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# Differential anatomical responses of tolerant and susceptible citrus species to the infection of '*Candidatus* Liberibacter asiaticus'

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ABSTRACT

Citrus Huanglongbing (HLB) is becoming the most devastating citrus disease worldwide. Although no known HLB-resistant citrus species or varieties have been identified, some citrus accessions such as rough lemon are reportedly tolerant. To better understand the HLB tolerance or susceptibility mechanisms in citrus, comparative anatomical analyses of tolerant rough lemon and sensitive sweet orange seedlings in response to HLB-associated bacterium, '*Candidatus* Liberibacter asiaticus', were performed on leaf, stem and root tissues using light microscopy and transmission electron microscopy. Phloem collapse, plugged sieve elements and accumulation of starch were observed in leaf petioles of symptomatic leaves from both HLB-diseased rough lemon and sweet orange, while not in the mock-inoculated controls. Interestingly, in symptomless leaves, significant anatomical changes (e.g. phloem cell collapse and starch accumulation) were found in HLB-diseased sweet orange, but not in rough lemon. Furthermore, starch depletion, phloem cell collapse and absence of phloem fibers were observed in secondary roots of only diseased sweet orange. In young green stems, a few plugged sieve elements were seen in both diseased rough lemon and sweet orange; whereas starch deposition only occurred in the latter. Taken together at the whole plant level, HLB infection induces fewer disruptive anatomical changes in rough lemon than in sweet orange. In particular, the absence of obvious changes in the rough lemon root system is suggested to be critical for sustaining plant growth after infection, and may contribute greatly to its HLB tolerance.



## 1. Introduction

The citrus industry in Florida (USA) and many other citrus-producing countries worldwide is being threatened by Huanglongbing (HLB, or citrus greening). HLB is presumably caused by '*Candidatus* Liberibacter spp.', a gram-negative phloem-limited α-Proteobacteria transmitted by the phloem-feeding Asian citrus psyllid (ACP), *Diaphorina citri*. There are three known HLB-associated *Ca.* L. species, namely *Ca.* L. asiaticus (CLas), *Ca.* L. africanus and *Ca.* L. americanus [1]. CLas, causing the most devastating HLB, was first found in Florida in 2005.

HLB-affected citrus plants often display typical symptoms such as yellow shoots and leaf blotchy mottle. Fruits on affected trees may be abnormally small, lopsided with color inversion or/and have aborted seed. Previously, Schneider [2] used light microscopy and observed massive starch accumulation, disruption of chloroplasts and phloem collapse in leaves of HLB-affected sweet orange. He suggested that the phloem block may impair the transport of photoassimilate, in turn leading to starch accumulation in diseased leaves, and leaf yellowing or mottling symptom [2]. Etxeberria et al. [3] investigated the distribution of starch throughout HLB-affected sweet orange plants using microscopy. They found that more starch accumulated in all aerial tissues from HLB-affected plants than those from HLB-negative control plants; by contrast, starch was depleted in roots of diseased trees while substantial starch deposits were observed in control ones. It is likely that the carbohydrate partitioning imbalance throughout the diseased tree may cause root death and eventually tree decline [3]. Achor et al. [4] proposed a sequence of HLB symptom development, i.e. phloem plugging and collapse followed by sugar backup, prior to starch accumulation in leaves with resulting chlorosis. Two types of phloem plugging materials, amorphous callose and filamentous phloem protein 2 (PP2), were identified in sieve elements of HLB-diseased citrus leaves [4,5].

*Abbreviations:* CLas, '*Candidatus* Liberibacter asiaticus'; HLB, Huanglongbing; LM, light microscopy; SEM, transmission electron microscopy.

