Different Sweet Orange–Rootstock Combinations Infected by *Candidatus Liberibacter asiaticus* under Greenhouse Conditions: Effects on the Roots

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Abstract. Grafting a scion onto a rootstock results in physical and physiological changes in plant growth and development, which can affect tree vigor, productivity, and tolerance to stress and disease. Huanglongbing (HLB) is one of the most destructive citrus diseases and has become endemic in Florida since its introduction in 2005. It is associated with the phloem-limited bacteria *Candidatus Liberibacter asiaticus* (CLAs), which cause severe metabolic disruptions in affected plants. Although most scion cultivars are highly susceptible, some rootstock cultivars are tolerant and allow the grafted tree to cope better with the disease. The objectives of this study were to identify rootstock traits that can be used to assess cultivars under controlled greenhouse conditions in advance of longer-term field trials. We used 10 commercially important rootstocks with different genetic backgrounds and known field performance in graft combination with ‘Valencia’ sweet orange scion. Trees were graft-inoculated with CLAs and compared against mock-inoculated trees. Tree health and CLAs populations were assessed regularly, and root growth was monitored using a minirhizotron imaging system. Plants were excavated and destructively sampled 21 months after inoculation to assess biomass distributions and other CLAs-induced effects. We found significant differences between healthy and infected trees for most variables measured, regardless of the rootstock. In contrast to leaf CLAs titers, root titers were significantly influenced by the rootstock, and highest levels were measured for ‘Ridge’ sweet orange and sour orange. Root growth and root biomasses were reduced upon infection but differences among rootstocks did not always agree with reported field performances. Despite severe biomass reductions plants maintained their relative distribution of biomass among different components of the root system, and no dead roots were observed. Root respiration was reduced by CLAs infection and was overall higher in tolerant cultivars suggesting its potential as a physiological marker. This study improves our knowledge about the strengths and weaknesses of assessing rootstock traits of grafted trees in a controlled greenhouse setting. Results from the study suggest that in addition to HLB tolerance, other rootstock traits will ultimately have major contributions to field survival and productivity of the grafted trees in an HLB endemic production environment.

Citrus huanglongbing (HLB), also known as “citrus greening,” is one of the most destructive diseases of citrus (Bové, 2006; da Graça et al., 2016; Wang, 2019). Phloem-limited bacteria in the genus *Candidatus Liberibacter* are the presumed agent causing HLB, but this has not yet been proven due to the unculturable nature of the bacterium (Merfa et al., 2019; Molki et al., 2020). In Florida, the pathogen associated with HLB is *Candidatus Liberibacter asiaticus* (CLAs), which is transmitted by the Asian citrus psyllid *Diaphorina citri*, a small insect that feeds on the phloem of citrus trees (Halbert and Manjunath, 2004; Hall et al., 2013).

Most commercially important citrus scion cultivars are susceptible to HLB (Alves et al., 2021; Bové, 2006), and management of the psyllid vector is one of the most effective strategies to control the disease. Vector control has not been successful in stopping the spread of infection, which has led to more than 70% decline in citrus production since the discovery of HLB in Florida in 2005; the disease has been considered endemic since 2013 (Graham et al., 2020). Other management strategies are now targeted at sustaining tree health and productivity and include irrigation and nutrient management and methods to improve root health (Atta et al., 2020; Kadyampakeni et al., 2014; Stansly et al., 2014).

In contrast to most scions, tolerance to HLB was observed in several citrus relatives commonly used as rootstocks, particularly in trifoliolate orange and some of its hybrids (Albrecht and Bowman, 2012; Boava et al., 2015). Using tolerant rootstocks is therefore one way to reduce the detrimental effects of HLB on the grafted scion (Bowman and McCollum, 2015; Bowman et al., 2016a, 2016b; Rodrigues et al., 2020; Shokrollah et al., 2011; Stover et al., 2016; Viteri et al., 2021).

Because of the severity of HLB-induced production losses and the lack of tolerant commercially relevant scion cultivars, there is an urgent need for superior rootstocks to mitigate disease impacts and maintain tree health and productivity. However, assessing rootstock performance in an HLB-endemic field environment takes many years and is hindered by the inability to directly compare infected with noninfected trees. Greenhouse testing in a controlled environment is a shorter-term approach that can provide valuable information on cultivar performance (Albrecht and Bowman, 2012, 2019; Alves et al., 2021; Folimonova et al., 2009; McCollum et al., 2016). Albrecht et al. (2014) documented the slower spread of CLAs infection in the natural field environment compared with the controlled environment of a greenhouse. Although some greenhouse studies have been successfully conducted using psyllids to inoculate experimental plants with CLAs (McCollum et al., 2016), graft inoculation requires fewer logistical challenges and has been used more often to assess cultivar performance and other HLB effects (Albrecht and Bowman, 2012, 2019; Folimonova et al.,

The rootstock, which provides the below-ground portion of a grafted tree, has had a significant role in the development of the Florida citrus industry. The physical and physiological changes that occur due to the grafting of a scion onto a rootstock can affect tree growth and development in many ways (Goldschmidt, 2014; Mudge et al., 2009). Grafting a desirable scion onto a specially selected rootstock can also help the tree be more tolerant to abiotic and biotic stresses including pests and diseases (Albacete et al., 2015; King et al., 2008).

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2009; Shokrollah et al., 2009). A direct comparison of the two inoculation methods found transmission of the bacteria by graft inoculation to be more efficient than by psyllid inoculation (Albrecht et al., 2014).

