INTERACTION BETWEEN \textit{PHYTOPHTHORA NICOTIANAE} AND \textit{CANDIDATUS LIBERIBACTER ASIATICUS} DAMAGE TO CITRUS FIBROUS ROOTS

By

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To my family, for their love
To my advisors, for their spirits of passing on the knowledge
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To those who helped me through the 4 ½ years I stayed at the University of Florida to finish the Ph. D. program.

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<td>CitCINV</td>
<td>Citrus Cytoplasmic invertase</td>
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<td>CitSUS</td>
<td>Citrus sucrose synthase</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>HLB</td>
<td>Huanglongbing</td>
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<tr>
<td>Laf</td>
<td><em>Candidatus</em> Liberibacter africanus</td>
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<td>Lam</td>
<td><em>Candidatus</em> Liberibacter americanus</td>
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<td>Las</td>
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<td>P.n.</td>
<td><em>Phytophthora nicotianae</em></td>
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<td>qPCR</td>
<td>Quantitative polymerase chain reactions</td>
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<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SLA</td>
<td>Specific leaf area</td>
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<tr>
<td>spp</td>
<td>Species plural</td>
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<tr>
<td>SPS</td>
<td>Sucrose-phosphate-synthase</td>
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<tr>
<td>SUT</td>
<td>Sucrose transporter</td>
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<tr>
<td>wk</td>
<td>Week</td>
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<tr>
<td>wpi</td>
<td>Week post inoculation</td>
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<tr>
<td>βFruct</td>
<td>Beta-fructosidase</td>
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INTERACTION BETWEEN *PHYTOPHTHORA NICOTIANAE* AND *CANDIDATUS LIBERIBACTER ASIATICUS* DAMAGE TO CITRUS FIBROUS ROOTS

By

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Huanglongbing (HLB) in Florida is caused by the phloem-limited bacterium, *Candidatus Liberibacter asiaticus* (Las), which is transmitted by the psyllid vector, *Diaphorina citri*, or by grafting infected tissue from hosts of the pathogen. *Phytophthora nicotianae* (*P.n.*) incites soil-borne diseases of citrus worldwide which reduces water and nutrient uptake and depletes carbohydrate reserves in citrus fibrous roots. Rapid yield loss of sweet orange trees at a low level of HLB canopy symptom expression was observed in Brazil. Meanwhile, unprecedented changes in population of the soil borne pathogen *Phytophthora* in citrus groves were documented as HLB spread throughout Florida. These findings led to the investigation and discovery that damage to fibrous roots was occurring before or at the same time as canopy symptoms developed and the root damage was caused by Las, and the interaction with soil-borne pathogen *P.n.*

In this study, the interaction between Las and *P.n.* was investigated. The results showed that 1) Las infection of citrus rootstocks predisposes fibrous roots to *P.n.* infection by increasing root leakage of exudates that attract zoospores and by disrupting host physiology that mediates the plant response to *P.n.*; 2) the roots of HLB seedlings
were damaged by Las and the combination of Las and \textit{P.n.}, and the root damage caused by co-inoculation was not greater than the damage from each pathogen alone.

Further investigation of root and shoot morphological changes caused by Las and \textit{P.n.} showed that: 1) Las damaged citrus roots by inducing faster root growth and turnover; 2) \textit{P.n.} damaged fibrous roots by causing rapid root collapse immediately after infection; 3) canopy development was reduced after root damage by both pathogens.

To explore disease development from the perspective of root carbohydrate metabolism responses to Las and \textit{P.n.} infection, sucrose metabolism related gene expression was investigated in two rootstocks (Cleopatra mandarin, susceptible to \textit{P.n.}; Swingle citrumelo, less susceptible to \textit{P.n.}). The results showed that sucrose metabolism was more disrupted by Las and \textit{P.n.} in \textit{P.n.} susceptible Cleopatra mandarin than in \textit{P.n.} tolerant Swingle citrumelo.
CHAPTER 1
LITERATURE REVIEW

1.1 Citrus Huanglongbing

Citrus is a worldwide fruit crop in tropical and subtropical regions, where favorable climate promotes tree vigor and production. These regions range approximately ±40°latitude of the equator in all six continents (Gottwald, 2010). The main commercial varieties include sweet orange, tangerine/mandarin, lemon/lime and grapefruit. Common varieties belong to three genera, *Citrus*, *Poncirus* and *Fortunella*, of tribe *Citreae*, plant family *Rutaceae* comprising 140 genera and 1300 species (Gottwald, 2010). In 2014, there were 515,147 grove acres, 68 million trees and 124 million boxes harvested in Florida, according to U.S. Department of Agriculture data. Citrus production is influenced by weather, soil, water, pest, and disease.

Huanglongbing (HLB) is one of the most devastating diseases of citrus known (Reinking, 1919), which causes catastrophic losses to affected citrus industries (Bassanezi et al., 2011; Roistacher, 1996). Typical symptoms of HLB include pre-symptomatic root decline, small and blotchy mottled leaves, dieback of shoots, uneven colored, acidic, bitter fruit and premature fruit drop (Capoor, 1963; Johnson et al., 2014). *Candidatus Liberibacter* spp. phloem-limited bacteria, which cause HLB (Jagoueix et al., 1994; Teixeira et al., 2005), are transmitted from tree to tree over short distance by the psyllid vector and over long distance by contaminated budwood and human activity (Garnier and Bové, 1983; Gottwald, 2010; Halbert and Manjunath, 2004; Ramadugu et al., 2008). The earliest record of HLB-like disease was in India in 1700 (Gottwald, 2010), followed by reports of HLB-like symptoms in other countries in Asia and Africa (Bové, 2006; Gottwald, 2010). HLB was first reported in Brazil, Florida, and Cuba in
2004, 2005 and 2008 respectively (Halbert, 2005; Luis et al., 2009; Teixeira et al., 2005). Since then HLB has spread to several other countries in the western hemisphere.

The bacterium that causes HLB belong to the alpha-subdivision of Proteobacteria based on their 16s rDNA sequence (Jagoueix et al., 1994). They are *Candidatus Liberibacter asiaticus* (Las), *Candidatus Liberibacter africanus* (Laf), and *Candidatus Liberibacter americanus* (Lam). Las is distributed throughout the humid subtropics in Asia and the Americas area, Laf exists in Southern Africa and Lam is restricted to Sao Paulo State, Brazil, but has recently been displaced by Las (Bové et al., 2008; Lopes et al., 2009). The leaf-hopper vectors that feed on phloem and transmit Liberibacter spp. are Asian Citrus Psyllid (ACP), *Diaphorina citri*, in Asia and the Americas, and African Citrus Psyllid, *Trioza erytreae*, in Africa. Due to the inability to culture Liberibacter spp., disease etiology has not been completely demonstrated.

Based on the symptoms developmental status and plant growth situation, sweet oranges, grapefruit, mandarins and tangelos are defined as susceptible, lemons and limes are field tolerant, while citranges and *Poncirus trifoliata* are the most tolerant (Folimonova et al., 2009). The variety’s susceptibility to HLB varies for different Las strains (Tsai et al., 2008).

HLB management requires a three pronged program of vector control, scouting and removal of diseased trees, and planting of pathogen-free nursery stock (Belasque et al., 2010; Grafton-Cardwell et al., 2013).
1.1.1 HLB History

Huanglongbing (hung-long-龙bing-病) is the Chinese name for this disease that means yellow dragon disease. HLB was named by a local grower and adopted by Lin in his work (Bové, 2006). Before the official name for the disease was accepted at the 13th Conference of the International Organization of Citrus Virologists (IOCV) in Fuzhou, China, the disease was most often referred to as citrus greening (Bové, 2006). The first description of the disease worldwide was in early 20th century with different names in different countries without gaining much attention (Bové, 2006). In the mid-20th century, the disease became a major problem in several Southeast Asian countries and extensive research was conducted in different countries on identification of the causal agent, potential vectors and comparison to diseases with similar symptoms (Martinez and Wallace, 1967; McClean and Oberholzer, 1965a; Salibe and Cortez, 1968; Schneider, 1968; Schwarz et al., 1973). Without specific disease symptoms, the disease was considered to be caused by mineral deficiency, soil disorders or virus infection (Bové, 2006; Capoor, 1963). With well-designed experiments, Lin (1956) proved the graft-transmissibility of the disease, which distinguished the cause of the disease symptoms from abiotic factors. Likewise, McClean and Oberholzer (1965b) demonstrated 10 years later that citrus greening is graft transmitted. Confirmation that the disease symptoms were caused by an infectious agent led to acceptance by IOCV of HLB as the official name for the disease. Another important finding by scientists in mid-20th century was transmission of HLB by psyllid vectors. In the 1980s, Garnier et al. (1984) demonstrated that the HLB causal agent is a Gram-negative bacterium, which was further confirmed in the 1990s by 16S rDNA sequence analysis (Jagoueix et al.,
1994). After more extensive genome sequencing, two species Las and Laf were classified. In 2004, a third species of Liberibacter infecting citrus and Murraya, Lam, was identified in Brazil (Lopes et al., 2009b).

1.1.2 Geographic Distribution

There are very good reviews that identify all the countries and areas that are infested by the psyllid and affected by HLB (Bové, 2014; Bové, 2006; Gottwald, 2010; Halbert and Manjunath, 2004). It is noteworthy that the vector always spreads into a new area before HLB is detected. In general, the areas that are still free of HLB are the Mediterranean basin, most of Western Asia, Australia, and the North and South Pacific islands. Psyllid management becomes an important step to control HLB spread. More information is available in the cited reviews.

1.1.3 Causal Agent and Vectors

Las and Lam are naturally transmitted by D. citri in Asia and the Americas and Laf is vectored by Trioza erytreae in Africa (Bové, 2006). Las and D. citri are both heat tolerant, as indicated by the worldwide disease distribution (Bové, 2013). The same vector distribution pattern as the disease indicates that the spread of Las is limited by vector movement (Zhao, 1981). In Reunion Island, severe HLB and D. citri occur in hot low altitude areas but not in high altitude locations (Bové, 2014). In contrast, Laf and T. erytreae are heat sensitive and do not occur below an altitude of approximately 600m (Bové, 2014). This finding was confirmed by a greenhouse study on the effect of temperature on HLB symptom expression. Plants infected with Laf showed severe symptoms below 27 °C at 30 weeks after inoculation, and symptomless growth was obtained after plants were transferred to a warm chamber (27/32°C) (Bové, 2006). By
comparison, no significant difference in temperature effect on symptom expression was
detected for Las. Lam is also heat sensitive, but less so than Laf (Lopes et al., 2009b).

The inability to culture Las made obtaining the complete DNA genome sequence
challenging. Duan et al. (2009) were the first to report sequencing of Las (GenBank
NC_012985.2). Genes involved in cell motility and active transport account for 4.5% and
8.0% of the total genes respectively, which might contribute to its virulence. Genes
involved in pathogenicity, such as type III and type IV secretion system components
were not found in this pathogen. Duan et al. (2009) suggested Las is a parasite rather
than pathogen of citrus. Las is predicted to be able to metabolize sugars like glucose,
fructose, xylulose and amino acid as energy supply based on the key enzymes it
encodes (Duan et al., 2009). Furthermore, evidence for a near complete set of glycolytic
enzymes was found in the gene sequence, which suggests that the pathogen is likely to
utilize carbon-glucose for its energy supply (Wang and Trivedi, 2013). Subsequent
research revealed that Las carries an excision plasmid prophage and a chromosomally
integrated prophage that becomes lytic in periwinkle, but the lytic stage of the prophage
was not observed in citrus (Wang and Trivedi, 2013; Zhang et al., 2011).

