTATGCAATACG-3′) and HLBr (5′-GCGTTATCCCGTAGAAAAAGG TAG-3′) and probe HLBp (5′-AGACGGGT GAGTAACGCG-3′) developed by Li et al.

Table 1. Cultivars, parentage, and previously reported huanglongbing (HLB) response.

Cultivar	Parentage	HLB response	Type
Assad citron	*Citrus medica*	Moderately tolerant[i]	Scion
Cleopatra mandarin	*C. reticulata*	Susceptible[i, ii, iii, iv, v]	Rootstock
Duncan grapefruit	*C. paradisi*	Susceptible[i]	Scion
Kinnow mandarin	*C. nobilis* 'King' × *C. deliciosa* 'Willow Leaf'	Susceptible[vi]	Scion
Microcitrus inodora	*Microcitrus inodora*	Tolerant[vii]	Germplasm
Olinda Valencia orange	*C. sinensis*	Susceptible[viii]	Scion
Rich 16-6 trifoliate orange	*P. trifoliata*	Tolerant[ix]	Rootstock
Sunburst mandarin	Robinson [*C. reticulata* × (*C. paradisi* × *C. reticulata*)] × Osceola [*C. reticulata* × (*C. paradisi* × *C. reticulata*)]	Susceptible[x, xi]	Scion
Triumph hybrid	*C. sinensis* × *C. paradisi*	Susceptible[xi]	Scion
US-897 citrandarin	*C. reticulata* 'Cleopatra' × *P. trifoliata* 'Flying Dragon'	Tolerant[iii, iv]	Rootstock
Valencia 1-14-19 orange	*C. sinensis*	Susceptible[xi]	Scion
W. Murcott mandarin	*C. reticulata*	Unknown[xii]	Scion

[i] Folimonova et al. 2009.
[ii] Albrecht et al. 2014.
[iii] Albrecht and Bowman 2012a.
[iv] Albrecht and Bowman 2011.
[v] Bowman et al. 2016.
[vi] Hussain et al. 2019.
[vii] Ramadugu et al. 2016.
[viii] No information found in the literature. It is expected that the HLB response will be similar to that of other Valencia clones.
[ix] No information found in the literature. It is expected that the HLB response will be similar to that of other trifoliate orange clones.
[x] Stover and McCollum 2011.
[xi] McCollum et al. 2016.
[xii] No information found in the literature.

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Table 2. Percentage of *C*Las-inoculated seedlings with Ct values ≤38 at 3, 8, and 12 months after inoculation (mai).

Cultivar	3 mai	8 mai	12 mai	Number of positive plants
Assad citron	8	13	17	4
Cleopatra	8	33	29	7
Duncan	4	33	25	5
Kinnow	0	13	20	5
M. inodora	0	17	33	6
Olinda Valencia	17	29	33	8
Rich 16-6	0	13	8	4
Sunburst	0	25	25	6
Triumph	0	25	33	8
US-897	0	8	17	4
Valencia 1-14-19	21	50	42	10
W. Murcott	0	25	50	12

(2006). Primers COXf and COXr and probe COXp were used for normalization. Amplifications were conducted using an AB7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and the QuantiTect Probe PCR Kit (Qiagen) according to the manufacturers' instructions. All reactions were performed in a 20-µl reaction volume using 5 µl of extracted DNA.

Plant growth measurements

Plant biomass. Plants were assessed at 3, 8, 12, and 18 mai. At 3 mai, one branch was selected to represent each quadrant on each tree, and other branches in the quadrant were removed so that new growth during the subsequent period could be measured. After each assessment, the selected branch in each quadrant was cut off at the third or fourth node, and the removed tissue was dried at 70 °C to a constant weight and weighed. During each subsequent assessment, two new branches grown from the previously cut branch were selected for symptom evaluation and leaf sample collection, subsequently cut off as described, and used to determine the dry mass. At 18 mai, plants were removed from the growth container and separated into canopy, trunk, and root system. The root system was washed and divided into large roots (>5 mm in diameter), medium roots (2–5 mm in diameter), and fibrous roots (<2 mm in diameter). Tissues were dried as described to determine biomass dry weights (g).

Trunk diameter. Trunk diameters were measured at 15 cm above the soil before inoculation and at each assessment using a digital caliper. Two measurements perpendicular to one another were obtained, and the averages were used for analysis. Trunk growth was calculated as the difference between the trunk diameter before inoculation and the trunk diameter at each timepoint.

Leaf measurements. All mature leaves were counted and leaves were scanned using a LI-3100C leaf area meter (LI-COR Biosciences, Lincoln, NE, USA); the total leaf area was determined at 18 mai.

Canopy health assessment

Foliar symptoms. Blotchy mottle and nutrient deficiency-like symptoms were scored in each canopy quadrant using a 5-point standard scale as previously described (Bowman et al. 2023). Blotchy mottle was scored as follows: 1 = no blotchy mottle; 2 = less than 25% of leaves with blotchy mottle; 3 = 26% to 50% of leaves with blotchy mottle; 4 = 51% to 75% of leaves with blotchy mottle; and 5 = more than 75% of leaves with blotchy mottle. Nutrient deficiency-like symptoms were scored as follows: 1 = no symptoms; 2 = less than 25% of leaves with symptoms; 3 = 26% to 50% of leaves with symptoms; 4 = 51% to 75% of leaves with symptoms; and 5 = more than 75% of leaves with symptoms.

Leaf nutrient content. Ten fully expanded leaves from the newest growth were collected from each tree at the end of the experiment (18 mai) and analyzed for leaf macronutrients [nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S)] and micronutrients [boron (B), zinc (Zn), manganese (Mn), iron (Fe), copper (Cu)] (Waters Agricultural Laboratories Inc., Camilla, GA, USA). The total nitrogen concentration was measured by combustion (Sweeney 1989). The other nutrients were analyzed using inductively coupled argon plasma atomic emission spectroscopy after acid digestion (Huang and Schulte 1985).