Most greenhouse studies used nongrafted seedlings to assess cultivar susceptibility to HLB; fewer studies investigated the rootstock influence on the grafted tree performance (Albrecht and Bowman, 2019; Bowman and Albrecht, 2020; Stover et al., 2018). These studies focused mostly on the aboveground effects of rootstocks without considering CLAs effects on the roots. Graham et al. (2013) and Johnson et al. (2014) reported that CLAs infection causes fibrous root decline before disease manifestation in the scion; since then, maintaining root health has become an important part of HLB management in Florida.

A typical citrus tree root system under present Florida growing conditions is composed mainly of lateral (structural) roots that provide anchorage, and fibrous roots that absorb water and nutrients (Pokhrel et al., 2021). The efficiency of the root system, especially the fibrous roots, can enhance the performance of the tree under disease stress. For example, Graham (1995) reported that under certain environmental conditions, the ability of a rootstock to regenerate roots is associated with its ability to tolerate root rot caused by *Phytophthora nicotianae*. Kumar et al. (2018) suggested that the pathological response to HLB differs among different root orders of a fibrous root system.

Several studies demonstrated substantial differences in citrus root system architectures among different rootstocks (Castle and Youtsey, 1977; Pokhrel et al., 2021; Savage et al., 1945). It is also well documented that the rootstock cultivar can have a strong influence on the vigor of a grafted citrus tree (Forner-Giner et al., 2014; Girardi and Murão Filho, 2006). Other root traits may also contribute to the different ability of rootstocks to reduce HLB-induced tree decline.

In contrast to assessing aboveground horticultural traits, assessing belowground traits is considerably more difficult, especially when the goal is to examine root growth

dynamics rather than root biomasses at the end of a study period. Using minirhizotrons is one methodology to assess root turnover *in-situ* and over long periods of time without destructive sampling (Majdi et al., 2005). Our study presented here uses this technology to determine its value for differentiating rootstocks' abilities to cope with HLB-induced growth reductions.

This study is the second part of a study investigating rootstock influence on the grafted citrus tree. In the first part, we examined rootstock and HLB effects on the grafted scion (Bodaghi et al., 2022). Here, we focus on the growth and performance of the root system in the healthy state and the HLB-affected state. The objective was to identify root traits that may be associated with rootstock influence on the grafted tree performance and that may be used as markers for rootstock selection to mitigate HLB-induced grafted tree decline. The availability of root trait markers that can be assessed under controlled greenhouse conditions would enable rootstock selection in advance of field testing and accelerating the release of new and superior cultivars for use in an HLB-endemic production environment.

Materials and Methods

Plant material. Ten commercial citrus rootstock cultivars with different sensitivity to CLAs were used in this study (Table 1); all these rootstocks are known to produce primarily nucellar seedlings that are genetically identical to the seed parent. Plants were grown from seed by planting one seed each into 3.8 cm × 21 cm cone cells (Containers; Stuewe and Sons, Tangent, OR) containing pre-moistened soilless potting mix (Pro Mix BX; Premier Horticulture, Inc., Quakertown, PA). After germination, plants were irrigated as needed and fertilized biweekly using a water-soluble fertilizer (20N–10P–20K; Peters Professional, The Scotts Company, Marysville, OH) at a dose of 400 mg·L⁻¹ N. Insecticides were applied as needed. Any off-types arising from zygotic embryos were identified based on their different leaf

morphology and growth habit and were discarded.

After 6 months, plants were transplanted into 19.7 cm × 31.8 cm plastic tree pots (Stuewe and Sons) and were grafted with FDACS DPI (Florida Department of Agriculture and Consumer Services, Division of Plant Industry) certified 'Valencia' (*Citrus sinensis*) budwood (clone 1-14-19) using the inverted T-method. All plants were grown under DPI-certified disease-free conditions in the US Horticultural Research Laboratory greenhouses (USDA, Fort Pierce, FL).

Six months after grafting, 12 plants of each graft combination were inoculated with buds from infected greenhouse-grown 'Valencia' orange plants that were PCR-positive for CLAs and symptomatic for HLB. Three 1–1.5 cm long bud pieces were used to inoculate each plant using the inverted T-method. Buds were inserted on different sides in the trunk at 4–6 cm above the graft union. Six plants of each genotype were mock inoculated with disease-free bud pieces obtained from healthy greenhouse-grown 'Valencia' orange plants for use as non-infected controls.

Nine months after inoculation (mai), plants were transplanted into 37 cm × 45 cm (48-L) plastic tree containers (Nursery Supplies, Chambersburg, PA) containing Pro Mix BX potting medium. Of the 12 CLAs-inoculated plants, only those with a cycle threshold (Ct) value of 32 or lower were included for a total of six replications. Ct-values for CLAs were determined using the methods described below.

Plants were arranged in a completely randomized design on the greenhouse benches and kept under natural light conditions at a temperature of 21 to 28 °C. Plants were irrigated and fertilized as described above, and insecticides were applied as needed. Tree size was managed by removing ≈30% of shoot growth every 6 months. The experiment was ended 21 months mai.

Root growth analysis. During the transplant into the 37 cm × 45 cm plastic tree containers (9 mai), one 6 cm × 50 cm clear acrylic minirhizotron tube (CID Bio-Science, Camas, WA) was inserted vertically and at a

Table 1. Rootstocks, parentage, and previously reported HLB response.