1.1.4 Psyllid Biology

In total, there are 13 different psyllid species occurring on citrus and citrus
relatives (Halbert and Manjunath, 2004). Asian Citrus Psyllid (ACP) in Asia and
Americas, and African Citrus Psyllid in Africa are the two known vectors that transmit
HLB-associated pathogens. The biology of *D. citri* was recently reviewed (Grafton-
Cardwell et al., 2013; Halbert and Manjunath, 2004). Typically, the life cycle ranges
from 15 to 47 days, with 2 to 4 days of egg hatching and 11 to 15 days for completion of
five instar stages. The duration of the cycle is influenced by temperature and humidity.
The temperature that allows for oviposition was estimated to range from 16°C to 41.6°C (Grafton-Cardwell et al., 2013), and temperature extreme for survival is -6°C. *D. citri* dispersal and transmission efficiency are two crucial factors in HLB spread. The investigations on *D. citri* flight showed that it is capable of moving 100 m within 3 days (Boina et al., 2009), at some times up to 400 m within 4 days (Tiwari et al., 2010), with the maximum dispersal distance of 2 km within 12 days (Lewis-Rosenblum, 2011), which is similar to the maximal distance for movement of the Africa citrus psyllid (Grafton-Cardwell et al., 2013). The peak of *D. citri* movement appears to occur after citrus spring flush.

Knowledge of the location of Las in the psyllid is required to evaluate the transmission efficiency of Las by *D. citri* (Grafton-Cardwell et al., 2013). A systemic presence of the bacterium within psyllids was reported using qPCR (Inoue et al., 2009), scanning electron microscopy, and fluorescence in situ hybridization techniques (Ammar et al., 2011). The transmission process consists of three stages: acquisition access period (AAP): acquiring the pathogen during feeding; period of latency: time for bacteria to enter the salivary gland and multiply; inoculation access period (IAP): introducing bacteria into the plant by feeding (Grafton-Cardwell et al., 2013). It was reported that AAP and IAP occurred within 24 and 7 h respectively. Based on the symptom development, the latency period ranged between 1 and 25 days. Molecular investigation using conventional polymerase chain reaction (PCR) or real-time PCR (qPCR) detected highly variable acquisition efficiencies for Las by adult *D. citri*, ranging from 13% to 90% (Inoue et al., 2009; Pelz-Stelinski et al., 2010). Furthermore, transovarial and sexual transmission were reported at a low rate of 3.6% (Pelz-Stelinski
et al., 2010) and 2–3% (Mann et al., 2011) respectively or as non-detectable (Hung et al., 2004). Furthermore, persistence of Las in *D. citri* adults after acquisition declined if there was no more pathogen supplied (Pelz-Stelinski et al., 2010). In conclusion, transmission efficiency varies depending on life stage of pathogen acquisition, nymphs versus adults, incidence of infected *D. citri* (Pelz-Stelinski et al., 2010), and distribution of the bacterium in the tree (Grafton-Cardwell et al., 2013).

### 1.1.5 HLB Disease Development

Damage caused by Las to citrus leaves, fruit and phloem (Bassanezi et al., 2009; Etxeberria et al., 2009; Fan, 2010; Schneider, 1968, 1967) has been widely reported with respect to sampling and identification of visual symptoms. HLB cytopathology studies carried out on severely infected field leaves identified necrotic phloem, phloem sieve plate is blocked by callose deposition, and the phloem blockage is associated with massive accumulation of starch in plastids and phloem proliferation (Achor et al., 2010; Schneider, 1968, 1967). These combined effects account for the blotchy mottle symptoms in fully expanded leaves and the gradual dieback of infected shoots (Achor et al., 2010). Thus, phloem blockage induces imbalanced carbohydrate partitioning which is considered as the major cause of the disease (Kim et al, 2009; Koh et al., 2012).

Schneider (1986) theorized that high starch accumulation in leaves of HLB-infected trees is a secondary effect of phloem blockage by examining the correlation between starch accumulations and yellowing mottle symptom on girdled branches. The starch build-up can be up to 20 times higher in HLB leaves than in healthy (Takushi, 2007) and induce disintegration of the chloroplast thylakoid system which is the cause of blotchy mottled leaf. In another work, starch accumulation was cited to be the cause of tree decline instead of secondary damage from phloem blockage (Etxeberria et al.,
Etxeberria et al. (2009) found in leaves and petioles of HLB positive trees that photosynthetic cells, phloem elements, vascular parenchyma, xylem parenchyma, and phelloderm all had excessive starch. In contrast, roots from HLB-positive trees were depleted of starch compared to roots from control trees. Etxeberria (2009) hypothesized that HLB systemically affects citrus tree carbohydrate metabolism and “root starch is consumed to sustain root metabolic activities when little sugar is translocated down from the leaves resulting in root death and eventually tree decline”. Etxeberria (2009) qualified his findings by stating that this evaluation was carried out at advanced stage of symptom development and that the examination of an early stage of HLB expression is still needed.

Folimonova and Achor (2010) studied disease development in 8-month-old seedlings in the greenhouse beginning one month after graft-inoculation to monitor the progression of bacterial colonization, anatomical aberrations and their relationship with symptom development for 1 year. They detected 71% of the asymptomatic leaves are Las positive at 3 months after graft inoculation (presymptomatic stage) and observed slight yellowing of leaves by 5 to 6 months. At 9 months after inoculation, mature leaves were small, thickened and severely mottled. As symptoms developed, 60% of the seedlings didn’t produce new shoots and, on the remaining seedlings, the new shoots became chlorotic upon maturity. Most of the infected seedlings declined over time. At the presymptomatic stage of young fully expanded leaves, no significant phloem disruption occurred, but significant swelling of the middle lamella between cell walls surrounding sieve elements and more extensive deposits of amorphous callose in sieve element was observed. By 6 months after inoculation, severe collapse of sieve
elements and companion cells and, occasionally, cambium cells was observed in the most mature symptomatic leaves. Surprisingly, the bacteria number was high in asymptomatic young, fully developed leaves and low in the mature symptomatic leaf which contradicted assessment of bacterial titer with PCR. It is noteworthy that, PCR methods do not discriminate between viable and dead DNA (Josephson, 1993) and only TEM can be used to observe live bacteria. Hence, Folimonova and Achor (2010) hypothesized that the majority of the pathogen population in a newly growing shoot of an infected tree is present as live bacteria for a short time. Later, most of the bacteria are occluded in phloem and presumed to be dead in the symptomatic tissue. In their studies, starch accumulation, which is widely accepted as the cause of blotchy mottle leaves and damage from HLB, was not detected. Liao and Burns (2012) suggested that development of HLB symptoms may directly result from the host response rather than as a consequence of carbohydrate starvation, by comparing the symptoms of HLB infected fruit and girdled fruit. The inconsistent finding about the relationship between phloem condition and carbohydrate partitioning indicates another mechanism other than disrupted phloem function is involved in disease development (Wang and Trivedi, 2013).

To get an integrated view of disease development, a few studies (Sagaram et al., 2008; Tatineni et al., 2008) examined the bacterial location in symptomatic trees to track bacteria movement. Bové (2006) stated that HLB symptom usually starts at one branch, and progress to the rest of the canopy. This symptom development observation was supported by bacteria movement (Johnson et al., 2014; Tatineni et al., 2008). With the help of qPCR, Tatineni et al. (2008) found systemic movement of bacteria from the
infection site to the rest of the tree, which included bark tissue, leaf midrib, roots, and different floral and fruit parts, but not in endosperm or embryo. Johnson et al. (2014) monitored bacterial titer in leaves and roots of greenhouse trees after initial infection and identified fibrous roots as the initial site for bacterial colonization which served as a reservoir for subsequent Las movement by phloem up into the shoot coincident with foliar flush. After initial infection by Las, root damage occurred before starch was depleted in roots and symptoms developed above ground with the causal mechanism unknown. Unprecedented *Phytophthora* population changes in Florida citrus groves were first reported in 2011 (Graham et al., 2011) after survey and documentation of root loss and *Phytophthora* populations on mature trees, it was hypothesized that Las damages root directly and interacts with other soil borne pathogens to cause additional root damage (Graham et al., 2013).

In a susceptible host, symptom development is the result of molecular, cellular and physiological changes (Albrecht and Bowman, 2008). To defend themselves, plants activate a series of responses to pathogens, such as hypersensitive reaction, structural alterations, and synthesis of pathogenesis-related proteins and phytoalexins (Hammond-Kosack and Jones, 1996). The transcriptional response of citrus to Las infection provides molecular information for the pathogenic process of HLB and the interaction between plant and bacterial infection, which could aid development of novel strategies for HLB management. Gene expression in leaves, fruit, stems and roots has been reported (Albrecht and Bowman, 2008; Aritua et al., 2013; Kim et al., 2009; Martinelli et al., 2012). Albrecht and Bowman (2008) reported a significant accumulation of transcripts for a phloem-specific lectin PP2-like protein, which is responsible for
phloem blockage together with callose, 13–17 weeks after graft inoculation. The blockage of phloem by PP2 and callose are considered as plant defense to inhibit bacterium spread within plant. This was confirmed later by Kim et al. (2009). Kim also found that expression of 624 genes in total was significantly influenced by Las infection. Encoded proteins were grouped into 18 categories according to function, which included sugar metabolism, plant defense, phytohormone, cell wall metabolism and 14 other categories. Furthermore, the excessive starch accumulation was hypothesized to be due in part to the up-regulation of starch synthesis genes, such as ADP-glucose pyrophosphorylase (AGPase), starch synthase, granule-bound starch synthase (GBSS) and the starch debranching enzyme (SDE) (Kim et al., 2009). The over 10% up-regulation of plant defense (PR) genes in the host indicated an activation of the defense mechanism. Another study in fruit gene expression (Martinelli, 2012) showed that regulation of pathways involved in photosynthesis, source-sink communication, sucrose and starch metabolism, hormone synthesis and signaling were affected by Las infection. Aritua et al. (2013) reported the different gene expression in stem and roots of two-year-old Valencia sweet orange (C. sinensis) on Swingle citrumelo (C. paradisi Macf. × P. trifoliata) rootstock 16 months after graft inoculation. The transcription of genes encoding an ADP-glucose pyrophosphorylase large subunit 3 (APL3) and a granule-bound starch synthase (GBSS) was up-regulated in stem but not in root tissue. Common genes and pathways were also up-regulated in roots 50 days post inoculation with Las (Zhong et al., 2014).

1.1.6 Rootstocks Susceptibility and Tolerance to Las Infection

Based on detectable Las level, shoot mass reduction and symptom development, trifoliate hybrid rootstocks are classified as tolerant and Cleopatra mandarin as
susceptible to Las in greenhouse study (Albrecht and Bowman, 2012a). However, no difference of disease incidence among rootstocks was detected in field trials, and all rootstocks are considerably damaged (Albrecht et al., 2012). By comparing the leaves, stems and roots anatomical response to Las infection between susceptible (sweet orange) and tolerant (rough lemon) citrus species, Fan et al. (2013) observed fewer anatomical changes in root of rough lemon than sweet orange. Transcriptional and anatomical analysis of rough lemon and sweet orange leaves response to Las infection revealed lower phloem transport activity and slower plant defense reaction in Rough lemon (Fan et al., 2012).