Statistical analysis

A one-way analysis of variance (ANOVA) was conducted with the disease state for each cultivar. Mean separation was performed by Tukey's honestly significant difference test. Differences were defined as statistically significant when $P < 0.05$. Data were analyzed using the statistical program R version 4.0.2 (The R Foundation for Statistical Computing, Vienna, Austria). Canopy health ratings were analyzed using a nonparametric aligned rank transformation ANOVA using ARTool (Wobbrock et al. 2011).

Results

CLas titer

The percentage of *C*Las-infected plants ranged from 0% to 21% at 3 mai, and from 8% to 50% at 8 mai and 12 mai (Table 2). 'Olinda Valencia' and 'Kinnow' had the highest percentage of plants infected at 3 mai, with 17% and 21%, respectively. However, in most cultivars, *C*Las was not detected in the leaves at 3 mai, and average Ct values were more than 33.9 (Table 3). The lowest Ct values and, therefore, highest *C*Las titers were measured in 'Olinda Valencia' (32.0) and in 'Valencia 1-14-19' (30.3). All cultivars had *C*Las detected in some leaves at 8 mai, with the average Ct value lowest in leaves from 'Cleopatra' (23.1), 'Olinda Valencia' (24.5), and 'Valencia 1-14-19' (25.2), and the highest in leaves from Rich 16-6 (38.0) and 'W. Murcott' (38.1). At 12 mai, the lowest average Ct values were found in leaves from 'Cleopatra' (22.6) and 'Valencia 1-14-19' (23.8), and the highest were from Rich 16-6 (38.7). The average Ct value at 18 mai was lowest in leaves from 'Cleopatra' (23.9) and highest in leaves from Rich 16-6 (41.0).

Root Ct values at 18 mai were generally higher than those in leaves. The lowest value (highest *C*Las titer) was measured in roots from 'Sunburst' (28.6), followed by 'Triumph' (31.6). Although 'Cleopatra', 'Duncan', 'Olinda Valencia', and 'Valencia 1-14-19' had relatively low leaf Ct values throughout the experiment, root Ct values for these cultivars were more than 32 at 18 months.

Plant growth

Pruned shoot dry biomass. There was no significant effect of *C*Las infection on the pruned shoot dry biomass of any of the cultivars at 3 mai (Fig. 2). *C*Las infection induced significant pruned shoot biomass reductions in 'Cleopatra' (64%), 'Duncan' (68%), 'Olinda Valencia' (38%), and 'Sunburst' (39%) at 8 mai. Pruned shoot biomass ranged from 24.9 g to 50.2 g for mock-inoculated plants and from 9 g to 26.7 g for infected plants of these cultivars at 8 mai (Supplemental Table 1). At 12 mai, significant pruned biomass reductions

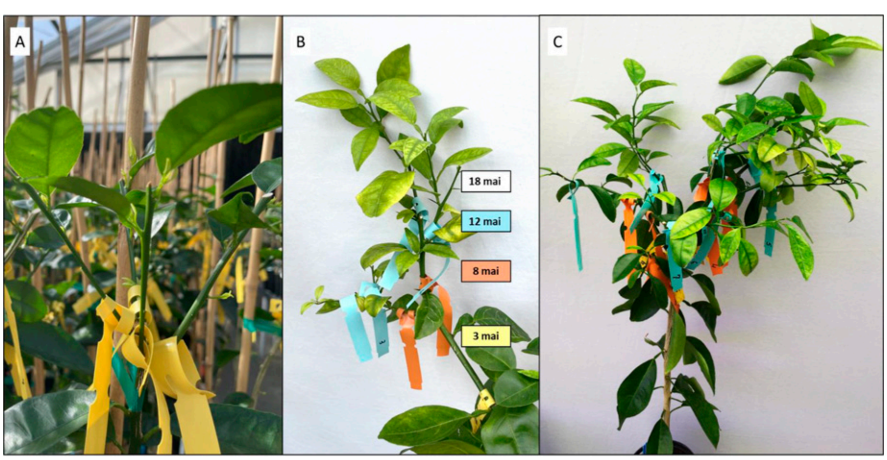

Fig. 1. (**A**) Assessment of seedling from 'Olinda Valencia' divided in four quadrants and cut back at the third bud after 3 months after inoculation (mai). (**B**) Assessment of one quadrant of the seedling at 18 mai, with selected branches tagged at assessments at 3, 8, and 12 mai. (**C**) Assessment of seedling at 18 mai.

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Table 3. Leaf and root Ct values of infected plants used for the assessment of horticultural parameters.

Cultivar	Leaves								Roots	
	3 MAI		8 MAI		12 MAI		18 MAI		18 MAI	
'Assad' citron	33.9	ab	29.2	a-c	30.2	a-c	31.6	a-d	35.0	a-d
'Cleopatra'	35.9	ab	23.1	c	22.6	c	23.9	d	32.4	b-d
'Duncan'	38.2	ab	26.5	a-c	26.5	bc	26.6	b-d	35.3	a-d
'Kinnow'	41.0	ab	32.3	a-c	30.2	a-c	32.0	a-d	37.3	a-d
M. inodora	41.0	a	36.8	ab	29.0	a-c	35.7	a-c	41.0	a
'Olinda Valencia'	32.0	ab	24.5	c	24.6	bc	26.4	b-d	32.4	b-d
Rich 16-6	40.7	ab	38.0	ab	38.7	a	41.0	a	41.0	ab
'Sunburst'	41.0	a	32.5	a-c	26.7	bc	25.1	cd	28.6	d
'Triumph'	40.8	a	25.9	bc	24.9	bc	26.2	b-d	31.6	cd
US-897	41.0	ab	33.4	a-c	28.4	a-c	33.9	a-d	40.0	a-c
'Valencia 1-14-19'	30.3	b	25.2	c	23.8	c	25.5	b-d	32.6	b-d
'W. Murcott'	41.0	a	38.1	ab	34.1	ab	35.9	ab	35.4	a-d
Average	38.1		30.5		28.3		30.3		35.2	
p-value	> 0.0000		> 0.0000		0.0004		> 0.0000		> 0.0000	