Rootstock	Parentage	HLB response
Ridge Pineapple sweet orange	<i>Citrus sinensis</i> (L.) Osbeck	Highly susceptible ^z
Cleopatra mandarin	<i>C. reticulata</i> Blanco	Susceptible ^{z,y,x,w,v}
Sour orange	<i>C. aurantium</i> (L.)	Susceptible ^v , moderately tolerant ^u
Carrizo citrange	<i>C. sinensis</i> 'Washington' × <i>Poncirus trifoliata</i> (L.) Raf.	Tolerant ^{y,v}
Swingle citrumelo	<i>C. paradisi</i> Macf. 'Duncan' × <i>P. trifoliata</i>	Moderately tolerant ^{w,v}
US-802	<i>C. maxima</i> 'Siamese' × <i>P. trifoliata</i> 'Gotha Road'	Moderately tolerant ^y
US-812	<i>C. reticulata</i> 'Sunki' × <i>P. trifoliata</i> 'Benecke'	Moderately tolerant ^y
US-897	<i>C. reticulata</i> 'Cleopatra' × <i>P. trifoliata</i> 'Flying Dragon'	Tolerant ^{y,x}
US-942	<i>C. reticulata</i> 'Sunki' × <i>P. trifoliata</i> 'Flying Dragon'	Tolerant ^{y,w}
US-1516	<i>C. maxima</i> (L.) Osbeck 'African' × <i>P. trifoliata</i> 'Flying Dragon'	Tolerant ^w

^zAlbrecht et al., 2014.

^yAlbrecht and Bowman, 2012.

^xAlbrecht and Bowman, 2011.

^wBowman et al., 2016.

^vFolimonova et al., 2009.

^uStover et al., 2016.

distance of about 15 cm from the stem into the potting medium of each pot. Fibrous root growth was measured monthly using a minirhizotron/camera (CI-600) system (CID Bioscience) starting 3 months after transplant (12 mai). The images were captured at a resolution of 100 DPI and were analyzed using WinRHIZO-Tron (Regent Instrument, Quebec, Canada).

Determination of CLas titers. Leaves and fibrous roots (<2 mm in diameter) were collected from each tree 6, 9, 15, and 21 mai. One hundred milligrams of ground tissue were used for DNA extraction. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Real-time PCR assays were performed using primers HLBas/HLBr and probe HLBp (Li et al., 2006). Primers COXf/COXr and probe COXp were used for normalization. Amplifications were performed using an Applied Biosystems QuantStudio 3 Real-Time PCR and the iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. All reactions were carried out in duplicate in a 20 μ L reaction volume using 5 μ L DNA. A subset of samples was analyzed using a primers/probe system designed specifically for CLas detection in citrus roots (Park et al., 2018). PCR results were identical, regardless of the primer/probe system used, and we continued our analyses using the primers designed by Li et al. (2006).

Plant biomass distribution. The plant biomass distribution was determined 21 mai by separating trees into leaves, stems, root crown, large-size roots (>5 mm in diameter), medium-size roots (2–5 mm in diameter), and fibrous roots (<2 mm in diameter). The potting mix was removed from the roots and roots were washed with tap water and blotted dry. Tissues were dried at 45°C until the weight was constant.

Root respiration. Immediately before excavation, a subset of fibrous roots was extracted from the soil of each tree, shaken to remove any loose potting medium, and washed with tap water. The fibrous roots

were separated into three orders based on their position in the root branch with the most distal roots numbered as first order roots (Pregitzer et al., 2002). Respiration was measured immediately after separation for each root order in a closed loop respiration chamber using an IR CO₂/H₂O analyzer (LI-850; LICOR, Lincoln, NE). The rate of CO₂ accumulation in the chamber was measured over a 5-minute interval. The root tissue was then scanned at 400 dpi on a flatbed scanner with a transparency unit (Epson Perfection v850 Pro; Epson America, Los Alamitos, CA) and dried at 45°C until constant weight. Root respiration rates were expressed relative to the total mass or length of root tissue.

Statistical analysis. Two-way analysis of variance (ANOVA) was conducted with disease state and rootstock as fixed effects. Monthly, root growth was analyzed using repeated measures ANOVA. Comparison of rootstock effects for each trait was by one-way ANOVA. Mean separation was performed by Tukey's honestly significant difference test. Differences were defined as statistically significant when the *P* value was <0.05. Data were analyzed using R 3.6.0 (The R Foundation for Statistical Computing).

Results

Root CLas titers. CLas titers were expressed as cycle threshold (Ct) values with lower Ct-values indicating higher CLas titers and vice versa. The average root Ct-values of infected plants were highest at 9 mai and 15 mai (35.0 and 34.4) and lowest at the end of the experiment (32.7) indicating an increase in root CLas titers over time (Table 2). For comparison, the average Ct-values of leaves were 28.0 (6 mai), 28.4 (9 mai), 26.3 (15 mai), and 24.7 (21 mai) (data not shown, see Bodaghi et al., 2022). Significant differences among rootstock cultivars were observed at 15 mai, when sour orange roots had the lowest Ct-value (31.0) and therefore highest level of bacteria and US-942 had the highest Ct-value (36.8) and lowest level of bacteria. No

significant differences among rootstocks were found at the other time points.

Minirhizotron root growth analysis. Regardless of rootstock and disease state, roots grew the most from September to October after the transplant into larger pots, and from March to April and May to July (Fig. 1). The root length measured in the visual field of the minirhizotrons was significantly reduced upon infection and there was a significant interaction between disease state and month of measurement. The rootstock cultivar had a significant effect on root length and there was a significant interaction with the month of measurement, but not with the disease state.

A significant rootstock effect was found for the root length measured in July just before the end of the experiment; the most roots were found for 'US-942', and the least were found for 'Ridge', and 'Cleopatra', and 'US-812' (Table 3). The average root length reductions at 21 mai were largest for 'Ridge' (73%) followed by 'US-802' (66%); for most of the other rootstocks, reductions were statistically not significant (Table 4).