1.1.7 HLB Detection

Historically, HLB was detected by field symptoms before the agent was confirmed by electron microscopy and DNA-based molecular detection methods (Bové, 2006; Halbert and Manjunath, 2004; Villechanoux et al., 1993). Because of the similarity between HLB symptom and disorders like zinc deficiency and other virus disease, and the long period of latency, field symptom expression is challenging for disease detection (Bové, 2006). Use of electron microscopy for detection was limited as it is time and labor consuming. After the advent of PCR, primers were developed for both Las and Laf (Hocquellet et al., 1997; Hocquellet et al., 1999; Hung et al., 2004; Hung et al., 1999; Teixeira et al., 2005b). Real-time PCR application improved sensitivity and stability of HLB detection compared to conventional PCR (Lin et al., 2010; Wang et al., 2006), and can be applied to quantify the titer of Las. With these benefits, qPCR is routinely used to detect Las, determine the disease status and pathogen distribution in trees and the insect vector (Kim et al., 2009; Lopes et al., 2009a; Lopes et al., 2009b; Pelz-Stelinski et al., 2010; Trivedi et al., 2009). The limitation of DNA-based methods is that they do not
distinguish live versus dead bacteria. To overcome this disadvantage, DNA-binding dyes, which can penetrate through membranes of dead cells more easily than live cells, was introduced into PCR protocols. Propidium iodide (PI), propidium monoazide (PMA) (Nocker et al., 2006), and ethidium monoazide (EMA) are three popular DNA-binding dyes. Compared to EMA, PMA is broadly used for its highly selective penetration of dead cells and less partial DNA loss (Nocker et al., 2006). Besides molecular methods, the iodine test has been used to detect starch accumulation in leaves for rapid detection with iodine in water to produce a darkish reaction in tissue (Chamberlain and Irey, 2008).

1.1.8 HLB Management

Efficient disease management should involve (i) preventing introduction of vector and pathogen; (ii) removing alternative hosts for the pathogen and vector; (iii) cultivation of tolerant citrus varieties; (iv) vector controls; (v) maintaining HLB tree health by providing good environmental condition for growth such as water and nutrient supply. For HLB free areas, spread of ACP needs to be monitored since the vector establishes in advance of the disease. Every effort needs to be taken to suppress the inoculum or the disease will increase exponentially to 100% incidence in as little as 5 years (Bové, 2006). When HLB was first detected in a region, the extent of disease spread needs to be surveyed. Usually, control of D. citri and T. erytreae are not well established since the direct damage by their feedings are minor (Halbert and Manjunath, 2004), which explained why HLB is spread so quickly after the first infected tree is found in the grove. To keep disease incidence low, stringent vector control and removal of infected trees are required using a 3 month tree inspection cycle to detect newly symptomatic trees
These methods are proven to decrease HLB incidence in Brazil (Belasque et al., 2010).

Environmental factors such as soil bicarbonate and high pH in irrigation water were also reported to reduce stress tolerance by increasing root loss caused by Las (Graham et al., 2014). A 20% reduction in root density was reported for a grove that was suffering from high bicarbonate stress compared to the grove that has had less bicarbonate stress. Acidification treatment of irrigation water or soil application of sulfur in prilled form was recommended to reduce bicarbonates in the rhizosphere which otherwise reduce uptake of Ca, Mg, and Fe. This acidification in the rhizosphere of soil may restore vigor and productivity of HLB-affected trees (Graham et al., 2014).

To date, no therapies have proven effective and HLB resistant citrus genotypes have not been found. Certain highly vigorous rootstocks, e.g. Volkamer lemon, may prolong productivity of younger trees after they are infected (Albrecht et al., 2012; Folimonova et al., 2009; Koizumi et al., 1993). Several disease control practices have been investigated to control HLB (Bové, 2006). Antimicrobial molecules have been screened to target the systemic Las (Zhang et al., 2012; Zhang et al., 2011a; Zhang et al., 2010a; Zhang et al., 2010b;). Without a disease resistant variety or curative methods available, vector control is the only short-term inoculum control option. A number of biological controls against the psyllid vector have been reported, including fungal entomopathogens, parasitoids and predators (Aubert et al., 1984; Aubert, 1990; Grafton-Cardwell et al., 2013). Insecticides are more effective when applied to new shoots in the winter when the population is low (Boina et al., 2010; Childers and Rogers,
1.2 *Phytophthora* spp. and Diseases

*Phytophthora*, eukaryotic fungus-like organisms, is a genus of Oomycete which is grouped with a variety of other protists within the Stramenopile cluster. They are organisms that “possess evenly spaced tripartite tubular hairs attached to the flagellum or other parts of the cell surface and species that have been derived from those organisms” (Adl et al., 2005, Judelson, 2005; Van de Peer et al., 1996). Oomycetes were classified with true fungi as they have similar morphology at several vegetative stages: filamentous growth pattern (mycelium) and reproductive propagules (spores), mode of nutrient acquisition and specialized infection structures (appressoria, infection hyphae and haustoria). The unique morphological, physiological, biochemical and genetic characteristics in *Phytophthora* that distinguishes it from true fungi include diploid and nonseptate hyphae, composition of the cell wall consisting mainly of 1,3-ß-glucans, some 1,6-ß-glucans and 1,4-ß-glucans, different from chitin in cell walls of most fungi, the structure of zoospores, different elicitors and gene encoding, the requirement for a source of sterols for sporulation and resistance to certain antibiotics, which are effective against true fungi (Latijnhouwers et al., 2003). The major difference between oomycetes and fungi is discussed in two good reviews (Judelson and Blanco, 2005; Latijnhouwers et al., 2003). This classification has been supported by several molecular studies (Cook et. al., 2000; Feng et al., 2011; Förster et. al., 1990; Tylor, 2006). The genus is further divided into 6 clades by Waterhouse (Stamps et al., 1990) which have been revised by Stamps et al. (1990), on the basis of host range, colony morphology, optimal growth temperature and other criteria. Kroon et al. (2012) divided
116 Phytophthora species into 10 clades within the genus by using genus wide phylogeny analysis.

As indicated by the name Phytophthora, plant destroyer, all the species in Phytophthora are pathogens to agricultural crops and plants in natural ecosystems and some cause great threats to human kind. A notable example is the Irish Potato Famine in the 1840s, which was caused by Phytophthora infestans, and is still a difficult pathogen to control (Haas et al., 2009). Some Phytophthora spp. can only attack a limited range of plants, such as P. infestans the causal agent of late blight of potato and P. sojae the causal agent of soybean root rot. Other Phytophthora spp. have a very broad host range, such as P. nicotianae and P. cinnamomi which can infect over 1000 species (Hardham, 2005; Meng et al., 2014). Phytophthora spp. cause several diseases of citrus worldwide, including damping-off of seedlings, root and crown rot in nurseries, and brown rot of fruit in groves (Graham, 1995; Graham et al., 1998). Pathogenic species on citrus worldwide are P. boehmeriae, P. cactorum, P. capsici, P. cinnamomi, P. citricola, P.citrophthora, P. dreschleri, P. hibernalis, P. megasperma, P. nicotianae, P. palmivora and P.syingae (Bawage et al., 2013; Erwin and Ribeiro, 1996). In Florida, P. nicotianae causes fruit brown rot, foot rot and root rot, and P. palmivora causes fruit brown rot and root rot (Graham et al., 1998; Graham and Feichtenberger, 2015; Widmer et al., 1998; Zitko and Timmer, 1994).

Phytophthora is a hemibiotroph, which requires a living organism to infect and reproduce but can survive for some time in the absence of the host on dead organic matter. The life cycle of Phytophthora consists of sexual and asexual phases. The sexual spore is called the oospore, which is produced by the fusion of antheridium and
oogonium. The oospores act like a resting structure, which can either produce hyphae to infect the plant directly or produce a sporangium. Motile biflagellate asexual zoospores are borne inside a multinucleate cell sporangium which is developed on the tip of a specialized hyphal structure called a sporangiophore. Zoospores are wall-less cells with the plasma membrane as the outer surface and they are instrumental in initiating plant infection. Another asexual spore is the chlamydospore. It is a thick-walled and multinucleated resting spore able to survive under unfavorable environment conditions. In most cases, pathogen infection begins with initial contact of a potential host by motile zoospores which are chemically attracted to the potential infection site, followed by successful penetration of the host surface and development of hyphae that penetrate cortical cells to absorb nutrition. These spores can be disseminated by water, rain, soil and human activity.

1.2.1 Diseases of Citrus Caused by *Phytophthora* spp.

Foot rot, also know as gummosis, is the most severe disease caused by *Phytophthora* spp. because it can lead to tree mortality. The typical symptoms of foot rot include damaged cambium and inner bark at the ground level, which can develop into a lesion that extends around the circumference of the trunk and girdles the tree cambium. Young trees can be killed by foot rot rapidly, whereas adult trees might survive with thin and weak canopy (Graham and Feichtenberger, 2015). *Phytophthora* spp. cause citrus root rot by infecting fibrous roots. Root rot induces soft and discolored cortex, white thread-like stele left without cortex, failure to form vigorous new growth and reduced yields (Feichtenberger, 1997; Sandler et al., 1989). Root damage caused by *Phytophthora* can be lethal on small seedlings as a result of large amount of fibrous root loss, and can reduce yield of fruit-bearing trees in a grove due to loss of canopy.
development (Feichtenberger, 1997; Sandler et al., 1989). *Phytophthora* spp. also causes brown rot of fruit when soil borne spores are splashed on or directly contact fruit near the soil surface under the tree. When rainfall coincides with early stages of fruit maturity, brown rot develops rapidly, resulting in light brown and leathery lesions on fruit.

Rootstock resistance/tolerance to *Phytophthora* spp. Rootstock’s resistance/tolerance to *Phytophthora* spp. is useful to reduce the crop loss. Rootstocks identified as tolerant to *Phytophthora* (Graham, 1995, 1990) are used for better management of disease. Tolerant rootstocks were defined as those that quickly replace roots at lower inoculum density to maintain tree health. Sour orange and Cleopatra mandarin were defined as susceptible to *P. nicotianae*, whereas trifoliate orange and its hybrids (Swingle citrumelo) were defined as resistant and tolerant of root rot, respectively. Interestingly, the rootstocks that are tolerant to *P. palmivora* are the opposite of those that are to *P. nicotianae*. Furthermore, rootstock tolerant to *Phytophthora* spp. varies with root age (Graham, 1995, 1990) and environmental conditions (Bright et al., 2004). For example, the lower rhizosphere population of *P. nicotianae* on mix-aged roots of tolerant rootstocks than susceptible rootstocks was not observed for newly regenerating roots (Graham, 1995). Furthermore, Swingle citrumelo lacks resistance to *Phytophthora* spp. under poorly drained soil series. Lower saturated hydraulic conductivity supported higher *P. nicotianae* populations in seasons when annual rainfall were high, but not in relatively dry years (Bright et al., 2004).

Host Pathogen interactions. Various studies have been carried out to elucidate the disease causal mechanism (Attard et al., Graham, 1995; Hardham, 2010, 2007;
Pathogenic effector molecules released by a pathogen into a plant cell play different roles in susceptible and resistant hosts. In susceptible hosts, these factors help with successful colonization such as cell wall degrading enzymes and proteins. In resistant hosts, these factors can trigger the plant defense system.

Environmental interactions. Soil texture, which determines aeration, drainage and nutrient availability, soil moisture and temperature, has major effects on Phytophthora infection, conversion of mycelium to sporangia and release of zoospores, disease development, and pathogen dissemination (Erwin and Ribeiro, 1956). Bright et al. (2004) reported that P. nicotianae populations are positively and negatively corelated with % clay and % of sand, respectively. Environmental conditions not only influence Phytophthora populations but also the rootstocks performance (Bright et al. 2004; Castle et al., 2004). The recommended management practices are use of disease free nursery stock; choice of a tolerant rootstock, proper soil drainage, irrigation, and a well-balanced soil applied fertilization program. In the event that cultural management practices do not control Phytophthora damage, systemic fungicides are recommended according to the Phytophthora propagule status of the grove (Graham, 2011; Graham, 2000; Graham and Menge, 1999).