Different letters within columns indicate significant differences within columns according to Tukey's honestly significant difference (HSD) test. Cell shading reflects the Ct value category, with darker blue indicating higher Ct values (lower CLas titers) and darker red indicating lower Ct values (higher *C*Las titers).

were measured for infected 'Cleopatra' (75%), 'Duncan' (42%), 'Olinda Valencia' (70%), 'Sunburst' (71%), 'Triumph' (32%), and 'Valencia 1-14-19' (61%), and biomass from mock-inoculated plants from these cultivars ranged from 39.7 to 48.8 g and from 9.9 to 33.3 g license-for infected plants. At 18 mai, the pruned shoot biomass was significantly reduced for infected 'Cleopatra' (76%), 'Kinnow' (46%), 'Olinda Valencia' (55%), and 'Sunburst' (66%) and mock-inoculated plant biomass from these cultivars ranged from 4 to 9.0 g and from 14.6 to 65.8 g for infected plants.

Root dry biomass. There was a substantial and significant reduction in biomass in response to infection for all root sizes measured for 'Cleopatra', 'Duncan', and 'Olinda Valencia' (Table 4) at 18 mai. 'Sunburst' also showed a significant reduction in root fibrous biomass, and 'Valencia 1-14-19' showed a significant reduction in medium biomass. The total root biomass reductions for these cultivars were 75% for 'Cleopatra', 45% for 'Duncan', 65% for 'Olinda Valencia', 57% for 'Sunburst', and 44% for 'Valencia 1-14-19'.

When comparing the entire plant dry biomass (root + trunk + canopy) at 18 mai to determine healthy and infected plants from each cultivar (Fig. 3), plants from 'Cleopatra', 'Duncan', 'Olinda Valencia', 'Sunburst', and 'Valencia 1-14-19' showed significant reductions.

Trunk growth. Growth of the trunk diameter of 'Cleopatra', 'Duncan', 'Olinda Valencia', 'Sunburst', 'Valencia 1-14-19', and 'W. Murcott' was significantly reduced in response to infection (Table 5), and the average trunk diameter growth from these cultivars ranged from 6.8 to 10.8 mm in mock-inoculated plants and from 4.6 to 7.7 mm in *C*Las-inoculated infected plants.

Leaves. The number of leaves on infected plants at 18 mai was significantly reduced for 'Cleopatra', 'Kinnow', and 'Sunburst' (Table 6). The total leaf area was reduced on most of the *C*Las-infected cultivars, except for Rich 16-6 and US-897, which showed no leaf area reduction. Reductions were 69% for 'Cleopatra', 26% for 'Duncan', 41% for 'Olinda Valencia', and 59% for 'Sunburst'. 'Cleopatra', 'Duncan', 'Olinda Valencia', and 'W. Murcott' also showed significant reductions in the area per leaf in the infected state.

Canopy health assessment

Foliar symptoms. Infected plants did not exhibit significant blotchy mottle symptoms until 12 mai (Table 7). At that time, significantly more blotchy mottle was observed for 'Assad' Citron, 'Cleopatra', 'Duncan', 'Olinda Valencia', and Rich 16-6. By 18 mai, infected 'Assad' Citron, 'Cleopatra', 'Duncan', 'Olinda Valencia', 'Sunburst', 'Triumph', and 'Valencia 1-14-19' displayed significant blotchy mottle. At 18 mai, all cultivars with Ct values less than 32 showed significant blotchy mottle symptoms.

Foliar nutrient deficiency-like symptoms were not observed in any of the plants until 8 mai. At that time, significantly more symptoms were observed for 'Cleopatra', 'Duncan', and 'Olinda Valencia' when they were infected (Table 8). At 12 mai, 'Cleopatra', 'Olinda Valencia', 'Sunburst', and 'Triumph' exhibited significant nutrient deficiency symptoms in response to infection; however, at 18 mai, only 'Cleopatra', 'Sunburst', and 'W. Murcott' showed deficiency.

Leaf nutrient content. Leaf macronutrients and micronutrients were measured at 18 mai. Among the macronutrients, significant reductions in response to *C*Las infection were found for Ca in 'Cleopatra', 'Olinda Valencia', 'Sunburst', and 'Valencia 1-14-19' (Table 9). Significant reductions in response to *C*Las infection were also found for magnesium in 'Cleopatra', 'Duncan', 'Olinda Valencia', 'Sunburst', 'Triumph', and 'Valencia 1-14-19'. Among the micronutrients, Mn was reduced in *C*Las-infected 'Cleopatra', Rich 16-6, 'Sunburst', and 'Valencia 1-14-19'.