Rootstock biomasses. CLas infection reduced all measured variables significantly and rootstock effect was significant for rootstock trunk, large-size roots, and medium-size roots (Table 5). On average, infection reduced root trunk weights by 44%, large-size roots by 60%, medium-size roots by 61%, and fibrous roots by 56%. The total root mass was reduced by 55%. A significant interaction between disease state and rootstock was found for the large-size size roots and for the total rootstock biomass (Table 6; Supplemental Table 1).

The largest root trunk mass reductions were found for 'Ridge' (59%); no significant reduction was found for 'US-1516'. 'Ridge', 'Cleopatra', and sour orange experienced the largest reductions of large-sized roots in response to CLas infection (66% to 78%), whereas 'US-897' and 'US-1516' did not experience any significant reductions. The largest medium-size root mass reductions were found for 'Cleopatra', 'Ridge', and 'US-812' (68% to 71%). No significant reductions of medium-size roots were found for 'US-1516', but the remaining rootstocks had reductions between 49% and 64%. Fibrous root mass reductions were largest in 'Ridge' and 'Cleopatra' (73%). No significant reductions were found for 'Swingle' and 'US-1516', whereas the remaining rootstocks experienced reductions of 50% to 62%. The largest reductions in total root biomass upon CLas infection were measured for 'Ridge' (71%), followed by 'Cleopatra' (66%). The least reductions were found for 'Swingle' and 'US-897' (43%); no significant total root mass reductions were found for 'US-1516'. A visual summary of root reductions is presented in Fig. 2.

Root size class distribution. Regardless of the disease state, large-size roots comprised the largest proportion of the belowground biomass (52% to 53%), followed by the fibrous roots (33%) and the medium-size roots (15%)

Table 2. Root Ct-values of infected 'Valencia' trees grafted on different rootstocks.

Factor	6 mai	9 mai	15 mai	21 mai	Avg
Time	33.6 ab	35.0 a	34.4 a	32.7 b	33.9
<i>P</i>				0.0034	
Rootstock					
Ridge	32.7	32.1	31.5 ab	30.9	31.8 b
Cleopatra	33.7	34.6	35.0 ab	30.7	33.5 ab
Sour orange	32.3	33.0	31.0 b	31.4	31.9 b
Carrizo	32.1	34.0	34.9 ab	33.0	33.5 ab
Swingle	36.4	35.4	35.0 ab	32.7	34.9 ab
US-802	33.0	35.9	34.2 ab	32.0	33.7 ab
US-812	32.9	34.9	34.2 ab	32.7	33.7 ab
US-897	35.3	37.5	36.0 ab	34.9	35.9 a
US-942	33.4	36.2	36.8 a	36.6	35.7 a
US-1516	34.3	36.0	35.6 ab	32.2	34.5 ab
<i>P</i>	0.6168	0.1481	0.0214	0.3354	0.0002
Time $\times$ rootstock					
<i>P</i>				0.9680	

Different letters within rows and columns indicate significant differences among months after inoculation (mai) and rootstocks, respectively, according to Tukey's honestly significant difference test.

Fig. 1. Root length of (A) healthy and (B) infected 'Valencia' trees grafted on different rootstocks measured monthly after transplant (12–21 mai). Error bars are not shown for clarity.

(Table 7). There were no significant reductions in the relative proportion of the different root size classes after CLAs infection.

In contrast to the disease state, the rootstock effect was significant. The largest proportion of large-size roots was found for 'Carrizo' (62%), followed by 'Cleopatra' (59%); the lowest proportion was found for 'US-1516', and 'US-897' (45%), followed by 'Swingle' (47%). The largest proportion of

medium-size roots was measured for sour orange (20%) and the lowest for 'Carrizo', 'US-812', and 'US-942' (11% to 12%). The fibrous root proportion was largest in 'US-897' (40%), followed by 'Swingle', 'US-802', 'US-812', 'US-942', and 'US-1516' (35% to 39%); it was lowest in sour orange (24%) followed by 'Cleopatra' and 'Carrizo' (25% to 26%). There were no significant interactions between disease state and root size class distribution.

Aboveground plant biomass. CLAs infection significantly reduced the leaf and stem biomass by 49% and 53%, respectively, with a total scion biomass reduction of 49% (Table 8). There was no significant rootstock effect on the scion biomass and no significant interaction between disease state and rootstock.

The average root to shoot ratio of plants was 0.69 and neither CLAs nor rootstock cultivar had any significant effect (data not shown).

Root respiration. Disease state, rootstock, and root order had a significant effect on the root respiration (Table 9) and there was a significant interaction between disease state and rootstock cultivar. The root respiration rate was higher in healthy 'US-897' roots, healthy and infected 'US-802' roots, and healthy sour orange, 'US-942', and 'US-1516' roots ($0.88\text{--}0.98\text{ mg}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) than in infected sour orange, 'Ridge', and 'Cleopatra' roots ($0.47\text{--}0.55\text{ mg}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$; Supplemental Table 2). The highest respiration rate was found for the first-order roots ($1.0\text{ mg}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) and the lowest for the third-order roots ($0.5\text{ mg}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$).

Within root orders and rootstocks, significant root respiration reductions in response to

CLAs infection were only found for 'Ridge', sour orange, and 'US-897' and ranged from 29% to 67% (Table 10). Across all three root orders, significant reductions were measured for 'Ridge', 'Cleopatra', sour orange, and 'US-897' with sour orange experiencing the highest reductions (50%); a moderate but significant increase in respiration was found for 'Swingle'.