Interactions with root pests. Besides impact of host performance, interaction with other soil pest is a critical consideration for effective management of Phytophthora diseases (Davis, 1981; Graham, 2002). The Phytophthora-Diaprepes abbreviatus complex illustrates that the interaction between an insect pest and Phytophthora can be mediated by increased plant exudation, as larvae feeding compromises rootstock’s
tolerance to *Phytophthora* spp. (Graham et al., 2002, 2003). In contrast, the antagonism between the citrus nematode *T. semipenetrans* and *P. nicotianae* was described for nematode susceptible citrus rootstocks (El-Borai et al., 2002). *T. semipenetrans* significantly reduced *P. nicotianae* infection and the damage that leads to root loss.

1.2.2 Phylogeny and Detection of *P. nicotianae* (Breda de Haan var. nicotianae Waterhouse)

In 1896, Van Breda de Haan named the fungus that is the causal agent of tobacco black shank as *Phytophthora nicotianae*, and *Phytophthora* morphotype was found in 1913 and named *P. parasitica* by Dastur. The name was further revised by Tucker and Waterhouse in 1963, and the priority of the name *P. nicotianae* was confirmed. Kroon et al. (2012) divided 116 *Phytophthora* species into 10 clades within the genus by using genus wide phylogeny analysis (Kroon et al., 2012). *P. nicotianae* is singularly classified in the clade 1, with *P. infestans* as its closest relative.

The isolation of *P. nicotianae* on citrus can be obtained from soil and plant material by use of pimaricin-ampicillin-rifampcin-pentachloronitrobenzene-hymexazol (PARPH) semi-selective agar medium or using leaf baiting procedures (Graham, 1998; Grimm and Alexander, 1973). The growth of *P. nicotianae* can be observed within 2-4 days on the semi-selective medium. The culture can be identified based on the morphology of the colony on the isolation medium, arachnoid branching of mycelium and the shape of papillate sporangia (Bush and Stromberg, 2006). Other criteria widely used to distinguish *P. nicotianae* from other species are cardinal growth temperature, growth rate, sexual and asexual morphology and mating behavior (Ashby, 1928; Erwin and Ribeiro, 1996). Molecular tools, isozyme analysis, serological techniques (Bonants et al., 2000, Grote et al., 2002; Ippolito et al., 2004; Man In't Veld et al., 1998; Pettitta et
al., 2002) have been developed to increase the accuracy of identification and sensitivity of detection and to reduce time and labor cost.

1.2.3 Life and Disease Cycle

*P. nicotianae* life cycle is comprised of sexual and asexual phases. Asexual structure includes sporangia, zoospores (predominant) and chlamydospores. The sexual structure is an oospore that requires mating of A1 and A2 type for the formation after a phase of vegetative growth (Randall et al., 2003). *P. nicotianae* prefers warm, moist and well aerated soil to continue its life cycle (Erwin and Ribeiro, 1996).

Sporangia are formed from oospores, chlamydospores and mycelium, and zoospores are released from sporangia into soil to infect and colonize fibrous roots. After mycelial growth in the root, sporangia are formed under favorable condition (20-30°C, abundant moisture) (Matheron and Porchas, 1996). Under unfavorable conditions (i.e., nutrient depletion, low oxygen levels and temperature below 15°C, oospore and chlamydospores are formed to survive until conductive conditions resume (Graham and Timmer, 1992).

Pre-penetration. *Phytophthora* spp. hyphae, sporangia and zoospores act to reach the host over a short and long distance. Zoospores swim at the rate of 200μm/s, and can disperse over a longer distance by water flow, rain splash or wind-blown rain (Declercq et al., 2012). Zoospores are attracted to infection sites such as the zone of root elongation and wounds by electrotaxis and chemotaxis (Tyler, 2002; Van West et al., 2002). A variety of sugars, amino acids, alcohols and calcium nonspecifically attract zoospores or isoflavones specifically attract zoospores (Tyler, 2002).

Penetration. After reaching the plant surface, zoospores encyst and adhere to the plant surface before penetration. The process of encystment includes detachment of
the flagellum, secretion of adhesive material and formation of a cell wall (Hardham and Shan, 2009). A number of physical and chemical factors, plant cell receptors, and the phospholipase D signal transduction pathway are involved in this process (Litijnhouwers et al., 2002). For example, an adhesion protein is secreted during zoospore encystment to orient and firmly attach the zoospore to the plant surface (Gubler and Hardham, 1990). Plant cell receptors determine plant response to Phytophthora spp. infection (Litijnhouwers et al., 2002). After adherence to the plant surface, an appressorium is formed at the tip of germ tubes for penetration (Kebdani et al., 2010; Wang et al., 2011). Appressoria penetrate the host cell wall by mechanical pressure or by cell wall degrading enzymes (Škalamera et al., 2004). Two sites on roots were reported to be preferred by Phytophthora pathogens, one is periclinal wall of the hypodermal cell and the other one is the groove formed by anticlinal walls of epidermal cells (Hardham, 2001).

Colonization. After initial infection, hyphae grow intracellularly or intercellularly inside the plant tissue to access resources (Enkerli, 1997; Widmer et al., 1998). Haustoria are finger-like hyphal structures inside the plant cell that absorb nutrients. The components that stimulate the formation of haustoria were reported to be an electron-dense chemicals produced by leaf and root cell walls (Coffey, 1983; Enkerli, 1997).

Reproduction, dispersal and survival. After colonization, Phytophthora spp. begin to produce spores, which may be sexual or asexual (Adrienne, 2001). Some Phytophthora spp. spores are produced on the surface of the leaves and dispersed by wind; some are produced inside the plant and can only be released when plant tissues die (Judelson and Blanco, 2005). Different factors trigger the sporulation, like reduced
nutrient and high humidity. Spores can be disseminated by seedbeds, rootstocks, nursery practices, irrigation water, and human activity, and can survive in soil, water and diseased plant material.

According to historical survey data from citrus groves, populations of *Phytophthora* are considered damaging to fibrous roots when counts exceed 10-15 propagules per cubic centimeter of rhizosphere soil (Graham, 2011). This threshold can be influenced by the interaction with other pathogens and pests, soil texture, rootstock resistance and/or tolerance to *Phytophthora* (Bright et al., 2004).

Genome sequencing. Genome sequencing provides information for pathogen lifestyle, host range, evolution, physiology, phylogeny, virulence mechanisms, identification and disease control. So far, sequences of six species *Phytophthora* spp. are published. They are *P. infestans* (Haas et al., 2009), *P. sojae* (Tyler, 2006), *P. ramorum* (Tyler, 2006), *P. capsici* (Lamou et al., 2012), *P. cinnamomi* (Li et al., 2000) and *P. nicotianae* (https://olive.broadinstitute.org/projects/phytophthora_parasitica). The gene numbers range from 14,451(*P. ramorum*) to 26,131 (*P. cinnamomi*), with the repetitive DNA sequence from 19% (*P. capsici*) to 74% (*P. infestans*) and genome size ranges from 64Mb (*P. capsici*) to 240Mb (*P. infestans*). The genome size of *P. parasitica* was estimated to be 95.5 Mb (Shan and Hardham, 2004), and includes about 23,121 predicted genes (Judelson, 2012). Study on interaction between *P. nicotianae* and *A. thaliana* suggested that salicylic acid, jasmonic acid and ethylene signaling pathways are involved in defense against *P. nicotianae* (Attard et al., 2010).

Gene numbers are implied to be the cause of variability of host range, which was shown by 26,131 genes of *P. cinnamomi* with over 1000 hosts. Ontology mapping
indicates the relationship between species by identifying around 1000 shared core genes (Seidl et al., 2011), which reveals the evolutionary relationships among *Phytophthora* spp. With the help of DNA mapping, gene expression during different stages of the life cycle, more information has been gained to understand physiology of *Phytophthora* spp. (Kunjeti et al., 2012). Genes for protein families expressed during adhesion, penetration and colonization help elucidate the mechanism of pathogenesis. For example, hydrolytic enzymes that degrade host cell walls are secreted during host penetration (Jiang and Tyler, 2012). The regulatory genes for the sterol pathway explain the resistance of *Phytophthora* spp. to sterol-inhibiting fungicides (Madoui et al., 2009).

**1.2.4 The Molecular Basis of Pathogenesis**

In the 1990s, molecular tools were developed for *Phytophthora* pathogenesis study, which also includes related pathogen biology. Such methods include DNA-mediated transformation (Bottin et al. 1999; Cvitanich and Judelson, 2003), homology-based gene-silencing strategies used to analyze gene function (Kamoun et al., 1998), marker-based maps and chromosome-walking projects in bacterial artificial chromosome (BAC) libraries (Randall et al., 2003). Expressed sequence tags (EST) analysis was used to analyze gene expression at different stages of the life cycle to understand pathogen’s biology and interaction with host, which includes vegetative growth-mycelium (Panabières et al., 2005), germinated cysts zoospore (Shan et al., 2004b; Škalamera et al., 2004), penetration and infection (Kebdani et al., 2010; Le Berre et al., 2008).

Effectors are defined as molecules that are secreted by pathogen to interfere with the host’s performance and physiology by formation of protein toxins, hydrolytic enzymes, enzyme inhibitors and cell entering signal peptides (Jiang and Tyler, 2012).
Effectors can disarm plant defense enzymes, manipulate host structure and function during infection and colonization process, kill host cells (Ali and Bakkeren, 2011; Kamoun, 2006; Meng et al., 2014), and favor the pathogen by accumulating auxin during penetration and inducing reactions that help the pathogen to attach to the plant surface (Evangelisti et al., 2013; Khatib et al., 2004). Several hundred proteins were estimated to manipulate host cell structure and function (Kamoun, 2006). Based on their location in the host, the effectors are grouped onto two categories- apoplastic effectors (located in plant extracellular space) and cytoplasmic effectors (inside the plant cell) (Kamoun, 2006). Readers are referred to these reviews for details (Hardham and Shan, 2009; Jiang and Tyler, 2012; Kamoun, 2006).

1.2.5 Interaction with other Organisms

A wide diversity of carbon-based compounds are released into the rhizosphere that mediate plant-microbe interaction (Bais et al., 2006). Sucrose is one of the chemicals that attracts *Phytophthora* zoospores and enhances root infection (Graham et al., 2003). In the *Phytophthora-D. abbreviatus* complex, larval damage to roots increased exudation of reducing sugar and promoted *Phytophthora* population (Graham et al., 2002, 2003). This damage broke the rootstock’s tolerance to *Phytophthora* spp., which increased *Phytophthora* spp. infection incidence and resulted in more severe damage. Conversely, prior colonization of fibrous root by a hypovirulent *P. nicotianae* isolate excluded the virulent isolate inoculated afterwards by out-competing the virulent isolate for colonization sites.

1.2.6 *Phytophthora* spp. Management

Details of management of *Phytophthora* were recently reviewed by Graham and Feichtenberger (2015). Management should be based on disease cycle and pathogen
physiology. Cultural management practices should strive to minimize as much as possible the environmental conditions that are favorable for disease development. Soil drainage and irrigation should be managed to reduce or eliminate conductive conditions for Phytophthora activity and damage. The recommended management practices in groves are use of disease-free nursery stock, choosing a resistant rootstock whenever possible, proper irrigation scheduling and a well-balanced fertilization program. In the case that cultural management does not control Phytophthora damage, systemic fungicides, mefenoxam metalaxyl and fosetyl-Al (phosphite) are recommended according to the Phytophthora propagule status of the grove (Feichtenberger, 1997; Graham and Menge, 1999; Sandler et al., 1989; Timmer et al., 1998). Soil applied mefenoxam and foliar-applied phosphites are rotated to reduce risk of fungicide resistance development (Graham et al., 2011). Application of fungicide is timed after the spring leaf flush and fall leaf flush coincident with root flushes. Use of registered fungicides is favored over nematicide when nematodes are present in the grove. Biological control was used to reduce the risk of ground water contamination and fungicide resistance. However, the field studies showed little success as a high level of the biocontrol agent is difficult to sustain (Nemec et al., 1996).