Discussion

Graft inoculation has been used frequently for controlled transmission to study the HLB host–pathogen interaction in citrus (Albrecht and Bowman 2008, 2012b; Bodaghi et al. 2022a, 2022b; Canale et al. 2020; Hilf and Lewis 2016; Lopes et al. 2009). During these studies, different results were observed for transmission efficiency, disease symptom severity, and other variables. The percentage of plants that became *C*Las-infected during our study was lower than that reported by Lopes et al. (2009), who studied graft transmission efficiencies and multiplication of *C*Las in Valencia and reported a transmission rate of 66.7%. However, the percentage of transmission was similar to that found by Cui et al. (2022), who compared different grafting methods for *C*Las transmission in sweet oranges. The infection and colonization by *C*Las in our study were affected by cultivar tolerance. For example, 'Valencia 1-14-19'sweet orange, which is considered HLB-susceptible (McCollum et al. 2016), had a higher infection rate than 'Assad' citron, Rich 16-6, and US-897, which are considered tolerant cultivars (Albrecht and Bowman 2011, 2012a; Folimonova et al. 2009). However, 'Cleopatra', also a well-known susceptible cultivar (Albrecht and Bowman 2011, 2012a; Albrecht et al. 2014; Bowman et al. 2016; Folimonova et al. 2009), had an infection rate less than 30%. The effectiveness of transmission can be compromised by temperature (Gasparoto et al. 2012), pathogen virulence, source of bud inoculum (Stover et al. 2016), and other factors. Therefore, there appears to be inconsistency and difficulty in *C*Las transmission using graft inoculation that can be confused with cultivar tolerance or resistance. To reduce incorrectly identified failures in inoculation for resistance, it is important to include well-known susceptible cultivars in studies as a control. In the present study, we used 'Cleopatra' and 'Valencia 1-14-19', which are known from both greenhouses studies and field observations as being susceptible and strongly symptomatic.

*C*Las is detectable by quantitative PCR at 2 to 3 months after graft inoculation (Albrecht and Bowman 2008; Coletta-Filho et al. 2010; Folimonova and Achor 2010; Lopes et al. 2009). In this study, 'Assad' citron, 'Cleopatra', 'Duncan', 'Valencia 1-14-19', and 'Olinda Valencia' had at least one plant with *C*Las detected in the leaves during the first assessment at 3 mai. Some plants from *M. inodora*, Rich 16-6, US-897, and 'W. Murcott' had *C*Las detected at 8 or 12 mai, but not at 18 mai. This inconsistency in *C*Las titers might be linked to the tolerance displayed by these cultivars because *M. inodora*, Rich 16-6, and US-897 were previously reported to be tolerant to *C*Las infection (Albrecht and

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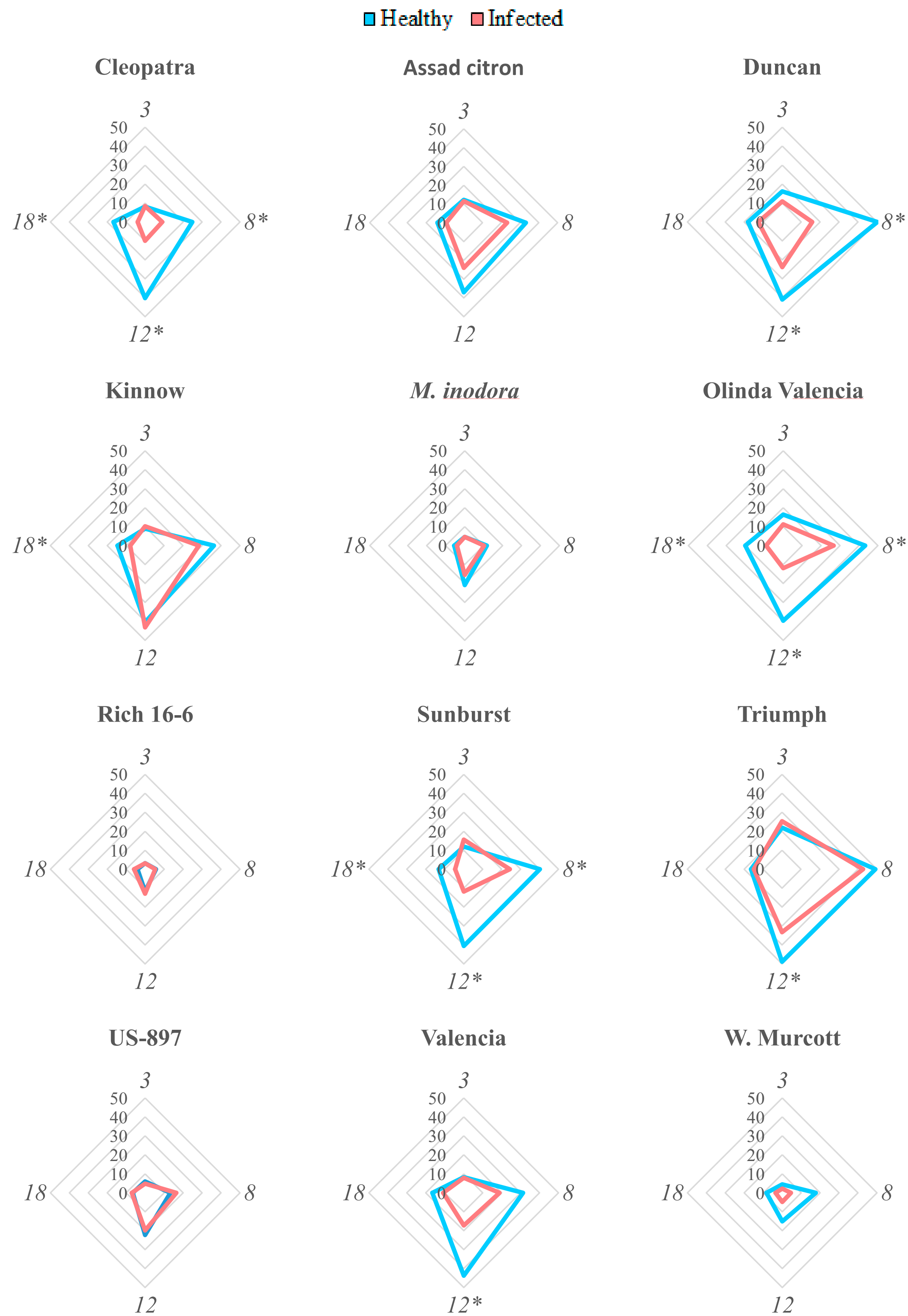

Fig. 2. Pruned shoot dry biomass (g) from healthy and infected plants at 3, 8, 12 and 18 months after *C*Las inoculation. *Significant differences between healthy and infected plants from each cultivar according to Tukey's honestly significant difference (HSD) test.