Discussion

Because of the devastating impact of HLB on citrus production and the lack of tolerance in commercially relevant scion cultivars, there is a high demand for tolerant rootstocks to mitigate disease effects and retain fruit production. Effective methods for assessing rootstock and HLB effects on the grafted tree tolerance quickly, and in advance of field testing, are therefore needed. This study is the second part of a larger study examining plant growth and physiological markers best suited to determine cultivar performance. Although the first part focuses on the aboveground portion, the grafted 'Valencia' scion (Bodaghi et al., 2022), this part focuses on the belowground portion, the roots.

The average root Ct_{CLAs} values of infected plants assessed at 6, 9, 15, and 21 months after inoculation did not fall below 33, which is considerably higher (thus signifying lower CLAs titers) than the average values measured for the leaves. Although differences in Ct-values (and therefore CLAs titers) among rootstocks were not consistent throughout the study, on average, 'US-942' and 'US-897' harbored the least bacteria in the roots, and

Table 3. Disease state and rootstock effects on root length of grafted 'Valencia' trees 21 months after CLAs inoculation using minirhizotrons.

Factor	Root length (mm)
Disease state	
HLT	172
INF	97
RD	44%
P	<0.0001
Rootstock	
Ridge pineapple	105 b
Cleopatra	107 b
Sour orange	117 ab
Carrizo	168 ab
Swingle	133 ab
US-802	132 ab
US-812	96 b
US-897	130 ab
US-942	211 a
US-1516	146 ab
P	0.0060
Disease state × rootstock	
P	0.3334

Different letters within columns indicate significant differences among rootstocks according to Tukey's honestly significant difference test. HLT = healthy, INF = infected, RD = reduction.

Table 4. Average live root length (mm) of healthy and infected Valencia' trees grafted on different rootstocks measured using minirhizotrons 21 months after inoculation.

	Ridge	Cleopatra	Sour	Carrizo	Swingle	US-802	US-812	US-897	US-942	US-1516
HLT	164	149	158	208	183	197	120	139	257	140
INF	45	65	77	128	84	66	71	120	165	152
RD	73%	56%	51%	38%	54%	66%	41%	14%	36%	-9%
P	<0.0001	0.0662	0.0385	0.1007	0.0827	0.0130	0.1189	0.5080	0.2094	0.7177

HLT = healthy, INF = infected, RD = reduction.

Table 5. Disease state and rootstock effects on root biomasses of grafted 'Valencia' trees 21 months after CLas inoculation.

Factor	Rootstock trunk (g)	Large-size roots (g)	Medium-size roots (g)	Fibrous roots (g)	Total (g)
Disease state					
HLT	50	79	23	49	201
INF	28	32	9	21	90
RD	44%	60%	61%	56%	55%
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Rootstock					
Ridge	43 abc	61 a	17 ab	41	154
Cleopatra	32 bc	69 a	20 ab	31	165
Sour orange	35 abc	67 a	25 a	31	157
Carrizo	36 abc	59 a	12 b	26	133
Swingle	47 ab	49 a	16 ab	38	156
US-802	50 a	57 a	18 ab	37	157
US-812	41 abc	56 a	14 b	37	147
US-897	30 c	42 a	14 b	37	127
US-942	37 abc	46 a	10 b	35	108
US-1516	37 abc	44 a	15 b	39	140
P	0.0014	0.0395	0.0002	0.2264	0.4945
Disease state × rootstock					
P	0.2257	0.0094	0.068	0.2974	0.0138

Different letters within columns indicate significant differences among rootstocks according to Tukey's honestly significant difference test.

HLT = healthy, INF = infected, RD = reduction.

Table 6. Root biomasses of healthy and infected 'Valencia' trees grafted on different rootstocks 21 months after CLas inoculation.

	Ridge	Cleopatra	Sour orange	Carrizo	Swingle	US-802	US-812	US-897	US-942	US-1516
Rootstock trunk (g)										
HLT	61	42	45	49	56	64	57	37	48	42
INF	25	22	25	23	38	37	26	24	26	33
RD	59%	48%	45%	53%	33%	41%	55%	36%	45%	21%
P	0.0012	0.0029	0.1110	0.0039	0.0795	0.0041	0.0012	0.0175	0.0173	0.1800
Large-size roots (g)										
HLT	100	106	100	87	66	80	83	52	63	51
INF	22	33	34	32	32	35	30	31	29	37
RD	78%	69%	66%	63%	52%	57%	64%	40%	54%	28%
P	<0.0001	0.0015	0.0063	0.0022	0.0442	0.0147	0.001	0.0738	0.0012	0.2160
Medium-size roots (g)										
HLT	25	30	37	17	22	26	22	19	15	17
INF	8	9	14	7	10	10	6	9	6	12
RD	68%	71%	63%	60%	55%	64%	71%	50%	62%	30%
P	0.0003	<0.0001	0.0117	0.0072	0.0448	0.0026	0.0085	0.0236	0.0028	0.2090
Fibrous roots (g)										
HLT	65	49	45	37	47	50	52	49	49	47
INF	18	13	17	14	30	24	23	25	20	31
RD	73%	73%	62%	62%	37%	52%	56%	49%	59%	35%
P	0.0002	0.0004	0.0095	0.0174	0.106	0.0057	0.0040	0.0098	0.0057	0.0868
Total root biomass (g)										
HLT	250	227	227	190	192	220	213	156	175	158
INF	73	77	89	76	109	106	85	89	81	113
RD	71%	66%	61%	60%	43%	52%	60%	43%	54%	28%
P	<0.0001	0.0004	0.0050	0.0034	0.0546	0.0057	0.0014	0.0190	0.0017	0.1430

Biomasses are expressed as dry weights. Large size roots were > 5 mm, medium-size roots 2–5 mm, and fibrous roots < 2 mm in diameter.