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To date, no known citrus species, varieties or combinations of scion and rootstock have been identified to be resistant to HLB. However, host response and symptom development are different among citrus genotypes in the greenhouse and field [6,7]. In general, sweet oranges and grapefruits are very sensitive; by contrast, some lemons, limes and trifoliate orange (*Poncirus trifoliata* L. Raf.) possess some levels of tolerance, showing less severe symptoms and much slower (or no) decline [6]. To understand the mechanism of HLB pathogenesis, much work has been done on sweet orange to reveal the changes of metabolites, gene expression profiles and anatomical structures upon *C*Las infection [3–5,8–14].

Despite the reports on transcriptional and metabolomic responses of tolerant and susceptible citrus to the infection of *C*Las [15,16], the HLB tolerance and susceptibility mechanisms are still not well known. In this study, the anatomical changes of rough lemon (tolerant) and sweet orange (sensitive) at whole plant level in response to *C*Las under controlled conditions in the greenhouse were compared. The results will contribute to a more complete understanding of HLB tolerance and susceptibility mechanisms in citrus.

## 2. Materials and methods

### 2.1. Plant materials

Two-year-old seedlings of rough lemon (*Citrus jambhiri* Lush.) and 'Madam Vinous' sweet orange (*Citrus sinensis* L. Osbeck) were graft-inoculated with bud-wood from *C*Las-infected Carrizo citrange (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.), and control plants were grafted with bud-wood from pathogen-free Carrizo. All plants were kept in a U.S. Department of Agriculture-APHIS/CDC-approved and secured greenhouse at University of Florida, Citrus Research and Education Center, Lake Alfred, FL. Quantitative real-time PCR was performed to confirm the presence of *C*Las [17]. One year after inoculation representing an advanced stage of HLB disease, *C*Las-inoculated sweet orange plants displayed severe symptoms such as leaf blotchy mottle and yellowing, and tree decline; although obvious leaf mottling symptom was observed, *C*Las-inoculated rough lemon plants continued flushing without stunted growth. Multiple leaf, young green stem and secondary root samples were collected from at least three individual plants of *C*Las- or mock-inoculated rough lemon or sweet orange. Leaves with or without HLB symptoms were sampled separately from *C*Las-inoculated plants.

### 2.2. Light microscopy (LM) and transmission electron microcopy (TEM)

Leaf petioles, stems and roots were collected from citrus plants in the greenhouse, and immediately cut into pieces approximately 2–3 mm square and fixed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2) for 4 h at room temperature, or overnight in the refrigerator. As described by Etxeberria et al. [3], the fixed samples were then post-fixed in 2% osmium tetroxide for 4 h at room temperature, dehydrated in an acetone series and embedded in Spurr's resin Ref. [18] with a modified formula (10 ml ERL 4221, 25 ml NSA, 6.5 ml DER 736, 0.3 ml DMAE). LM and TEM sections were prepared and stained as described [3]. Briefly, 1 μm sections were cut with glass knives and stained with methylene blue/azure A and basic fuchsin for LM [19]. A Leitz Laborlux S compound microscope (Germany) attached with a Canon Powershot S31S digital camera (Tokyo, Japan) was used to take LM micrographs. For TEM, 100 nm sections were cut with a diamond knife, stained with 2% aq. uranyl acetate and poststained with lead citrate [19]. An AMT (Advanced Microscopy Techniques Corp., Danvers, MA) digital camera on a Morgagni 268 (FEI Company, Hillsboro, OR) transmission electron microscope was used to generate TEM micrographs. Since multiple sections sampled from at least three individual plants were carefully examined, sampling error leading to differential microscopic responses between rough lemon and sweet orange has been reduced to a minimum.