Bowman 2011, 2012a; Ramadugu et al. 2016). Several factors could contribute to the irregular *C*Las titers observed in our study. These include the uneven distribution of the pathogen within the plant, a delayed recolonization of *C*Las in new growth tissues, and potential resistance mechanisms leading to *C*Las clearance from specific plant parts over time, followed by phloem regeneration. Previous research has indicated that *C*Las-tolerant germplasm, such as the cultivar Sugar Belle (*Citrus reticulata*) × (*Citrus paradisi* × *Citrus reticulata*), might exhibit more robust phloem regeneration in response to *C*Las infection as compared with susceptible germplasm like 'Valencia' (Deng et al. 2019; Ribeiro et al. 2023).

In our study, *C*Las was not detected in any plants from Rich 16-6, a trifoliate orange (*P. trifoliata*) cultivar, at 18 mai (in April), despite showing severe dieback in some old branches from infected plants, whereas new branches from the same plants exhibited vigorous growth. Similarly, Folimonova et al. (2009) reported inconsistent infection and

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Table 4. Fibrous, medium and large root dry biomasses from healthy and *C*Las-infected plants and the percentage of total root biomass reduction in infected plants at 18 months after inoculation.

Cultivar	Fibrous roots (g) Healthy		Fibrous roots (g) Infected		Medium roots (g) Healthy		Medium roots (g) Infected		Large roots (g) Healthy		Large roots (g) Infected		Total root reduction (%)
Assad citron	30.3	a	27.8	a	9.2	a	8.0	a	25.5	a	21.6	a	12
Cleopatra	11.7	a	2.7	b	6.6	a	1.7	b	12.0	a	3.1	b	75
Duncan	15.8	a	8.6	b	4.0	a	1.6	b	19.2	a	11.2	b	45
Kinnow	14.2	a	11.8	a	3.9	a	2.7	a	8.5	a	9.0	a	12
M. inodora	1.1	a	1.1	a	0.6	a	1.1	a	5.9	a	5.3	a	2
Olinda Valencia	16.3	a	5.9	b	7.2	a	2.9	b	16.6	a	5.3	b	65
Rich 16-6	2.9	a	3.7	a	1.3	a	1.4	a	3.1	a	4.6	a	−34
Sunburst	13.5	a	3.6	b	4.9	a	2.7	a	10.5	a	6.2	a	57
Triumph	12.9	a	10.5	a	7.2	a	5.4	a	16.3	a	16.3	a	12
US-897	5.0	a	6.0	a	2.3	a	2.1	a	6.4	a	6.2	a	−5
Valencia 1-14-19	11.3	a	6.8	a	5.2	a	2.4	b	12.1	a	6.8	a	44
W. Murcott	9.3	a	4.9	a	1.9	a	1.4	a	7.6	a	6.0	a	35

Different letters within rows indicate significant differences between healthy and infected plants from each cultivar according to Tukey's honestly significant difference (HSD) test. Large roots (>5 mm in diameter), medium roots (2–5 mm in diameter), and fibrous roots (<2 mm in diameter).

Table 5. Trunk diameter growth (mm) from healthy and *C*Las-infected plants from *C*Las inoculation to 18 months after inoculation and percentage of trunk diameter reduction.

Cultivar	Trunk diam (mm) Healthy		Trunk diam (mm) Infected		Reduction (%)
Assad citron	8.9	a	8.4	a	5
Cleopatra	8.9	a	4.6	b	48
Duncan	10.8	a	7.4	b	31
Kinnow	8.3	a	7.9	a	5
M. inodora	5.3	a	5.0	a	6
Olinda Valencia	9.8	a	6.5	b	34
Rich 16-6	4.1	a	4.8	a	−18
Sunburst	9.7	a	7.7	b	21
Triumph	10.4	a	9.9	a	5
US-897	6.1	a	6.5	a	−6
Valencia 1-14-19	9.2	a	6.6	b	29
W. Murcott	6.8	a	5.0	b	27

Different letters within rows indicate significant differences between healthy and infected plants from each cultivar according to Tukey's honestly significant difference (HSD) test. Reduction (%) is the percentage of the total leaf area reduction from healthy to infected plants.

low *C*Las titers in *P. trifoliata*. The inconsistency observed in Rich 16-6 might be linked to the plant dormancy during the winter for *P. trifoliata* and some of its hybrids. US-897, a trifoliate orange hybrid, showed similar inconsistencies in our study. Previous research of Carrizo (*P. trifoliata* × *C. sinensis*) by Hilf and Luo (2018) found lower *C*Las populations during cooler months, suggesting that temperature fluctuations could impact bacterial growth, especially considering that *P. trifoliata* is deciduous and undergoes dormancy during winter.

Leaf *C*Las titers were higher than those of the roots for all the cultivars studied. These results are in accordance with the results of greenhouse studies by Bodaghi et al. (2022a, 2022b), who reported higher *C*Las titers in leaves compared with roots in young graft-inoculated Valencia trees with varying rootstocks. A field study by Tardivo et al. (2023) also observed that *C*Las titers remained lower in the roots than in the leaves, regardless of the rootstock. However, the progression of HLB in the plant after inoculation is still not fully understood, and there are conflicting findings regarding root colonization by *C*Las. A field study performed in Texas reported that *C*Las was detected more in the roots than in the leaves (Braswell et al. 2020). It is important to highlight that when dealing with larger trees in field conditions, the possibility of encountering false-negative results arises because of the irregular dispersion of *C*Las within the tree canopy. Occurrences of these "false"-negative results have also been identified during previous research (de Gracia Coquerel et al. 2023; Li et al. 2021; Louzada et al. 2016), and it was proposed that these occurrences might stem from the limited sensitivity of quantitative PCR or the uneven distribution of *C*Las within the tree.