HLT = healthy, INF = infected, RD = reduction.

'Ridge' and sour orange the most. This is in accordance with the previously reported higher tolerance of these two cultivars compared with sweet orange when grown as seedling trees (Albrecht and Bowman, 2012); however, variable results were reported for sour orange (Folimonova et al., 2009; Stover

et al., 2016). A similar trend for the grafted tree tolerance was found in a recent study where 'US-942' exhibited the lowest and 'Ridge' the highest root CLas titers (Bowman and Albrecht, 2020). In that study, root CLas titers were also considerably lower than leaf CLas titers, validating the findings presented

here. This suggests that CLas preferentially colonizes the leaves and not the roots, and that the grafted tree tolerance is determined predominantly by the scion rather than the rootstock. This has also been demonstrated in a study on reciprocally grafted tolerant and susceptible citrus cultivars (Albrecht and

Fig. 2. Percent reductions (%) of root trunk, large, medium, and fibrous roots and total root biomass of 'Valencia' trees grafted on different rootstocks.

Bowman, 2019). These results suggest that using roots instead of leaves for HLB diagnosis, as suggested by Park et al. (2018), may not be a suitable approach for all situations. Furthermore, Johnson et al. (2014) reported that CLAs could be detected in infected roots of grafted sweet orange trees before it could be detected in the leaves. In our study, in contrast to leaves, root CLAs titers rarely exceeded the threshold at which plants may be truly considered infected. Different developmental stages affecting source-sink relations of the trees at the

time of CLAs inoculation could have contributed to these different findings.

The monthly evaluation of root growth by minirhizotrons showed consistent root growth during the time of evaluation regardless of the disease state, albeit growth was diminished in the infected state; however, root decline as reported by Johnson et al. (2014) for field-grown trees was not observed. The controlled environment of the greenhouse and the absence of field-associated secondary pathogens and other stresses may be possible reasons for this discrepancy.

The largest increase in root length measured with the minirhizotron system occurred from September to October after the transplant of trees into larger pots, and from March to July of the following year, which corresponded to the most active time of leaf and shoot growth. Minirhizotron field studies by Pokhrel et al. (2021) found the largest net increase in live root length between June and July after the spring leaf flush. Bevington and Castle (1985) suggested that root growth follows shoot elongation and is stimulated by increases in soil temperature when water is not a limitation. The increase in root growth commencing in spring observed in our study is likely because of the more favorable and controlled conditions in the greenhouse than in the open field during that period. Neither Bevington and Castle (1985) nor Pokhrel et al. (2021) found differences in the root growth dynamics among different rootstock cultivars. In this study, root growth was determined by the interaction between rootstock and disease state and between rootstock and month of assessment; regardless of the disease state, 'US-942' had the longest root length at the end of the study. 'US-942' is currently the most propagated rootstock in Florida because of its excellent field performance (Bowman and McCollum, 2015; Rosson, 2020). A longer root length is usually associated with a better ability to uptake nutrients and water and may be partially responsible for the superior commercial performance of 'US-942'. Other citrandarins (hybrids of mandarin and trifoliate orange), especially those with 'Sunki' mandarin in the parentage, like 'US-942', have also been reported as field tolerant to HLB and their use as an important management tool to combat HLB was suggested (Boava et al., 2015).

One of the reported impacts of HLB is a decline in fibrous root biomass (Johnson et al., 2014). Here, we studied HLB effects on the total root biomass and its different components, namely, rootstock trunk and large, medium, and fibrous roots. Across all root system components, biomass reductions were substantial (44% to 61%) upon CLAs infection. However, the root to shoot ratio as well as the relative proportion of root size classes remained unaffected by infection. This suggests that the root loss associated with HLB is not caused by the death or decline of roots, but rather by a reduced production, at least in the absence of secondary opportunistic pathogens. This is supported by the minirhizotron results where no dead roots were found.

Among the different rootstocks, differences were found for some of the root traits such as the root trunk and the medium-sized root biomass and the relative distribution of large, medium, and fibrous roots. In general, largest reductions were found for 'Ridge' sweet orange, which is consistent with the high sensitivity of sweet orange to HLB (Folimonova et al., 2009; McClean and Schwarz, 1970) and of the cultivar Ridge, in particular (Albrecht and Bowman, 2012). The fibrous root mass did not vary among the

Table 7. Disease state and rootstock effects on root size class distribution of grafted 'Valencia' trees 21 months after CLAs inoculation.

Factor	Large-size roots (%)	Medium-size roots (%)	Fibrous roots (%)
Disease state			
HLT	53	15	33
INF	52	15	33
P	0.2637	0.3278	0.5700
Rootstock			
Ridge pineapple	52 bcde	16 ab	32 bc
Cleopatra	59 ab	17 ab	25 cd
Sour orange	56 abc	20 a	24 d
Carrizo	62 a	12 b	26 cd
Swingle	47 de	15 ab	38 ab
US-802	50 cde	16 ab	34 ab
US-812	52 bcde	12 b	36 ab
US-897	45 e	15 ab	40 a
US-942	54 bcd	11 b	35 ab
US-1516	45 e	16 ab	40 ab
P	<0.0001	<0.0001	<0.0001
Disease state × rootstock			
P	0.6348	0.3638	0.22

Different letters within columns indicate significant differences among rootstocks according to Tukey's honestly significant difference test. HLT = healthy, INF = infected.

Table 8. Disease state and rootstock effects on the grafted ‘Valencia’ scion biomass’ 21 months after CLas inoculation.