1.3 Interaction between P. nicotianae and Candidatus Liberibacter asiaticus

The interaction between Las and Phytophthora on citrus was first and only reported by Ann (Ann et al., 2004). The damage on plant growth, 6 months after inoculated with each pathogen or both was evaluated by measuring trees height and biomass. Greater reduction of growth and symptom development was found for trees infected with both pathogens than either one alone. Previous study in our lab showed that 1) Las infection on citrus rootstocks predisposed Phytophthora infection on fibrous
root by attracting zoospore (more leakage from root), breaking down their resistance to 
*Phytophthora* (sugar mediated plant response to *P.n.* infection) or both; 2) the 
combination of Las and *Phythophthora* does not cause greater damage than either 
alone; 3) the interaction between *Phytophthora* and Las on fibrous root damage over 
time is mediated by available root biomass and their interaction.

### 1.4 Citrus Root Biology

#### 1.4.1 Citrus Root Function and Growth Pattern

The citrus root system consists of primary roots and lateral roots (≤2mm 
diameter). The fibrous root growth was believed to be periodic with 2-3 growth cycles 
(Febarury to early April, May to June, and August to October) per year with alternating 
growth of shoot and root (Bevington and Castle, 1985). Most citrus fibrous roots exist in 
0-30cm depth in soil near the trunk (Castle, 1980; Mattos et al., 2003). The optimal 
environment for healthy fibrous root growth is often associated with temperature above 
27°C, high water potential in soil (>0.02MPa) and available carbohydrate (Bevington 
and Castle, 1985; Mattos et al., 2003). Unfavorable conditions for citrus fibrous root 
growth include high salinity (Ruiz et al., 1997), leaching loss of soluble nutrient through 
irrigation (Mattos et al., 2003), unbalanced nutrient absorption caused by high 
bicarbonate (HCO₃⁻) at pH range from 6.5 to 8.5 (Graham et al., 2014).

Citrus trees absorb water and mineral nutrients in soil through fibrous roots 
(diameter < 2mm). In addition to acquiring water and nutrients, fibrous roots store 
carbohydrate and synthesize hormones and secondary metabolites (Flores et al., 1999; 
Walker et al., 2003). Fibrous root bunches develop by branching, elongating and 
maturation. As they age, citrus fibrous roots change color from white to brown (Fitter, 
2002; Wells and Eissenstat, 2003). The median life span of fibrous roots can exceed
100 d (Eissenstat and Yanai, 1997), during which they change biochemically and structurally in response to normal development and environmental conditions (Duncan et al., 1993; Eissenstat and Achor, 1999). New root growth serves to extend the root system and replace dead roots, and its life span is important for plant growth (Eissenstat, 2000). Longevity of citrus fibrous roots results from management of the tree’s root system to minimize cost (i.e. root construction and respiration) and maximize benefit (i.e. nutrient and water absorption) (Eissenstat, 2000). Citrus species vary widely in root longevity (Eissenstat and Yanai, 1997).

1.4.2 Citrus Root Architecture

Linkage of root traits to root functions has previously been used to predict plant adaptation to environmental conditions (Eissenstat et al., 2000; Eissenstat and Achor, 1999). Specific root length (SRL) is defined as root length per unit root biomass. Higher SRL is often associated with smaller root diameter, higher respiration and hydraulic conductivity, and shorter lifespan (Eissenstat and Achor, 1999; Graham and Syvertsen, 1985). Citrus species vary widely in SRL (Graham and Syvertsen, 1985). Root color has been used to predict root life span and provide the most current information about root growth and viability (Eissenstat and Achor, 1999; Fitter, 2002).

Branch order (the most distal root without other root branching from it is order 1, and the one has only one branching root is order 2…) is another trait used to link to root functions. Lower root order is associated with smaller root diameter, higher SRL, respiration and hydraulic conductivity, and shorter lifespan (Eissenstat and Achor, 1999; Pregitzer et al., 1998; Wells and Lissenstat, 1997). Between different species, first order diameters differ according to cell size rather than cell number (Eissenstat and Achor, 1999).
1.4.3 Citrus Fibrous Root Anatomy

Citrus fibrous root ultrastructure has been studied to assist explaining plant resistance/tolerance to pathogen (i.e. oomycete and nematode) infection (Duncan et al., 1993), extreme environmental conditions (i.e. high chloride) (Walker et al. 1984), and relationship between root architecture and function (Eissenstat and Achor, 1999). Citrus root anatomy plays an important role at different stages of root growth as structure is connected with water, mineral elements and nutrient flow within roots (Peterson and Enstone, 1996). Walker et al. (1984) reported that citrus fibrous root, from outside to inside, consist of epidermis (single layer of cells), hypodermis (single layer of cells), cortex (large thin-walled cells), endodermis (single layer of cells), pericycle (single layer of cells), phloem, cambium and xylem. Single passage cells occur in between hypodermal cells and cortex cells.

As roots age, plasmodesmata are blocked by suberin and lignin in the hypodermis and endodermis, and a casparian strap forms in almost all endodermis cells (Walker et al., 1984). The symplastic transport way is through passage cells, which connect hypodermal, endodermal and cortex cell through plasmodesmata. Passage cells have delayed in the development compared to other cell types (Peterson and Enstone, 1996).

Though root can modify structure such as cell thickening by developing lignin and suberin layer to provide protection from soil organism attack (Duncan et al., 1993), the main resistance might be due to other mechanisms. Eissenstat and Achor (1999) reported that the tolerant rootstock (trifoliate orange) to citrus nematode and *P. nicotianae* has thinner exodermal walls than the susceptible rootstock (sour orange).
CHAPTER 2
THE INTERACTION BETWEEN PHYTOPHTHORA NICOTIANAE AND CANDIDATUS LIBERIBACTER ASIATICUS DAMAGE TO CITRUS FIBROUS ROOTS

2.1 Introduction

Huanglongbing (HLB), also known as citrus greening (in Africa) or likubin (in Taiwan) (Ann et al., 2004), is the most devastating disease of citrus known (Bové, 2006). HLB in Florida is caused by the phloem-limited bacterium, Candidatus Liberibacter asiaticus (Las) (Jagoueix et al., 1994; Teixeira et al., 2005), which is transmitted from tree to tree by the psyllid vector, Diaphorina citri, or by grafting infected tissue from other hosts of the pathogen (Garnier and Bové, 1983; Halbert and Manjunath, 2004).

Symptoms of HLB on leaves, fruits and in phloem have been widely reported in previous studies (Bassanezi et al., 2009; Etxeberria et al., 2009). Root loss of HLB trees was believed to be secondary damage after carbohydrate metabolism in canopy and phloem was disrupted (Etxeberria et al., 2009). However, rapid yield loss of sweet orange trees at a low level of canopy symptom expression was observed in Brazil (Bassanezi et al., 2011). This finding indicated that unobserved damage was occurring before or at the same time as canopy symptoms developed (Johnson et al., 2014). Johnson et al. (2014) investigated roots as a site for Las multiplication at an early stage of the infection process by tracking movement of the pathogen in graft-inoculated greenhouse trees. Root infection and loss was detected before phloem was blocked and leaf symptoms developed which led to the prediction that Las root infection may directly damage root or interact with other soil borne pathogens to cause damage (Graham et al., 2013; Johnson et al., 2014). Meanwhile, unprecedented changes in population of the soil borne pathogen Phytophthora in citrus groves were documented
as HLB spread throughout Florida (Graham et al., 2011). These findings led to the investigation of fibrous root status and the causal agents of root loss at the early stage of HLB symptom development.

*Phytophthora* spp. cause soil-borne diseases of citrus worldwide by damaging fibrous roots, which reduces water and nutrient uptake and depletes carbohydrate reserves in roots (Graham, 1995). The interaction of *Phytophthora* with other root pathogens and pests is important for developing the most effective management of fibrous root health (Davis, 1981; Graham and Feichtenberger, 2015). In the study of the complex between *Phytophthora* spp. and the root weevil *Diaprepes abbreviatus*, the interaction between *Phytophthora* and root damage caused by larval feeding was shown to be mediated by root exudates (Graham et al., 2003, 2002), because larval damage increases leakage of sugars from wounds of fibrous roots. In addition, the quantity of available carbohydrates for production of defense compounds is an important determinant of host susceptibility or tolerance to *Phytophthora* (Angay et al., 2014; Jönsson, 2006).

The interaction between Las and *Phytophthora* has been proposed to contribute to pre-symptomatic root loss on HLB trees (Graham et al., 2013; Johnson et al., 2014). The interaction was first recognized by Ann et al. (2004) who evaluated in the greenhouse the effect on tree growth 6 months after inoculation with each pathogen individually or together. HLB symptom development and reduction in citrus seedling growth was greater on trees infected with Las and *Phytophthora* than with either pathogen alone.
Greater understanding of the interaction between Las and *Phytophthora* will provide insight into how roots are damaged at the early stage of HLB symptom development. This knowledge will contribute to more effective management of root health on HLB-affected trees. Based on preliminary studies, we hypothesize: 1) Las infection of citrus rootstocks predisposes fibrous roots to *Phytophthora* infection by increasing root leakage of exudates that attract zoospores or by breaking down host resistance, or both mechanisms; 2) the combination of Las and *Phytophthora* causes greater damage than each pathogen alone.

To test these hypotheses, greenhouse studies of the interaction between Las and *P. nicotianae (P.n.)* were conducted with seedlings of the citrus rootstocks, Cleopatra mandarin (*Citrus reticulata*), Swingle citrumelo (*C. paradisi x Poncirus trifoliata*), sour orange (*C. aurantium*) and X639 (*C. reticulata X P. trifoliata*). Swingle citrumelo is recognized as tolerant to *P.n.*, while sour orange and Cleopatra mandarin and X639 are considered to be susceptible (Graham, 1995). To quantify the interaction, *P.n.* infection of roots, fibrous root biomass, root exudation of sucrose, starch and total sugar (starch+sucrose) concentration in roots were evaluated for seedlings that were non-inoculated, inoculated with Las, inoculated with *P.n.*, or inoculated with Las and *P.n.*

### 2.2 Materials and Methods

#### 2.2.1 Experimental Design and Inoculation with *Candidatus Liberibacter asiaticus*

Seeds of Cleopatra mandarin were sown in Metro Mix 500 (Hummert International, MO, USA) in 120 cm³ containers in the greenhouse. Seedlings were fertilized with Jack’s Pro 20-20-20 (liquid) (A.M. Leonard, Inc., OH, USA) and Harrell’s 18-5-10 (Harrell’s, FL, USA) every other week and every 6 mo, respectively, and
watered three times per week. One hundred and twenty 6-mo-old seedlings were graft inoculated with budwood from Las-infected trees. The budwood used for inoculation was from a greenhouse grown 'Madam Vinous' sweet orange (C. sinensis) tree infected with Las isolate FC6 as previously described (Folimonova et al., 2009). The bud was inserted into the seedling approximately 12-17 cm above the soil line. Six months after inoculation (trial 1: April 8, 2013; trial 2: May 13, 2014), 60 Las-infected seedlings (PCR confirmed; see methods below) from 120 Las-inoculated seedlings, and 60 non-inoculated seedlings were selected for uniform size, and transferred into autoclaved Candler fine sand in 120 cm$^3$ containers for four treatments P.n. +/- and Las+/- (10 replicates for each treatment) and 3 harvest times. In 2014, this protocol was repeated for Swingle citrumelo (August 12, 2014), sour orange (March 15, 2014) and X639 (August 5, 2014).