## 3. Results

### 3.1. Differential microscopic changes occurred between symptomless leaves of HLB-diseased rough lemon and sweet orange

To find out why HLB-affected rough lemon can sustain growth albeit typical leaf symptoms can be observed as in HLB-affected sweet orange, the anatomical changes within leaf petioles from HLB-affected symptomless and symptomatic leaves were compared with mock-inoculated controls. In diseased rough lemon, large quantities of starch accumulated in pith, cortex, and xylem cells of leaf petioles from symptomatic leaves (Fig. 1C and F). Phloem plugging material presumed to be callose (blue arrows in Fig. 1F and its left-lower inset) and excessive phloem formation (Fig. 1C and F) were also observed in these samples. Interestingly, no obvious changes were seen in petioles of symptomless leaves (Fig. 1B and E), except very few plugged phloem cells (Fig. 1E, blue arrows). By contrast, petioles of symptomless leaves from diseased sweet orange displayed dramatic anatomical changes, that included starch accumulation in pith, xylem parenchyma and phloem parenchyma (Fig. 1H and K), phloem plugging (Fig. 1K) and thickened phloem tissue (Fig. 1H and K). More severe microscopic disorders were found in symptomatic leaves of sweet orange (Fig. 1I and L), especially starch deposition in phloem parenchyma and phloem distortion and excessive formation (Fig. 1L), which are consistent with previous microscopic results [2,3,5]. TEM micrographs displaying the phloem area further confirmed that callose-plugged sieve elements, collapsed phloem (asterisks in Fig. 2B, C, E, F) and starch deposition in phloem parenchyma were commonly found in leaf petioles of diseased rough lemon (Fig. 2B and C) and sweet orange (Fig. 2E and F).

### 3.2. Differential anatomical changes in stems and roots of HLB-diseased rough lemon and sweet orange

Upon infection and disease progression, the microscopic structure of stems underwent obvious changes in both HLB-affected rough lemon (Fig. 3B) and sweet orange (Fig. 3F) when compared with their mock-inoculated controls (Fig. 3A and E, respectively). A few plugged phloem sieve elements were observed in young green stems of diseased rough lemon (Fig. 3B, blue arrows), while a number of starch granules formed in xylem and pith cells of diseased sweet orange (Fig. 3F, yellow arrows). Under TEM, small starch granules were found in some sieve elements of mock-inoculated rough lemon stems (Fig. 4A), whilst callose-plugged sieve elements were displayed in diseased stems (Fig. 4B). In the phloem area of sweet orange stems, no significant change was detected in either control or diseased samples (Fig. 4E and F).

In healthy rough lemon or sweet orange, a large amount of starch was stored in roots (Fig. 3C and G, yellow arrows). Surprisingly, remarkable starch depletion was observed in xylem and phloem cells of diseased sweet orange roots, although there were some starch granules in cortex cells (Fig. 3H). Furthermore, phloem appeared collapsed and no well-organized phloem fibers were observed in these samples (Fig. 3H). Stored starch was occasionally found in phloem and xylem parenchyma cells of root samples from diseased sweet orange (data not shown), but collapsed phloem cells were commonly detected (Fig. 3H). It is suggested that root phloem