Plants from *M. inodora* and Rich 16-6 did not exhibit any detectable *C*Las in their roots. The rootstocks US-897, US-942, and US-802 are all hybrids of trifoliate orange and have demonstrated tolerance to *C*Las infection when grown as seedlings (Albrecht and Bowman 2012a). During studies in which grafted rootstocks were used, *C*Las titers were lower in the roots of US-896, US-802, US-812, US-897, and US-942 at 50 weeks after inoculation, even with high leaf *C*Las titers of infected sweet orange trees, in contrast to more sensitive rootstocks (Bowman and Albrecht 2020). It can be noted that *P. trifoliata* is often used in citrus rootstock breeding programs because of its tolerance to several pests and pathogens of citrus, including *C*Las. Nevertheless, *P. trifoliata* is typically excluded from scion breeding programs because of its propensity to yield hybrids with off-flavor or poorly flavored fruits. Although many *Poncirus* hybrids produce juice with a bitter flavor and aftertaste, a recent investigation identified four hybrids resulting from crosses between *Citrus* and *P. trifoliata* with a favorable taste (Deterre et al. 2023). *Microcitrus* species have also been used in rootstock and scion breeding programs, partly because of their tolerance against HLB (Bowman et al. 2023; Ramadugu et al. 2016). However, *Microcitrus* hybrids generally have not performed well as rootstocks (Bowman et al. 2023), and their fruits are generally unsuitable for juicing or fresh consumption (Bowman et al. 2019; Shaw et al. 2000), likely requiring several generations of backcrossing to obtain suitable fruit quality in progeny for use as a commercial scion.

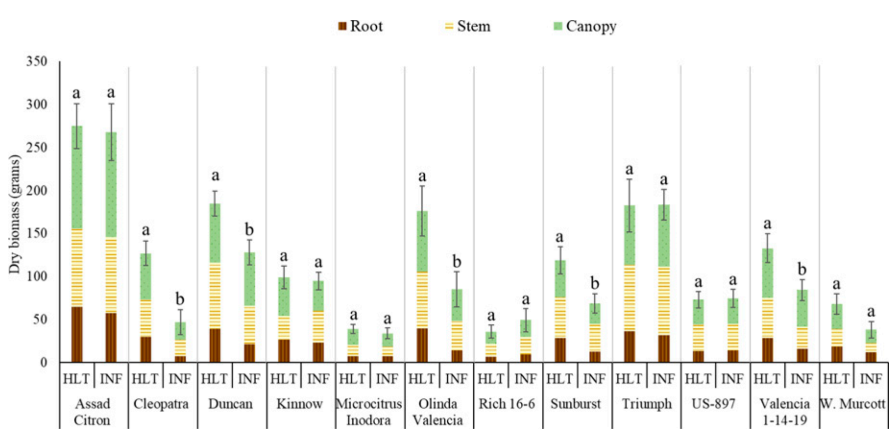

Fig. 3. Plant total dry biomass (mm) from healthy (HLT) and *C*Las-infected (INF) plants at 18 months after inoculation (mai). Different letters indicate significant differences in total dry biomass between healthy and infected plants from each cultivar according to Tukey's honestly significant difference (HSD) test. Vertical bars indicate the *SE* of the total dry biomass mean.

HLB is characterized by massive starch accumulations (Etxeberria et al. 2009), which result in the typical blotchy mottled appearance of affected leaves. Despite high *C*Las titers in the leaves, particularly in 'Cleopatra', 'Duncan', 'Olinda Valencia', and 'Valencia 1-14-19', the HLB foliar symptoms were sometimes not evident during our study. In general, blotchy mottle symptoms on the leaves were mild, even in plants with high *C*Las titers. Throughout the evaluation period, leaves typically showed mild foliar HLB disease symptoms, although symptom severity increased during the later stages of the study. Bodaghi et al. (2022a), working with graft-inoculated plants in the greenhouse, also reported only mild HLB symptoms. Studies conducted by Albrecht and Bowman (2012a), Albrecht et al. (2014), and Shokrollah et al. (2011) have indicated that plants can become infected, but HLB foliar

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Table 6. Number of leaves, area per leaf, and total leaf area from healthy and infected plants at 18 months after *C*Las inoculation.

	Number of leaves				Total leaf area (cm^2)				Area per leaf (cm^2)				Total leaf area
Cultivar	Healthy		Infected		Healthy		Infected		Healthy		Infected		Reduction (%)
Assad citron	121	a	126	a	5168	a	4986	a	43	a	40	a	4
Cleopatra	192	a	114	b	2804	a	871	b	15	a	7	b	69
Duncan	136	a	136	a	4563	a	3384	b	34	a	25	b	26
Kinnow	185	a	129	b	3147	a	2583	a	17	a	21	a	18
M. inodora	129	a	92	a	1322	a	895	a	10	a	9	a	32
Olinda Valencia	149	a	126	a	3987	a	2372	b	29	a	21	b	41
Rich 16-6	98	a	113	a	566	a	622	a	6	a	6	a	−10
Sunburst	221	a	114	b	3734	a	1519	b	17	a	12	a	59
Triumph	155	a	171	a	3596	a	3524	a	23	a	21	a	2
US-897	159	a	177	a	868	a	920	a	6	a	5	a	−6
Valencia 1-14-19	153	a	149	a	3388	a	2796	a	22	a	18	a	17
W. Murcott	77	a	62	a	2256	a	1289	a	28	a	21	b	43
Average	150		124		2970		2111		21		17		29

Different letters within rows indicate significant differences between healthy and infected plants from each cultivar according to Tukey's honestly significant difference (HSD) test. Reduction (%) is the percentage of the total leaf area reduction from healthy to infected plants.

disease symptoms may not manifest until several months after inoculation. Likewise, Stover et al. (2018) found that typical foliar blotchy mottle symptoms were not identified until 1 year after pathogen exposure, regardless of whether plants were graft-inoculated or psyllid-inoculated.