2.2.2 Inoculation with Phytophthora nicotianae

P. nicotianae (P.n. 198) isolated from declining Cleopatra mandarin in the greenhouse in 1999 was maintained on clarified V8 juice agar. Chlamydospores were produced by the method of Tsao (1971) for inoculation. Chlamydospores harvested from liquid culture were blended, added to Candler fine sand soil and mixed manually for inoculum preparation. Soil inoculum was diluted 1:10 in water agar, incubated at room temperature in dark for 2 d on pimaricin-ampicillin-rifampcin-pentachloronitrobenzene-hymexazol (PARPH) semi-selective agar medium for assay of propagule density. An inoculum density of 20 propagules/cm$^3$ of soil was obtained by mixing the inoculum concentrate with autoclaved Candler fine sand. Seedling roots were pruned to a uniform length (8cm) to induce root growth. Thirty Las-infected and 30 non-inoculated seedlings were transplanted into soil infested with P.n. and 30 Las-
infected and 30 non-infected seedlings were transplanted into autoclaved soil.

Seedlings inoculated and non-inoculated with \textit{P.n.} were located on different benches in the greenhouse (27ºC-32ºC), watered 3 times a week and fertilized with Jack’s Pro 20-20-20 every other wk.

2.2.3 Assessment of \textit{Phytophthora} Root Infection and Collection of Exudates

At 5, 8 and 11 wk post inoculation with \textit{P.n.} (wpi), seedlings roots were thoroughly watered and harvested 24 h after irrigation. During harvest, seedlings from each treatment were lifted out of the container and tapped 20 times in plastic bags. The dead roots were separated from the rhizosphere soil, and the soil was dried for 48 h at 70 ºC and stored at -20ºC. Roots were washed free of soil and 20 fibrous root tips were randomly selected from \textit{P.n.} inoculated and non-inoculated seedlings and plated on PARPH medium. Plated root tips were incubated in the dark for 3-4 d, and the percentage of \textit{P.n.} positive tips was recorded as infection incidence (%). Two leaves from top and bottom position on the stem and 25-50 mg of fresh roots were collected randomly for Las detection in roots and shoots. Fibrous roots (diameter ≤ 2mm) were separated, dried and weighed (70ºC for 48 h).

2.2.4 \textit{Candidatus Liberibacter asiaticus} Detection

To detect Las in seedlings, leaf midribs were chopped into square segments, placed in 2 mL screw cap tubes with one 5-mm stainless steel bead (Qiagen, Valencia, CA, USA). The tubes were stored in a -80ºC freezer for at least 2 h and after removal from the freezer immediately ground twice at 30 revolutions per sec in a Tissuelyzer II (Qiagen, Valencia, CA, USA). Ground midrib tissue was then processed for DNA extraction using the Qiagen (Valencia, CA, USA) DNeasy Plant Mini kit according to the manufacturer’s instructions, except that DNA was eluted twice with 50 µl AE buffer.
instead of once by 100μl (Johnson et al., 2014). Randomly selected fibrous roots were cut and ground as described above for the midrib tissue. Root DNA was extracted using the Mo Bio PowerSoil DNA Isolation kit (Mo. Bio. Laboratories, Carlsbad, CA, USA) as previously described (Johnson et al., 2014). The first step of the protocol was modified as initial buffer, garnet and solution C1 was added into 2 ml screw cap tubes which were used for grinding and then vortexed for 5 sec instead of 10 min (Johnson et al., 2014). Purified DNA solution was stored at -20°C for later analysis.

Quantitative polymerase chain reactions (qPCR) were carried out using primers and probes as previously described (Wang et al., 2006) on an Applied Biosystems 7500 Fast Real-Time PCR System. The master mix included Qiagen Hot Star Taq, ROX passive reference dye (Bio-Rad), primers, probe and buffer in a concentration ratio as described by the manufacturer’s protocol. 4 μL of template DNA and 16 μL master mix were loaded into each well of a 96 well micro plate, and each sample was replicated. Samples were run in triplicate if significant variation was found between 2 replicates in the initial assay. The standard curve included concentrations of Las plasmids from $1 \times 10^1$ to $1 \times 10^6$ copies.

**2.2.5 Quantification of Starch in Fibrous Roots**

A 25 mg subsample of dried fibrous root was randomly selected for starch analysis, and processed as previously described (Rosales and Burns, 2011) with modifications. Fibrous roots were cut and ground in 2 ml tubes as described above for DNA extraction. A 900 μl aliquot of DI water was mixed with the root powder. The solution of ground roots or standard was vortexed and boiled for 10 min. Samples were centrifuged for 2 min at 2500 x G. A 300 μl aliquot of supernatant was stored at -20°C for sucrose assay. Another 300 μl aliquot of supernatant was transferred into a 2ml tube
and mixed with 900 μL ethanol. The mixture was vortexed for 5 sec, and centrifuged for 10 min at 10,000 x G. The supernatant was discarded and the pellet was suspended in 1.0 mL DI water by vortexing for 4 min. A 50 μl aliquot of KI: I₂ solution (8 mM: 50 mM) was added, vortexed for 5 sec and centrifuged for 2 min at 10,000 x G. The mixture was transferred into a 96-well micro plate and the absorbance at 594 nm was measured. Rice starch (Sigma-Aldrich) was used as the standard with a concentration range from 0 to 1.0 mg/ml. Sample starch concentration was calculated from absorbance reading according to the standard curve.

2.2.6 Quantification of Sucrose in Root Exudates and Fibrous Roots

A 50 mg sample of rhizosphere soil collected from Las (+) P.n. (-) and Las (-) P.n. (-) seedlings above was suspended in 7.5 ml DI water to obtain a 15% soil moisture concentration. The mixture was incubated at 4 °C for 2 h and centrifuged for 10 min at 2,700 x G in a filter bottle. The volume of filtrate was recorded and the filtrate was stored at -20 °C. For sucrose assay, the root exudate was diluted 1:20, and centrifuged at 10,000 x G for 2 min. A 4μl aliquot of supernatant from each sample was processed according to the manufacturer’s protocol for the Glucose and Sucrose Colorimetric/Fluormetric Assay (Sigma-Aldrich, St. Louis, MO, USA). Sucrose concentration in fibrous root was expressed as µg sucrose per mg root dry weight.

2.2.7 Statistical Analysis

Fibrous root biomass, root starch, sucrose in root exudates and P.n. infection incidence were analyzed by one-way ANOVA using PROC GLM (SAS v. 9.4). The interaction between Las and P.n. was analyzed by two-way ANOVA using PROC GLM (SAS v. 9.4). The significance level was set at P≤ 0.05. The relationship between
fibrous biomass and *P. n.* infection incidence, total sugar and *P. n.* infection incidence was analyzed by linear regression.

2.3 Results

2.3.1 Las Effect on *P. n.* Infection Incidence

Significant increase (*P*≤0.05) in *P. n.* infection incidence of Cleopatra mandarin seedlings inoculated with Las compared to seedlings not inoculated with Las was detected at 11 wpi in the 2013 trial (Figure 2-1A) and at 5 wpi in the 2014 trial (Figure 2-1B). In 2013, *P. n.* infection incidence was lower (*P*≤0.05) for Las-inoculated seedlings at 5 and 8 wpi (Figure 2-1A). In 2014, no significant effect of Las on *P. n.* infection incidence was detected at 8 and 11 wpi (Figure 2-1B).

Similar to Cleopatra mandarin, temporal interactions of Las with *P. n.* infection were observed for Swingle citrumelo, X639 and sour orange seedlings. Las increased *P. n.* infection incidence at 5 wpi for Swingle citrumelo (*P*≤0.05) (Figure 2-2A), X639 (*P*≤0.05) (Figure 2-2B) and sour orange (*P*≤0.05) (Figure 2-2C), and at 11 wpi for X639 (Figure 2-2B). Seedlings of Swingle citrumelo (*P*≤0.05) and X639 (*P*≤0.05) inoculated with Las exhibited lower *P. n.* infection incidence at 8 wpi compared to seedlings not inoculated with Las (Figure 2-2A, B, C).

2.3.2 Relationship between Fibrous Root Damage and *P. n.* Infection Incidence

Cleopatra mandarin root damage due to Las, *P. n.* and their interaction was assessed by measurement of fibrous root biomass at 5, 8 and 11 wpi. Non-inoculated control seedlings continuously increased in root biomass in the 2013 and 2014 trials (Figure 2-3A, B). A significant interaction between Las and *P. n.* for root biomass was detected at 8 and 11 wpi in 2014 trial (*P*≤0.05). Overall, root biomass accumulation for seedlings inoculated with Las, *P. n.* or both was lower by 11 wpi compared to the
controls (Figure 2-3A, B). In the 2013 trial, root biomass was 42% \( (P \leq 0.05) \), 19% and 46% \( (P \leq 0.05) \) lower for the seedlings inoculated with Las, \( P.n. \) or both, respectively compared to non-inoculated controls at 11 wpi (Figure 2-3A). In the 2014 trial, root biomass was 51% \( (P \leq 0.05) \), 29% \( (P \leq 0.05) \) and 39% \( (P \leq 0.05) \) lower for seedlings inoculated with Las, \( P.n. \) or both, respectively compared to non-inoculated controls at 11 wpi (Figure 2-3B). Las infection, with or without \( P.n. \) inoculation, reduced greater root biomass accumulation compared to \( P.n. \) infection in 2013 trial.

The temporal relationship between \( P.n. \) infection incidence and fibrous root growth was evaluated by linear regression analysis. In the 2013 trial, for seedlings inoculated with \( P.n. \) only, the slope of regression of fibrous root biomass on \( P.n. \) infection incidence was negative (Figure 2-5B), whereas, for seedlings with Las infection the slope was positive at 5 wpi (Figure 2-5A). At 8 wpi in 2013 trial, the relationship between fibrous root biomass and \( P.n. \) infection incidence was positive for seedlings with or without Las infection (Figure 2-5C, D). At 11 wpi in 2013 trial, the relationship between fibrous root biomass and \( P.n. \) infection incidence was negative for seedlings with or without Las infection (Figure 2-5E, F). In the 2014 trial, the slope of regression of fibrous root biomass on \( P.n. \) infection incidence was negative (Figure 2-6A), whereas, for seedlings with Las infection the slope was positive (Figure 2-6B). At 11 wpi in 2014 trial, the relationship between fibrous root biomass and \( P.n. \) infection incidence was positive for seedlings with or without Las infection (Figure 2-6C, D).

Fibrous root biomass of non-inoculated control seedlings of Swingle citrumelo and X639 increased from 5 to 11 wpi (Figure 2-4A, B). Unexpectedly, fibrous root biomass of non-inoculated sour orange seedlings decreased at 8 wpi and then
increased at 11 wpi (Figure 2-4C). A significant interaction between Las and \( P.n. \) for fibrous root biomass occurred at 8 wpi for sour orange \((P \leq 0.05)\). Swingle citrumelo infected with Las, \( P.n. \) or both pathogens had 47\% \((P \leq 0.05)\), 68\% \((P \leq 0.05)\) and 64\% \((P \leq 0.05)\) lower fibrous root biomass compared to non-inoculated controls at 11 wpi, respectively (Figure 2-4A). X639 seedlings infected with Las, \( P.n. \) or both pathogens had a 6\%, 88\% \((P \leq 0.05)\) and 90\% \((P \leq 0.05)\) lower root biomass compared to non-inoculated controls at 11 wpi, respectively (Figure 2-4B). Sour orange infected with Las, \( P.n. \) or both pathogens had a 67\% \((P \leq 0.05)\), 60\% \((P \leq 0.05)\) and 80\% \((P \leq 0.05)\) lower root biomass compared to non-inoculated controls at 11 wpi, respectively (Figure 2-4C). Las, \( P.n. \) or both pathogens reduced similar fibrous root accumulation on Swingle citrumelo and sour orange at 11 wpi (Figure 2-4A, B).