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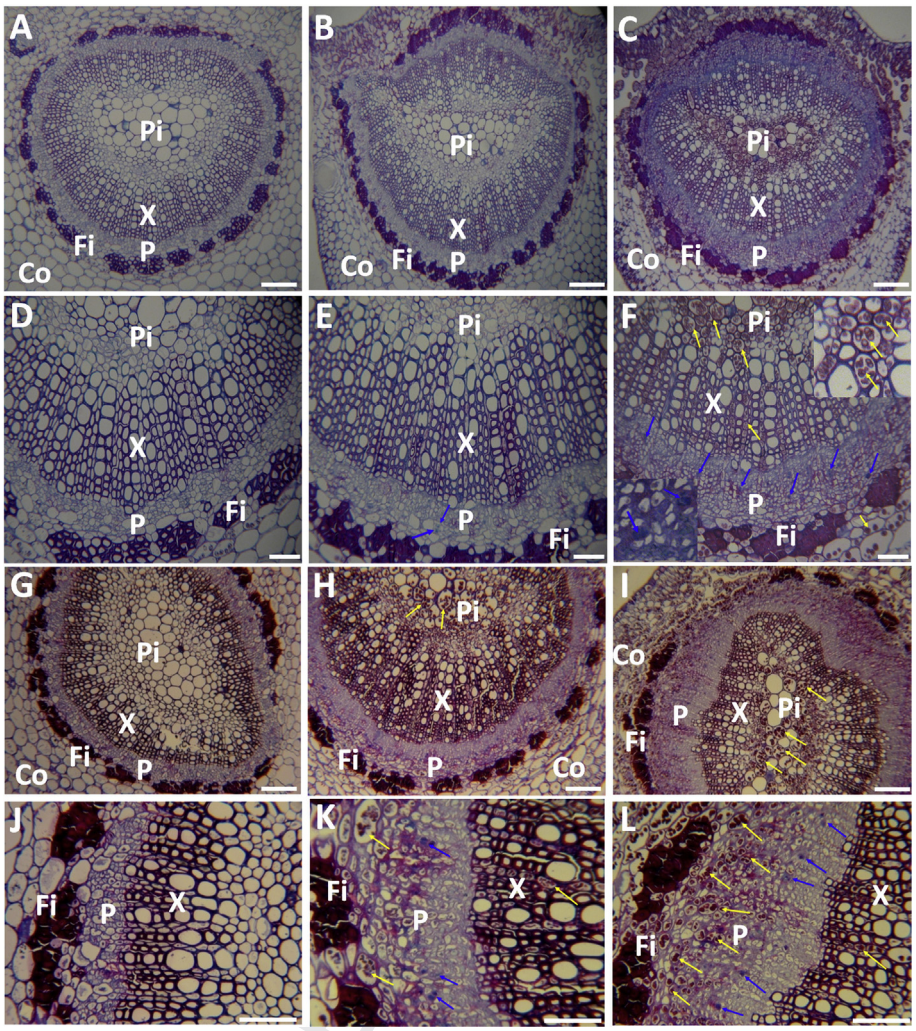


**Fig. 1.** Light micrographs of cross sections of leaf midribs from rough lemon and sweet orange. (A), (B), (C) represent midribs from rough lemon healthy control leaves, HLB-diseased symptomless leaves, symptomatic leaves, respectively. (D), (E), (F) are close-ups of (A), (B), (C) respectively. (G), (H), (I) represent midribs from sweet orange healthy control leaves, HLB-diseased symptomless leaves, symptomatic leaves, respectively. (J), (K), (L) are close-ups of (G), (H), (I) respectively. The right-upper and left-lower inset in (F) indicates starch deposition in pith parenchyma cells and plugging material in phloem sieve elements, respectively. Co, cortex; Fi, fiber; P, phloem; Pi, pith; X, xylem. Non-lignified/cellulose cell walls were stained red or purple, cytoplasm blue, and starch granules red. The blue spots indicated by blue arrows represent phloem plugging. The red granules indicated by yellow arrows represent starch. Bars = 100 μm (A–C, G–L) and 50 μm (D–F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sieve element and companion cell collapse is prior to starch depletion in the disease progression of *C*Las-infected sweet orange. By contrast, no obvious changes were seen in LM micrographs of diseased rough lemon roots, and stored starch was mainly remained in xylem (Fig. 3D, yellow arrows). TEM observations showed that some starch granules accumulated in phloem parenchyma cells of both control roots (rough lemon and sweet orange) and diseased rough lemon roots (Fig. 4C, D, G), but not in diseased sweet orange roots (Fig. 4H). In addition, distorted sieve elements were found in diseased sweet orange samples (Fig. 4H, asterisks), and swelling of middle lamella between cell walls surrounding sieve elements were sometimes detected in disease rough lemon (Fig. 4D, triangles).