The method we used, which involved periodic pruning of the plants, was intended to limit the size of the growing potted trees and allow a comparison of the amount of new growth on healthy and infected plants for each evaluation cycle. For instance, during the assessment in October (12 mai), after the warmest and sunniest months of the year, a significant reduction in plant biomass for infected trees was observed in 'Duncan', 'Triumph', and 'Valencia 1-14-19'. However, by the next assessment in April (18 mai), after the winter season, when even healthy plants did not grow well, there was no difference in biomass between healthy and infected plants from these cultivars. The method we used to prune plants for growth management proved to be overly limiting on new growth in some parts of the growth cycle. This demonstrates the importance of the phase of the growing period when conducting cultivar assessments for HLB screening. Moreover, temperature has been shown to influence plant development and HLB disease progression (Lopes et al. 2017; Nagano et al. 2019; Thapa et al. 2021). In fact, significant variations in the plant transcriptome across the four seasons were reported by Ribeiro et al. (2023). During greenhouse studies examining *C*Las-plant interactions, the seasons appeared to exert a significant influence on the disease effects on growth and biomass accumulation; therefore, they may have a significant effect on cultivar assessments.

During their greenhouse study, Bodaghi et al. (2022b) found an average reduction of 55% in root system biomass caused by *C*Las infection, although the results varied by rootstock. In the present study, we observed a severe reduction in total root biomass from infected plants for the most susceptible cultivars that ranged from 44% to 75%. The largest loss of root biomass was measured for the fibrous roots. This severe loss in fibrous biomass caused by *C*Las infection has been previously documented by Graham et al. (2013), Johnson et al. (2014), and Kumar et al. (2018). During the present study, we noted that fibrous roots from severely *C*Las-infected plants were "mushy" and dark brown, especially for 'Cleopatra' and 'Sunburst'; this observation was similar to what Kumar et al. (2018) described as "spongy" and black to dark brown in color.

Fibrous roots play a crucial role in water and nutrient absorption. The reduction of fibrous roots caused by *C*Las infection can consequently compromise the uptake of nutrients in the plant. During our present study, we observed significant reductions in Ca, Mg, and Mn in the infected plants. Similar results were reported by Nwugo et al. 2013, who performed a greenhouse study and found significant reductions in Ca, Mg, and Mn levels in HLB-affected plants, and Shahzad et al. (2020), using a hydroponic system, also observed significant reductions in Ca and Mg. Bodaghi et al. (2022a) studied 'Valencia' grafted on different rootstocks in a greenhouse and reported a significant reduction in Ca in *C*Las-infected plants grafted on Ridge, 'Cleopatra', Carrizo, and US-942. Additionally, da Silva et al. (2020), who worked with sweet orange trees, noted a decrease in Mg in sap extracts. Deficiencies of N, S, Fe, Zn, and Cu were also reported for *C*Las-infected citrus plants (da Silva et al. 2020; Nwugo et al. 2013), but they were not identified during our study. The lack of observed differences in nutrient levels in our study could be attributed to the short duration of *C*Las colonization, the timing of leaf sample collection, and the optimal nutritional conditions that we provided to the greenhouse potted seedlings. Few nutrient deficiencies were also reported by a previous greenhouse study (Bodaghi et al. 2022a).

'W. Murcott', a scion cultivar, was grown as seedlings in this experiment. However, seedlings from this cultivar did not exhibit a well-developed root system, and the plants displayed severe nutrient deficiency symptoms and some branch dieback even among the noninfected control plants. Because of these factors, the accurate assessment of 'W. Murcott' tolerance to *C*Las may have been compromised in this study. Throughout the experiment, the leaf Ct values from this cultivar were consistently high, suggesting tolerance to *C*Las infection. Until now, no literature evidence has been found regarding the *C*Las response from 'W. Murcott'. However, 'Tango' mandarin, a variety developed from gamma-irradiated 'W. Murcott' budwood, has been reported to exhibit relative tolerance to HLB (Stover et al. 2016; Wilcox 2018).

In general, young seedlings from previously reported susceptible cultivars exhibited clear susceptibility to *C*Las infection under controlled inoculation. Notably, infected seedlings from 'Cleopatra', 'Olinda Valencia', and 'Sunburst' were severely affected by *C*Las infection, as indicated by the different variables measured in the present study, which is in alignment with previous studies (Albrecht and Bowman 2011, 2012a; Albrecht et at. 2014; Bowman et al. 2016; Folimonova et al. 2009). Conversely, seedlings from cultivars known for their tolerance to HLB, such as 'Assad' Citron, *M. inodora*, Rich 16-6, and US-897, showed only mild effects when infected with

Table 7. Foliar blotchy mottle symptoms of healthy and *C*Las-infected citrus cultivars at 12 and 18 months after inoculation.

	12 mai				18 mai			
Cultivar	Healthy		Infected		Healthy		Infected	
Assad citron	1.0	a	1.9	b	1.0	a	1.9	b
Cleopatra	1.0	a	3.0	b	1.0	a	4.3	b
Duncan	1.0	a	2.0	b	1.0	a	1.9	b
Kinnow	1.0	a	1.6	a	1.0	a	1.5	a
M. inodora	1.0	a	1.1	b	1.0	a	1.1	a
Olinda Valencia	1.0	a	2.9	b	1.0	a	2.6	b
Rich 16-6	1.0	a	1.6	b	1.0	a	1.0	a
Sunburst	1.0	a	1.9	a	1.0	a	3.3	b
Triumph	1.0	a	1.3	a	1.0	a	1.8	b
US-897	1.0	a	1.8	a	1.0	a	1.0	a
Valencia 1-14-19	1.0	a	1.8	a	1.0	a	2.3	b
W. Murcott	1.0	a	1.6	a	1.0	a	1.2	a

Different letters within rows indicate significant differences between healthy and infected plants from each cultivar according to the aligned rank transformation analysis of variance (ANOVA). No blotchy mottle was observed at 3 and 8 months after inoculation.