For Swingle citrumelo, the relationship between root biomass and \( P.n. \) infection incidence fluctuated over time. At 5 wpi for seedlings inoculated with \( P.n. \), the slope of regression between fibrous root biomass and \( P.n. \) infection incidence was negative, whereas, for seedlings with Las infection the slope was positive (Figure 2-7). At 8 wpi, the relationship between fibrous root biomass and \( P.n. \) infection incidence was negative for seedlings with Las infection and positive for seedlings without Las infection. At 11 wpi, the relationship between fibrous root biomass and \( P.n. \) infection incidence was positive for seedlings with Las infection and negative for seedlings without Las infection.

In contrast to Swingle citrumelo, the relationship between fibrous root biomass and \( P.n. \) infection incidence did not change over time for X639. At 5, 8 and 11 wpi, \( P.n. \) infection and fibrous root biomass were negatively correlated with or without Las
infection (Figure 2-8). The relationship between Las and \textit{P.n.} infection incidence for sour orange was similar to X639, i.e., negative at 5 and 8 wpi (Figure 2-9).

2.3.3 Effect of Las on Root Exudation of Sucrose

Sucrose in root exudates collected from the rhizosphere soil was assayed to determine why Las may have increased \textit{P.n.} infection of fibrous roots. Cleopatra mandarin seedlings showed the trend of higher sucrose concentration in root exudates in Las-infected seedlings than in the non-inoculated controls at all three harvests in the 2013 and 2014 trials (Figure 2-10A, B).

For Swingle citrumelo, X639 and sour orange, sucrose concentration in root exudates was higher in Las-infected seedlings than in non-inoculated controls at 8 wpi for Swingle citrumelo \((P \leq 0.05)\) (Figure 2-11A) and X639 \((P \leq 0.05)\) (Figure 2-11B), and 11 wpi for Swingle citrumelo (Figure 2-11A).

2.3.4 Effect of Las and \textit{P.n.} on Root Starch Concentration

Starch concentration in non-inoculated Cleopatra mandarin roots ranged from 34 to 40 mg/g in the 2013 trial and 9 to 26 mg/g in the 2014 trial. A significant interaction between Las and \textit{P.n.} for fibrous root starch was detected at 8 and 11 wpi in 2013 and 2014 trial respectively. Control seedlings showed higher starch concentration in fibrous roots at 8 \((P \leq 0.05)\) and 11 \((P \leq 0.05)\) wpi than seedlings that were inoculated with each pathogen or both (Figure 2-12A) in 2013. Continuous increase in starch concentration from 5 wpi to 11 wpi was detected for non-inoculated Cleopatra roots in 2014 (Figure 2-12B). Las roots with or without \textit{P.n.} had lower starch than seedlings inoculated with \textit{P.n.} at 11 wpi in 2013 \((P \leq 0.05)\) and 2014 trial (showed a trend) (Figure 2-12A, B). Each pathogen alone significantly \((P \leq 0.05)\) reduced starch concentration in fibrous roots compared to non-inoculated controls in 2013 trial (Figure 2-12A).
Starch concentration of non-inoculated roots ranged from 10 to 115 mg/g for Swingle citrumelo, 35 to 165 mg/g for X639 and 15 to 31 mg/g for sour orange up to 11 wpi. A significant interaction between Las and P.n. for fibrous root starch was detected at 11 wpi for Swingle citrumelo ($P \leq 0.05$), at 8 wpi for sour orange ($P \leq 0.05$), and at 5 and 11 wpi for X639 ($P \leq 0.05$). By 11 wpi, Las reduced ($P \leq 0.05$) starch concentration compared to controls for all rootstocks. (Figure 2-13A, B, C). Overall, X639 and Swingle citrumelo showed higher starch concentration in P.n. infected seedlings compared to non-inoculated seedlings (Figure 2-13A, B). Las infected sour orange seedlings with and without P.n. had lower ($P \leq 0.05$) starch than seedlings inoculated with P.n. (Figure 2-13C).

2.3.5 Relationship between P.n. Infection Incidence and Total Sugar in Fibrous Roots

For Cleopatra mandarin at all harvest times in both trials, the linear regression between P.n. infection incidence and total sugar concentration was positive for Las infected roots and negative for seedlings not inoculated with Las at all harvest times in both trials (Figure 2-14, 2-15). The exception was at 8 wpi in 2013 when seedlings infected with Las showed a negative linear relationship between P.n. infection incidence and total sugar concentration, whereas seedlings not inoculated with Las showed a positive relationship between P.n. infection incidence and total sugar concentration (Figure 2-14C,D).

For Swingle citrumelo, the opposite (positive and negative) linear relationship between P.n. infection incidence and total sugar concentration caused by Las occurred at 5 and 8 wpi (Figure 2-16A, B, C, D). For X639, the opposite linear regression between P.n. infection incidence and total sugar concentration caused by Las occurred
at 5 wpi (Figure 2-17A, B). The opposite linear relationship between \textit{P.n.} infection incidence and total sugar concentration caused by Las occurred at 11 wpi for sour orange (Figure 18C, D).

**2.4 Discussion**

As previously hypothesized in this study, Las significantly altered \textit{P.n.} root infection incidence. Moreover, Las increased \textit{P.n.} root infection incidence at certain time points after inoculation, but also significantly reduced infection incidence at other time points after inoculation. Assessment of fibrous root biomass confirmed that each pathogen reduced root biomass accumulation compared to non-inoculated controls, and \textit{P.n.} reduced root biomass faster than Las. Suprisingly, the combination of Las and \textit{P.n.} did not significantly reduce the fibrous root biomass more than each root pathogen alone as hypothesized. The significant interaction of Las and \textit{P.n.} on root biomass shows that reduction in available roots was produced by each pathogen alone, no additional root damage was observed after inoculation with both pathogens.

Linear regression between fibrous root biomass and \textit{P.n.} infection incidence revealed that these two parameters interacted in two ways. First, fibrous root biomass decreased with increased \textit{P.n.} infection as more damage was expressed. Second, \textit{P.n.} infection had a positive relationship with fibrous root growth as the reproduction of \textit{P.n.} depends on root availability. These relationships indicate \textit{P.n.} infection either preceded or lagged behind changes in root biomass depending on the timing of the infection process (Figure 19).

To determine the basis for how Las increases \textit{P.n.} infection incidence, root exudation of sucrose and starch concentration in roots were assessed at each harvest. Sucrose concentration in root exudates from Las-infected seedlings was always higher
than from non-inoculated controls. Greater sucrose exudation into the rhizosphere enhances attraction of zoospores and promotes root penetration because this sugar is the key carbohydrate source for *P.n.* (Duncan et al., 1993). Starch is the storage form of non-structural carbohydrate in roots. Lower starch concentration in roots of Las-infected seedlings indicates a loss of root vitality which increases susceptibility to *P.n.* infection.

Jonsson (2006) proposed that elevation of sugar concentration increases *Quercus robur* tolerance to *Phytophthora quercina*, whereas Angay et al. (2014) found a positive relationship between *Phytophthora quercina* infection and availability of non-structural sugar in *Quercus robur*. Sucrose is the main form of transport sugar and starch is the main carbohydrate reserve in roots. Therefore, these carbohydrates may be used to evaluate the relationship between non-structural sugars and the susceptibility to *P.n.* infection. In this study, the relationship changed over time, indicating a complex interaction between the pathogens, alone or combined, and root susceptibility to infection. Cleopatra mandarin seedlings inoculated with *P.n.* had a higher sugar concentration with lower *P.n.* infection incidence, which is consistent with Jonsson’s model that available sugar makes the host more tolerant to *Phytophthora* infection. In contrast, when Las interacted with *P.n.* there was a positive relationship between *P.n.* infection and total sugar in co-inoculated Cleopatra mandarin seedlings. Higher sugar concentration was related with higher *P.n.* infection incidence in Las-inoculated seedlings, which is consistent with the finding of Angay et al. (2014) that higher sugar concentration is a benchmark for plant susceptibility to pathogen infection. However, the relationship between *P.n.* infection and sugar concentration was not always positive or negative when Las was or was not present. This indicates that the
sugar availability is too dynamic to be used as a predictor of plant tolerance to *P.n.* infection at different disease development stages. Based on these results, we conclude that Las increase of root susceptibility is attributed to the greater release of root exudates that attract *P.n.* zoospores and/or to Las-mediated disruption of sugar metabolism that interferes with plant response to *P.n.* infection.

Rootstocks are defined as tolerant or susceptible to *P.n.* based on whether their root biomass is or is not maintained by replacement root growth in the presence of *P.n.* (Graham, 1995). Four rootstocks that range from susceptible to tolerant to *P.n.* were screened to determine the effect of Las on *P.n.* infection of citrus. *P.n.* infection incidence for four rootstocks was around 60%. Slightly higher *P.n.* infection incidence was detected for Cleopatra mandarin than Swingle citrumelo, X639 and sour orange, but no significant difference was found among rootstocks. However, temporal changes of *P.n.* infection incidence of Las-infected seedlings differed for Swingle citrumelo and sour orange compared to Cleopatra mandarin and X639. *P.n.* infection incidence rebounded at 11 wpi compared to 8 wpi for Cleopatra mandarin and X639. In contrast, Swingle citrumelo and sour orange decreased in *P.n.* infection incidence by 11 wpi, which suggests that these two rootstocks showed some tolerance to *P.n.* infection when infected with Las. Cleopatra mandarin showed a steeper slope (Figure 2-14, 15) of the linear regression between *P.n.* infection incidence and sugar concentration for seedlings with and without Las compared to Swingle citrumelo (Figure 2-16), followed by X639 (Figure 2-17) and sour orange (Figure 2-18), which indicates that the *P.n.* infection is more responsive to sugar concentration in roots for Cleopatra mandarin than for Swingle citrumelo, X639 and sour range. Additionally, Las consistently altered the
relationship between *P. n.* infection incidence and sugar concentration for Cleopatra mandarin seedlings. By comparison, this condition was not repeatedly observed in other rootstocks, indicating the higher susceptibility of Cleopatra mandarin to Las and *P. n.*

Comparison of the four pathogen inoculation conditions, +/- *P. n.* or +/- Las, demonstrated that the damage caused by the interaction of the two pathogens did not exceed that of either Las or *P. n.* alone depending on the stages of disease development. Greater *P. n.* infection incidence of Las-infected roots at 5 wpi suggests that Las predisposes the roots to *P. n.* A decline in *P. n.* infection following reduction in root biomass and root replacement indicates there is a lag between *P. n.* infection and fibrous root loss. The contrasting relationship between sugar concentration and *P. n.* infection for *P. n.*-infected and co-inoculation seedlings indicates that Las disrupts carbohydrate metabolism in roots and increases root susceptibility to *P. n.*

Based on these results we hypothesize (Figure 20):

1) Early in disease development, Las increases susceptibility to *P. n.* infection by increasing zoospore attraction, facilitating penetration.

2) Las and its interaction with *P. n.* increases root loss resulting in a temporary drop in *P. n.* population by reducing available infection sites and/or available carbohydrate.