Furthermore, the root systems of rough lemon and sweet orange seedlings were examined (Fig. 5). Compared with the vigorous root system of mock-inoculated sweet orange seedlings (Fig. 5C), the secondary roots of HLB-diseased sweet orange were partially rotted (Fig. 5D) or completely degraded (Fig. 5E). By contrast, no obvious root rot or degradation was found in HLB-affected rough lemon seedlings (Fig. 5B). Together with the anatomical results, it is suggested that starch depletion and phloem cell collapse in secondary roots (Fig. 3H) are closely related to the severe damage of root system in HLB-diseased sweet orange seedlings (Fig. 5D and E).

## 4. Discussion

The putative causal agent of HLB, *C*Las, is a phloem-inhabiting gram-negative bacterium, of which the nature of pathogenicity remains unclear. Substantial evidence indicates that phloem necrosis and phloem plugging often occur in HLB-diseased citrus plants [2,4,5], which likely leads to the impairment of photoassimilate transport from source organs (i.e. mature leaves) to sink organs (e.g. roots), in turn resulting in metabolism imbalance and tree decline [3,20]. However, different citrus species and varieties exhibit varied sensitivities to *C*Las infection [6]. Once infected, sweet orange plants display severe leaf symptoms, stunted growth and tree decline; whereas, rough lemon can continue flushing without obvious growth inhibition, although leaf symptoms can be expressed. It was then hypothesized that the phloem system in diseased rough lemon seedlings may be less affected than

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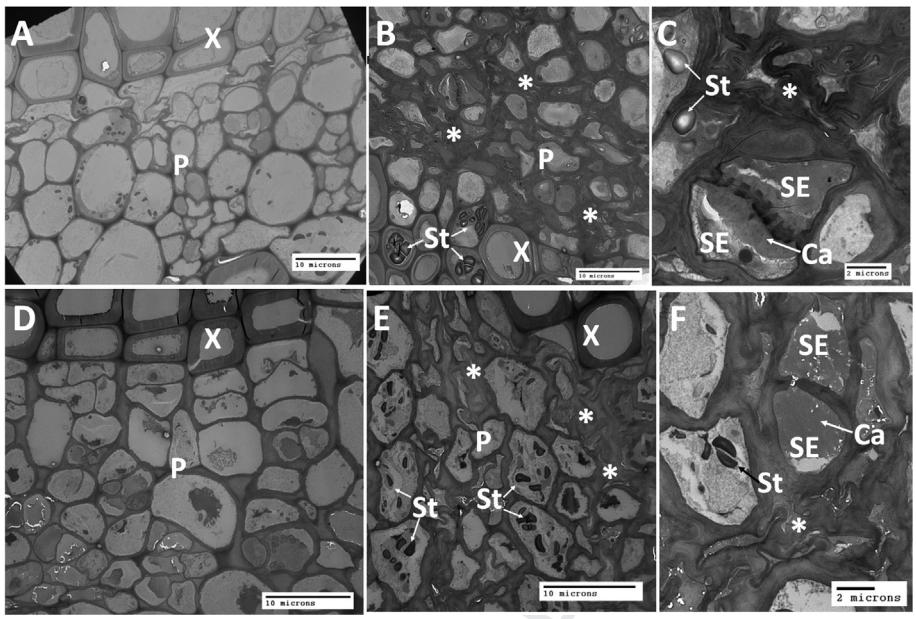


**Fig. 2.** Transmission electron micrographs of cross sections of leaf midribs from rough lemon and sweet orange displaying the phloem area. Phloem cells were well-organized in healthy control samples of rough lemon (A) and sweet orange (D). Collapsed phloem cells, plugged sieve elements and starch accumulation were commonly found in HLB-diseased symptomatic leaf samples of rough lemon (B, C) and sweet orange (E, F). Ca, callose (presumed); SE, sieve element; St, starch; P, phloem; X, xylem; *, phloem cell collapse. Bars = 10 μm (A, B, D, E) and 2 μm (C, F).

in diseased sweet orange seedlings. In this work, differential anatomical changes of rough lemon and sweet orange infected with CLas were revealed (Figs. 1 and 2).