Factor	Leaves (g)	Stem (g)	Total plant biomass (g)
Disease state			
HLT	119	193	513
INF	58	103	250
RD	49%	53%	49%
<i>P</i>	<0.0001	<0.0001	<0.0001
Rootstock			
<i>P</i>	0.2045	0.3597	0.5761
Disease state × rootstock			
<i>P</i>	0.2666	0.1242	0.1188

HLT = healthy, INF = infected, RD = reduction.

rootstock cultivars. However, except for ‘Swingle’ and ‘US-1516’, fibrous root masses were significantly reduced in infected plants. ‘Swingle’ was described as moderately tolerant to HLB (Folimonova et al., 2009) and ‘US-1516’ was the best performing rootstock in a field trial under HLB-endemic conditions, followed by ‘US-942’ (Bowman et al., 2016). Despite the larger root length measured for ‘US-942’ with the minirhizotron system, root mass analysis did not suggest the superiority of this rootstock over the other ones in our study. One possible reason for these different results could be differences in diameter and density of the fibrous roots among rootstocks. As finer and longer roots generally have a higher capacity

for absorption of nutrients and water (Comas et al., 2013), root length as measured with the minirhizotron system may be a better indicator of root efficiency than the fibrous root weight. Another reason for the variable results for root length and fibrous root masses among rootstocks are the limitations of the minirhizotron, which allows only a subset of the whole root system (determined by the surface area of the minirhizotron tube) to be examined (Majdi, 1996). Considering these limitations and the large amount of work associated with the image analysis, the minirhizotron method may not be well suited for large-scale evaluation of different rootstock cultivars unless root growth dynamics are the primary study objective.

In addition to the absolute root masses, we compared the relative distribution of the different root size classes. In contrast to the significant root mass reductions, we did not find any changes in the relative root class distribution in response to CLas infection, although distributions varied among rootstocks. Different studies reported root responses to CLas infection ranging from anatomical alterations to physiological changes (Kumar et al., 2018; Shahzad et al., 2020; Wu et al., 2018). Shahzad et al. (2020) identified multiple genes upregulated in infected roots associated with nutrient uptake. They found that the CLas-positive fibrous roots are highly efficient in water and nutrient uptake and concluded that the limiting factor of water and nutrient uptake in infected plants is the smaller root mass after infection because of an HLB-induced lower root-to-shoot ratio. This contrasts with our study where the root to shoot ratio was not changed upon infection. Differences in experimental conditions such as use of a hydroponic system and artificially induced nutrient deprivation at the onset of that study may have contributed to these differences.

In addition to root growth and root biomass distributions, we examined fibrous root respiration of the first three orders of roots to provide a better understanding of feeder root function (Pregitzer et al., 2002). Root respiration is related to root morphology, nitrogen content, soil temperature, and season (Jia et al., 2013) and provides an important measure of nutrient uptake efficiency (Lambers et al., 2008; Lynch, 2015). It has also been suggested that different fibrous root orders exhibit different physiological responses to

HLB (Kumar et al., 2018). In this study, the root respiration rate was significantly influenced by root order and the interaction of rootstock cultivars and disease state. The highest respiration rate was found in first-order roots as observed for other plant systems (Jia et al., 2013 and references therein). Despite the interaction of cultivar and disease state, lower respiration rates were found in infected roots of most cultivars, but higher respiration rates were found in some of the trifoliate hybrid cultivars, regardless of the disease state. This suggests a better ability of these rootstocks to take up water and nutrients than the unifoliate rootstocks under healthy and infected conditions and may be one of the reasons for their superior influence on the grafted tree tolerance in an HLB-endemic field environment. Across all root orders, reductions in respiration due to CLas infection were largest for sour orange roots; large reduction were also found for ‘Cleopatra’ and ‘Ridge’, which is in accordance with the higher susceptibility of these cultivars to HLB. An exception was ‘US-897’, which despite being a trifoliate orange hybrid exhibited relatively large reductions in respiration. This may be because ‘US-897’ also contains ‘Cleopatra’ in its parentage. However, the respiration rate of ‘US-897’ was among the highest measured, which may contribute to its higher tolerance to HLB compared with its non-trifoliate counterpart ‘Cleopatra’ (Albrecht and Bowman, 2011).

Although HLB tolerance may be an important attribute for citrus rootstocks, we suspect that other rootstock traits associated with generalized tree health, effective water and nutrient utilization, and broad stress tolerance are also critical for good performance of the grafted tree in an HLB-endemic environment. Results of this study suggest that differences among rootstocks for simple tolerance to HLB do not adequately explain the observed larger differences in field performance.

Conclusions

This study improves our knowledge about rootstock and root responses to CLas and rootstock influence on the grafted tree tolerance to HLB. Artificial CLas inoculation under the conditions of this greenhouse study caused considerable biomass reductions of infected compared with healthy plants regardless of the rootstock cultivar. With some exceptions, disease effects were generally more severe in trees grafted on known susceptible rootstocks than on known tolerant rootstocks. Among the root traits studied, CLas titers and root respiration exhibited differences among rootstocks that were most closely associated with the known disease responses of the selected cultivars. Notable was the lack of evidence for CLas to induce fibrous root decline as a primary consequence of infection. Rather, infected trees adjusted their root mass relative to the scion biomass in accordance with the genetic potential of the rootstock and maintained the same relative distribution of fibrous roots to larger-size-class roots. Taken

Table 9. Disease state and rootstock effects on root respiration of grafted ‘Valencia’ trees 21 months after CLas inoculation.