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Table 8. Foliar nutritional deficiency symptoms in healthy and *C*Las-infected citrus cultivars at 8, 12 and 18 months after inoculation (mai).

	8 mai				12 mai				18 mai			
Cultivar	Healthy		Infected		Healthy		Infected		Healthy		Infected	
Assad citron	1.0	a	1.3	a	1.5	a	1.9	a	1.8	a	2.4	a
Cleopatra	1.1	a	1.7	b	1.0	a	3.5	b	1.0	a	3.5	b
Duncan	1.1	a	1.5	b	1.0	a	1.3	a	1.0	a	1.5	a
Kinnow	1.3	a	1.6	a	1.5	a	1.6	a	1.4	a	1.7	a
M. inodora	1.6	a	1.8	a	2.4	a	2.9	a	1.4	a	2.2	a
Olinda Valencia	1.0	a	1.9	b	1.0	a	2.5	b	1.3	a	2.0	a
Rich 16-6	1.4	a	1.1	a	1.9	a	2.0	a	1.0	a	1.1	a
Sunburst	1.0	a	1.0	a	1.0	a	2.4	b	1.0	a	2.7	b
Triumph	1.0	a	1.5	a	1.0	a	1.4	b	1.1	a	1.2	a
US-897	1.2	a	1.1	a	1.4	a	1.8	a	1.2	a	1.6	a
Valencia 1-14-19	1.0	a	1.2	a	1.0	a	1.3	a	1.0	a	2.0	a
W. Murcott	2.0	a	1.8	a	2.4	a	4.2	a	1.5	a	4.2	b

Different letters within rows indicate significant differences between healthy and infected plants from each cultivar according to the aligned rank transformation analysis of variance (ANOVA). No blotchy mottle was observed 3 mai.

Table 9. Disease state and cultivar effect on calcium (Ca), magnesium (Mg), and manganese (Mn) concentrations of healthy and infected plants at 18 months after *C*Las inoculation.

	Ca (%)				Mg (%)				Mn (ppm)			
Cultivar	Healthy		Infected		Healthy		Infected		Healthy		Infected	
Assad citron	2.7	a	2.5	a	0.4	a	0.4	a	28.5	a	23.8	a
Cleopatra	2.4	a	1.5	b	0.2	a	0.2	b	44.5	a	33.1	b
Duncan	2.7	a	2.6	a	0.3	a	0.2	b	35.0	a	30.7	a
Kinnow	2.2	a	2.1	a	0.3	a	0.3	a	27.0	a	19.8	a
M. inodora	2.0	a	1.9	a	0.2	a	0.2	a	20.0	a	18.0	a
Olinda Valencia	2.9	a	2.4	b	0.3	a	0.2	b	26.0	a	25.4	a
Rich 16-6	1.7	a	2.0	a	0.1	a	0.1	a	20.0	a	30.0	b
Sunburst	3.0	a	2.2	b	0.3	a	0.2	b	42.2	a	30.8	b
Triumph	2.5	a	2.1	a	0.3	a	0.2	b	27.2	a	20.3	a
US-897	2.4	a	2.7	a	0.2	a	0.2	a	30.0	a	33.8	a
Valencia 1-14-19	2.8	a	2.3	b	0.3	a	0.2	b	36.0	a	25.4	b
W. Murcott	2.5	a	2.1	a	0.3	a	0.3	a	21.8	a	15.8	a

Different letters within rows indicate significant differences between healthy and infected plants from each cultivar according to Tukey's honestly significant difference (HSD) test.

*C*Las, which is also consistent with previous reports (Albrecht and Bowman 2011, 2012a; Folimonova et al. 2009; Ramadugu et al. 2016).

Experiments involving young plants, controlled inoculation, and greenhouse conditions serve as valuable tools to support citrus breeding programs and provide an understanding of specific plant responses to disease infection. However, it is important to acknowledge that these experiments may not fully capture the complexities of field conditions. In the field, citrus trees are subjected to a multitude of interacting factors that can influence their response to *C*Las infection. These factors include varying environmental conditions, diverse soil types, natural variations in pathogen populations, and the presence of other pests and diseases. Additionally, factors such as tree age, scion–rootstock interactions, and overall orchard management practices can also play a significant role in influencing the outcome of *C*Las infection in field-grown citrus trees. It is through an integrated approach—combining greenhouse-controlled experiments with large-scale field trials—that we can make the most efficient progress in developing resilient citrus varieties for sustainable citrus production.

Conclusions

Results from this study demonstrate that greenhouse experiments using controlled *C*Las inoculation are profitable for evaluating the citrus germplasm response and support citrus breeding programs in the development of new tolerant or resistant citrus cultivars. *P. trifoliata*, *M. inodora* [F.M. Bail.] Swing., *C. medica* [L.], and some selections of *C. reticulata* appeared to be of potential value for breeding for tolerance to *C*Las; however, further studies with long-term field assessments are necessary to fully understand the potential of these species and their hybrids in commercial use. *P. trifoliata*, *M. inodora*, and *C. medica* have fruit traits that are far from suitable for commercial use as scions, and that make introgression of *C*Las tolerance with suitable fruit traits likely to require several generations. Future studies that involve graft inoculation with *C*Las need to consider the inconsistency and often low rates of disease transmission and include a suitably large number of inoculated plants for each of the species or hybrids to be evaluated.

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