No significant anatomical change was detected in leaf petioles of symptomless leaves from HLB-affected rough lemon (Fig. 1B and E), compared with mock-inoculated controls (Fig. 1A and D). It is suggested that these leaves may still carry out normal functions as healthy leaves, such as transport of photoassimilates in the phloem from source leaves to other plant parts. This implication is supported by a recent work that functional phloem transportation was observed in midribs of HLB-diseased rough lemon leaves, surprisingly including symptomatic leaves [21]. By contrast, leaf petioles of symptomatic and some symptomless leaves in sweet orange underwent dramatic phloem plugging and phloem cell collapse (Fig. 1H, I, K, L), resulting in the inhibition of phloem transport [21]. Consequently, the carbohydrate partitioning in the whole plant could be impaired in diseased sweet orange [3,8], causing more severe damages than in diseased rough lemon.

Intriguingly, starch depletion and phloem cellcollapse were observed in secondary roots from HLB-diseased sweet orange (Fig. 3H) but not in those from diseased rough lemon (Fig. 3D). Roots in healthy plants are important repositories for carbohydrates, usually in the form of starch granules, providing energy for plants surviving stress and dormancy [22]. Depletion of starch in roots could lead to root death and ultimately to tree decline, which has been observed in HLB-diseased sweet orange trees grown on

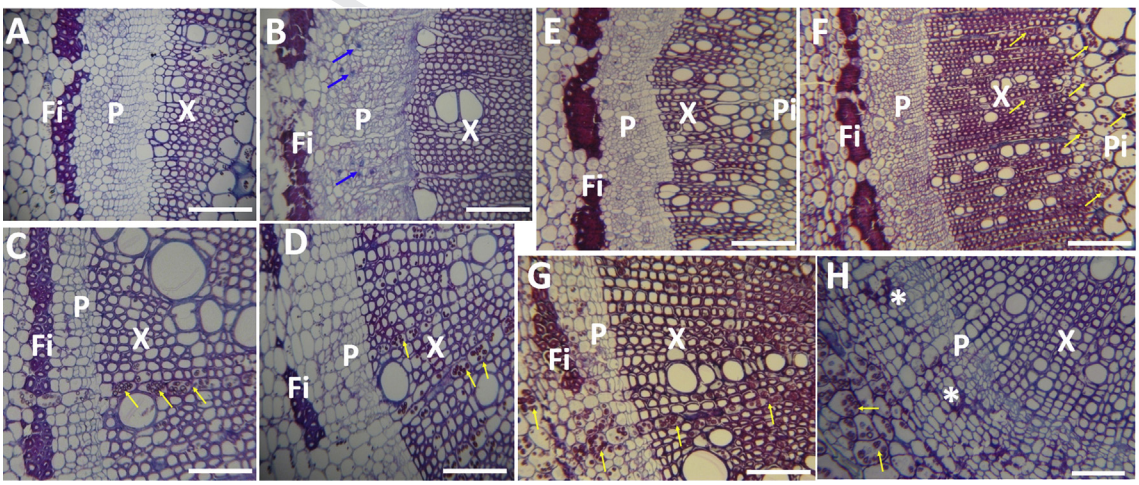


**Fig. 3.** Light micrographs of cross sections of stems and roots from rough lemon (A, B, C, D) and sweet orange (E, F, G, H). Young (green) stems were sampled from mock-inoculated controls (A, E) and HLB-diseased plants (B, F); secondary roots were sampled from mock-inoculated controls (C, G) and HLB-diseased plants (D, H). Fi, fiber; P, phloem; X, xylem; Pi, pith; *, degraded phloem cells. Non-lignified/cellulose cell walls were stained red or purple, cytoplasm blue, and starch granules red. The blue spots indicated by blue arrows represent phloem plugging. The red granules indicated by yellow arrows represent starch. Bars = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