Factor	Root respiration (mg·g ⁻¹ ·h ⁻¹)
Disease state	
HLT	0.84
INF	0.70
RD	17%
<i>P</i>	<0.0001
Rootstock	
Ridge pineapple	0.63 c
Cleopatra	0.66 bc
Sour orange	0.69 bc
Carrizo	0.81 abc
Swingle	0.74 abc
US-802	0.93 a
US-812	0.76 abc
US-897	0.81 abc
US-942	0.83 ab
US-1516	0.81 abc
<i>P</i>	<0.0001
Root order	
First	1.00 a
Second	0.76 b
Third	0.54 c
<i>P</i>	<0.0001
Disease state × rootstock	
<i>P</i>	0.0002
Disease state × root order	
<i>P</i>	0.7075
Rootstock × root order	
<i>P</i>	0.9646
Disease state × rootstock × root order	
<i>P</i>	0.9915

Different letters within columns indicate significant differences among rootstocks and root orders, respectively, according to Tukey’s honestly significant difference test.

HLT = healthy, INF = infected, RD = reduction.

Table 10. Root respiration rate ($\text{mg}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) of healthy and infected ‘Valencia’ trees grafted on different rootstocks.

	Ridge	Cleopatra	Sour orange	Carrizo	Swingle	US-802	US-812	US-897	US-942	US-1516
First-order roots										
HLT	0.9	1.0	1.6	1.1	1.0	1.1	1.0	1.3	1.2	1.2
INF	0.6	0.8	0.5	1.1	1.0	1.4	0.9	0.9	1.0	1.0
RD (%)	33%	17%	67%	4%	−3%	−19%	8%	29%	17%	17%
P	0.0582	0.1781	0.0014	0.3953	0.4042	0.208	0.2745	0.0398	0.2048	0.1734
Second-order roots										
HLT	0.7	0.7	0.8	0.8	0.7	0.9	0.8	1.0	0.9	0.9
INF	0.5	0.6	0.4	0.8	0.7	0.9	0.8	0.7	0.8	0.7
RD (%)	29%	22%	48%	−1%	−12%	1%	−3%	32%	5%	26%
P	0.0246	0.2	0.0024	0.4698	0.2728	0.4807	0.4137	0.0651	0.397	0.1728
Third-order roots										
HLT	0.6	0.5	0.8	0.5	0.4	0.8	0.5	0.7	0.6	0.6
INF	0.4	0.4	0.3	0.6	0.5	0.7	0.6	0.5	0.5	0.5
RD (%)	42%	30%	61%	−10%	−21%	18%	−10%	23%	19%	4%
P	0.0219	0.0715	0.0002	0.3105	0.1868	0.1993	0.3381	0.1737	0.1997	0.4082
Total²										
HLT	0.7	0.8	0.9	0.8	0.7	1.0	0.8	1.0	0.9	0.9
INF	0.5	0.6	0.5	0.8	0.8	0.9	0.8	0.7	0.8	0.8
RD (%)	26%	30%	50%	0%	−13%	6%	0%	32%	14%	14%
P	0.0394	0.0072	<0.0001	0.4639	0.0475	0.4217	0.4859	0.0009	0.1796	0.1218

²Measured in combined root samples including first-, second-, and third-order roots.

HLT = healthy, INF = infected, RD = reduction.

together, this study demonstrates that controlled greenhouse studies are valuable for assessing citrus rootstock response to HLB before long-term field studies. However, field evaluations are essential to determine the true ability of a rootstock to improve tree health and productivity under high disease pressure in different production environments where secondary stresses interact with HLB.

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Supplemental Table 1. Large and total root biomass–disease state and rootstock interactions.

Factor	Large root biomass (g)	Total root biomass (g)
Disease state × rootstock		
Healthy sour orange	100 abc	241 a
Healthy Ridge	100 ab	233 ab
Healthy Cleopatra	106 a	227 abc
Healthy US-802	80 abcde	204 abcd
Healthy US-812	30 f	199 abcde
Healthy Carrizo	87 abc	190 abcdef
Healthy Swingle	66 abcdef	186 abcdef
Healthy US-1516	51 cdef	143 abcdef
Healthy US-897	52 abcdef	141 abcdef
Infected US-1516	37 def	136 abcdef
Infected Swingle	32 ef	126 abcdef
Healthy US-942	63 abcdef	119 abcdef
Infected US-897	31 f	112 bcdef
Infected US-802	35 def	111 bcdef
Infected Cleopatra	33 ef	103 cdef
Infected US-812	83 abcd	95 def
Infected US-942	29 f	84 def
Infected Ridge	22 f	76 ef
Infected Carrizo	32 f	76 ef
Infected sour orange	34 ef	73 f
<i>P</i>	0.0094	0.0138

Different letters within columns indicate significant differences among treatments according to Tukey's honestly significant difference test

Supplemental Table 2. Root respiration–disease state and rootstock interactions.

Factor	Root respiration rate (mg g ⁻¹ ·h ⁻¹)
Disease state × rootstock	
Healthy US-897	0.98 a
Healthy US-802	0.96 ab
Healthy sour orange	0.93 ab
Infected US-802	0.90 ab
Healthy US-942	0.90 ab
Healthy US-1516	0.88 ab
Healthy Carrizo	0.81 abc
Infected Carrizo	0.81 abc
Healthy Cleopatra	0.79 abc
Infected Swingle	0.78 abc
Infected US-942	0.77 abc
Infected US-1516	0.76 abcd
Healthy US-812	0.76 abcd
Infected US-812	0.76 abcd
Healthy Ridge	0.74 abcd
Healthy Swingle	0.69 abcd
Infected US-897	0.67 bcd
Infected Cleopatra	0.55 cd
Infected Ridge	0.54 cd
Infected sour orange	0.47 d
<i>P</i>	0.0002

Different letters within columns indicate significant differences among treatments according to Tukey's honestly significant difference test.