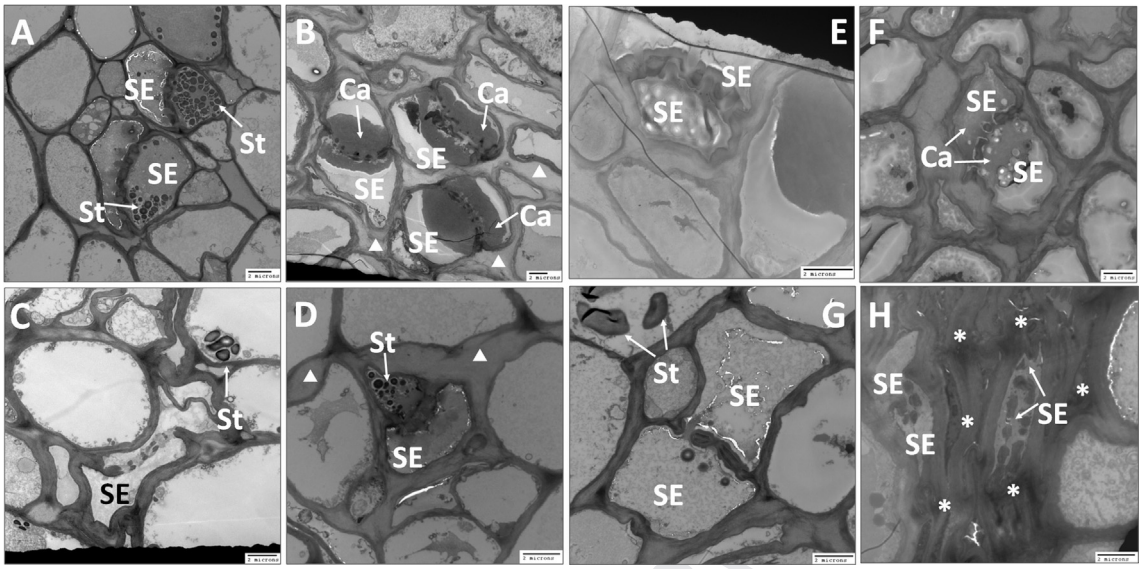


**Fig. 4.** Transmission electron micrographs of cross sections of stems and roots from rough lemon A, B, C, D) and sweet orange (E, F, G, H) displaying the phloem area. Young (green) stems were sampled from mock-inoculated control (A, E) and HLB-diseased plants (B, F); secondary roots were sampled from mock-inoculated control (C, G) and HLB-diseased plants (D, H). Ca, callose; SE, sieve element; St, starch; *, collapsed phloem cells; white triangles indicate swollen middle lamella between cell walls surrounding sieve elements. Bars = 2 μm.

various rootstocks in the field. It is suggested that the root starch is consumed to maintain root metabolism when little carbohydrate is transported down from the source leaves due to impaired phloem transportation in HLB-diseased sweet orange [3,21]. However, it should be noted that the extent of starch depletion in diseased roots is associated with the stage of HLB infection. For instance, root starch may not be totally depleted in the infected plants before getting to the advanced stage of infection characterized by tree decline and root rot.

Root phloem fiber consists of sclerenchymatous cells and functions as the physical supporting structure of the root. The absence of well-organized phloem fiber could cause damage to the structure

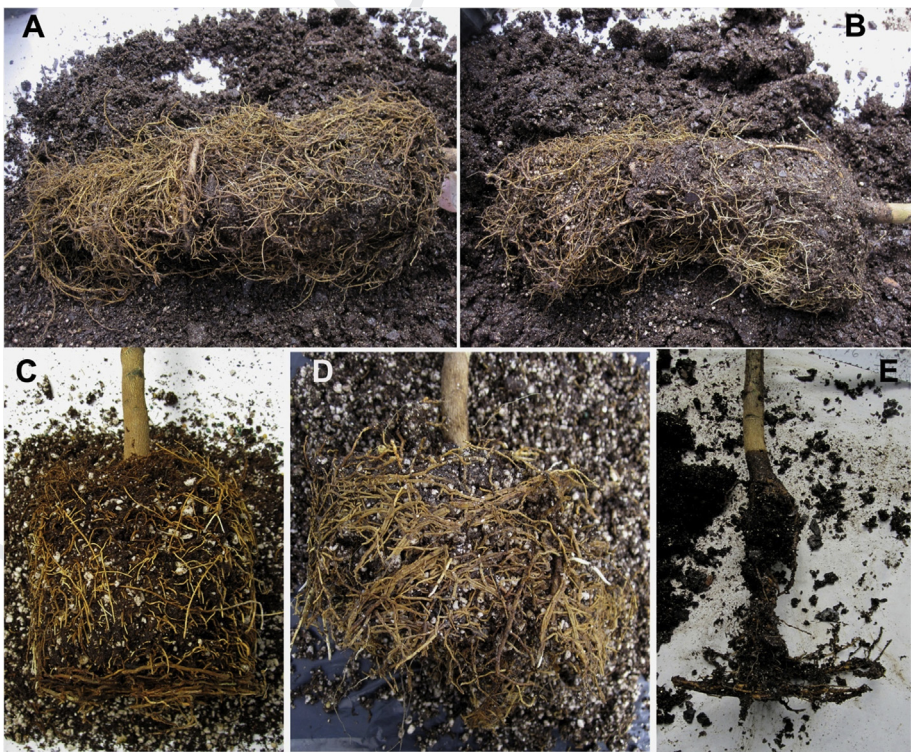


**Fig. 5.** Visual observation of the root system of rough lemon and sweet orange seedlings infected with *C*Las. The plants were pulled out from the pots one year after graft-inoculation of *C*Las, and their root systems were photographed. (A) and (B), roots of mock-inoculated and *C*Las-inoculated rough lemon, respectively. (C), roots of mock-inoculated sweet orange. (D) and (E), roots of CLas-inoculated sweet orange plants. Partially rotted (D) or completely degraded (E) roots were observed in diseased sweet orange, but not in diseased rough lemon (B).

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of roots, leading ultimately to the decline of the root system. Since the formation of phloem fiber requires a large amount of carbohydrates, high expression levels of sugar transporter genes are usually detected in root tissue; e.g. *Medicago truncatula MtSt1* (a sugar transporter gene) is highly expressed in root phloem fibers [23]. In HLB-diseased sweet orange, it is believed that it is difficult for adequate amounts of sugars to be translocated from leaves to roots due to the plugged phloem system, which may inhibit the development of phloem fibers in roots and eventually result in disruption of the root system (Fig. 5D and E). Conversely, the absence of starch depletion and phloem collapse in roots of HLB-diseased rough lemon suggests that the root system of rough lemon may be still functional. As observed visually in the greenhouse grown seedlings, the root system of infected rough lemon plants was comparable to that of mock-inoculated controls (Fig. 5A and B). Thus, the root metabolic activities in diseased rough lemon are likely to be sufficiently sustained to support plant growth. It has been reported that HLB can restructure the bacterial community associated with sweet orange roots and in the rhizosphere [24,25]. These results should be expected according to the findings in the present study that the root system of HLB-affected sweet orange seedlings underwent damage and death (Fig. 5D and E). However, it is uncertain whether the bacterial community associated with roots and rhizosphere of rough lemon seedlings is restructured by HLB, as no clear symptoms of root damage and root death were found in diseased rough lemon seedlings (Fig. 5B). The sustained vigorous root system of rough lemon upon *C*Las infection may be a critical factor of its tolerance to HLB disease.

In conclusion, comparative microscopy analysis of rough lemon and sweet orange in response to *C*Las infection indicates that much less phloem damage occur in rough lemon than in sweet orange, especially in leaf petioles of symptomless leaves and secondary roots. These differences likely contribute to HLB tolerance of rough lemon or susceptibility of sweet orange.

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