3) As new root flushes occur, Las moves down to the root tips with the phloem flow, *P. n.* repeats the infection cycle until there is a complete loss of fibrous root system.
Figure 2-1. *Phytophthora nicotianae* (*P.n.*) infection incidence of Cleopatra mandarin seedlings at 5, 8 and 11 weeks post inoculation (wpi). A) 2013 trial, B) 2014 trial. Treatments: inoculation with *P.n.* (*Las-Pn+*); co-inoculation of *Candidatus Liberibacter asiaticus* (*Las*) and *P.n.* (*Las+Pn+*). Different letters denote significant difference at $P \leq 0.05$. Bars represent the mean of 4-8 replicates.
Figure 2-2. *Phytophthora nicotianae* (*P.n.*) infection incidence of A) Swingle citrumelo, B) X639 and C) sour orange seedlings at 5, 8 and 11 weeks post inoculation (wpi). Treatments: inoculation with *P.n.* (Las-Pn+); co-inoculation of *Candidatus Liberibacter asiaticus* (Las) and *P.n.* (Las+Pn+). Different letters denote significant difference at $P \leq 0.05$. Bars represent the mean of 4-8 replicates.
Figure 2-3. Fibrous root biomass of Cleopatra mandarin seedlings at 5, 8 and 11 weeks post inoculation (wpi). A) 2013 trial, B) 2014 trial. Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotiana (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). Different letters denote significant difference at $P \leq 0.05$. * Denotes significant interaction at $P \leq 0.05$. Bars represent the mean of 5-8 replicates.
Figure 2-4. Fibrous root biomass of A) Swingle citrumelo, B) X639 and C) sour orange seedlings at 5, 8 and 11 weeks post inoculation (wpi). Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn+); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). Different letters denote significant difference at $P \leq 0.05$. * Denotes significant interaction at $P \leq 0.05$. Bars represent the mean of 5-8 replicates.
Figure 2-5. Cleopatra mandarin 2013. Linear regression of *Phytophthora nicotianae* (*P.n.*) infection incidence and fibrous root biomass at 5, 8 and 11 weeks post inoculation (wpi). Treatments: co-inoculation of *Candidatus Liberibacter asiaticus* (Las) and *P.n.* (Las+Pn+); inoculation with *P.n.* (Las-Pn+). 5 wpi: A) Las+Pn+, B) Las-Pn+; 8 wpi: C) Las+Pn+, D) Las-Pn+; 11 wpi: E) Las+Pn+, F) Las-Pn+. Points represent the value of 6-9 replicates.
Figure 2-6. Cleopatra mandarin 2014. Linear regression of *Phytophthora nicotianae* (*P.n.*) infection incidence and fibrous root biomass at 5 and 11 weeks post inoculation (wpi). Treatments: co-inoculation of *Candidatus Liberibacter asiaticus* (Las) and *P.n.* (Las+Pn+); inoculation with *P.n.* (Las-Pn+). 5 wpi: A) Las+Pn+, B) Las-Pn+; 8 wpi: C) Las+Pn+, D) Las-Pn+. Points represent the value of 6-9 replicates.
Figure 2-7. Swingle citrumelo. Linear regression of *Phytophthora nicotianae* (*P.n.*) infection incidence and fibrous root biomass at 5, 8 and 11 weeks post inoculation (wpi). Treatments: co-inoculation of *Candidatus Liberibacter asiaticus* (Las) and *P.n.* (Las+Pn+); inoculation with *P.n.* (Las-Pn+). 5 wpi: A) Las+Pn+, B) Las-Pn+; 8 wpi: C) Las+Pn+, D) Las-Pn+; 11 wpi: E) Las+Pn+, F) Las-Pn+. Points represent the value of 6-9 replicates.
Figure 2-8. X639. Linear regression of *Phytophthora nicotianae* (*P.n.*) infection incidence and fibrous root biomass at 5, 8 and 11 weeks post inoculation (wpi). Treatments: co-inoculation of *Candidatus Liberibacter asiaticus* (Las) and *P.n.* (Las+Pn+); inoculation with *P.n.* (Las-Pn+). 5 wpi: A) Las+Pn+, B) Las-Pn+; 8 wpi: C) Las+Pn+, D) Las-Pn+; 11 wpi: E) Las+Pn+, F) Las-Pn+. Points represent the value of 4-6 replicates.
Figure 2-9. Sour orange. Linear regression of *Phytophthora nicotianae* (*P.n.*) infection incidence and fibrous root biomass at 5 and 8 weeks post inoculation (wpi). Treatments: co-inoculation of *Candidatus Liberibacter asiaticus* (Las) and *P.n.* (Las+Pn+); inoculation with *P.n.* (Las-Pn+). 5 wpi: A) Las+Pn+, B) Las-Pn+; 8 wpi: C) Las+Pn+, D) Las-Pn+. Points represent the value of 5-7 replicates.
Figure 2-10. Sucrose exudation of Cleopatra mandarin seedlings at 5, 8 and 11 weeks post inoculation (wpi). A) 2013 trial, B) 2014 trial. Treatments: inoculation with *Candidatus* Liberibacter asiaticus (Las+); non-inoculated (Las-). Different letters denote significant difference at $P \leq 0.05$. Bars represent the mean of 4-7 replicates.
Figure 2-11. Sucrose exudation of A) Swingle citrumelo, B) X639 and C) sour orange seedlings at 5, 8 and 11 weeks post inoculation (wpi). Treatments: inoculation with *Candidatus* Liberibacter asiaticus (Las+); non-inoculated (Las-). Different letters denote significant difference at $P \leq 0.05$. Bars represent the mean of 4-7 replicates.
Figure 2-12. Fibrous root starch concentration of Cleopatra mandarin at 5, 8 and 11 weeks after inoculation with Phytophthora nicotianae (P.n.). A) 2013 trial, B) 2014 trial. Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with P.n. (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn -). Different letters denote significant difference at $P \leq 0.05$. * Denotes significant interaction at $P \leq 0.05$. Bars represent the mean of 4-8 replicates.
Figure 2-13. Fibrous root starch concentration of A) Swingle citrumelo, B) X639 and C) sour orange at 5, 8 and 11 weeks post inoculation with *Phytophthora nicotianae* (*P.n.*). Treatments: Inoculation with *Candidatus Liberibacter asiaticus* (Las) (Las+Pn-), inoculation with *P.n.* (Las-Pn +); co-inoculation of Las and *P.n.* (Las+Pn+); non-inoculated (Las-Pn-). Different letters denote significant difference at $P \leq 0.05$. * Denotes significant interaction at $P \leq 0.05$. Bars represent the mean of 4-8 replicates.
Figure 2-14. Cleopatra mandarin 2013. Linear regression of *Phytophthora nicotianae* (*P.n.*) infection incidence and Total available sugar at 5, 8 and 11 weeks post inoculation (wpi). Treatments: co-inoculation of *Candidatus Liberibacter asiaticus* (Las) and *P.n.* (Las+Pn+); inoculation with *P.n.* (Las-Pn+). 5 wpi: A) Las+Pn+, B) Las-Pn+; 8 wpi: C) Las+Pn+, D) Las-Pn+; 11 wpi: E) Las+Pn+, F) Las-Pn+. Points represent the value of 5-9 replicates.
Figure 2-15. Cleopatra mandarin 2014. Linear regression of *Phytophthora nicotianae* (*P.n.*) infection incidence and Total available sugar at 5 and 11 weeks post inoculation (wpi). Treatments: co-inoculation of *Candidatus Liberibacter asiaticus* (Las) and *P.n.* (Las+Pn+); inoculation with *P.n.* (Las-Pn+). 5 wpi: A) Las+Pn+, B) Las-Pn+; 8 wpi: C) Las+Pn+, D) Las-Pn+. Points represent the value of 5-7 replicates.
Figure 2-16. Swingle citrumelo. Linear regression of *Phytophthora nicotianae (P.n.)* infection incidence and Total available sugar at 5, 8 and 11 weeks post inoculation (wpi). Treatments: co-inoculation of *Candidatus Liberibacter asiaticus* (Las) and *P.n.* (Las+Pn+); inoculation with *P.n.* (Las-Pn+). 5 wpi: A) Las+Pn+, B) Las-Pn+; 8 wpi: C) Las+Pn+, D) Las-Pn+; 11 wpi: E) Las+Pn+, F) Las-Pn+. Points represent the value of 6-8 replicates.
Figure 2-17. X639. Linear regression of *Phytophthora nicotianae* (*P.n.*) infection incidence and Total available sugar at 5, 8 and 11 weeks post inoculation (wpi). Treatments: co-inoculation of *Candidatus Liberibacter asiaticus* (Las) and *P.n.* (Las+Pn+); inoculation with *P.n.* (Las-Pn+). 5 wpi: A) Las+Pn+, B) Las-Pn+; 8 wpi: C) Las+Pn+, D) Las-Pn+; 11 wpi: E) Las+Pn+, F) Las-Pn+. Points represent the value of 3-7 replicates.
Figure 2-18. Sour orange. Linear regression of *Phytophthora nicotianae* (*P.n.*) infection incidence and Total available sugar at 5 and 8 weeks post inoculation (wpi). Treatments: co-inoculation of *Candidatus Liberibacter asiaticus* (Las) and *P.n.* (Las+Pn+); inoculation with *P.n.* (Las-Pn+). 5 wpi: A) Las+Pn+, B) Las-Pn+; 8 wpi: C) Las+Pn+, D) Las-Pn+. Points represent the value of 4-6 replicates.
Figure 2-19. Model for the relationship between *Phytophthora nicotianae* (*P.n.*) infection and root biomass. The dashed line represents fibrous root biomass, and the solid line represents *P.n.* infection incidence.
Figure 2-20. Hypothesis for the interaction between *Candidatus* Liberibacter asiaticus (Las), *Phytophthora nicotianae* (*P.n.*) and root. Blue dot represents Las; red dot represents *P.n.* in rhizosphere; red strap represents root. Step 1: Las moves down to the root, root structure and root exudation was changed, and *P.n.* was attracted to swim to root surface and penetrated through outer cortex. Step 2: *P.n.* infection increased because of enhanced penetration and decreased plant tolerance to *P.n.*; Step 3: Root decline out of damage caused by the interaction, both Las and *P.n.* population on root were decreased with root loss; Step 4: During root replacement, Las moves down to root with phloem sap and *P.n.* infection and interaction with Las cycle repeated; Step 5 and 6 repeated step 3 and 4, until there is a complete loss of fibrous root system.
CHAPTER 3
COMPARISON OF FIBROUS ROOT DAMAGE ON CITRUS ROOTSTOCKS CAUSED
BY CANDIDATUS LIBERIBACTER ASIATICUS AND PHYTOPHTHORA NICOTIANAE

3.1 Introduction

Citrus trees absorb water and mineral nutrients in soil through fibrous roots (diameter < 2mm). Fibrous root bunches develop by branching, elongating and maturation. As they age, citrus fibrous roots change color from white to brown (Fitter, 2002; Wells and Eissenstat, 2003). Roots change biochemically and structurally in response to normal development and environmental conditions (Duncan et al., 1993; Eissenstat and Achor, 1999). Phytophthora spp. are rhizosphere-inhabiting pathogens that can rapidly infect and kill citrus fibrous roots (Kosola et al., 1999). To maintain water and mineral nutrients uptake, the tree produces new roots for replacement of roots lost due to biotic and abiotic stresses (Graham, 1995).

Whereas, root loss and replacement might cause lower fruit yield and quality as a larger amounts of photoassimilate are allocated for root replacement (Eissenstat et al., 2000). Hence, understanding the relationship between fibrous root damage and canopy development in relation to pathogen infection may provide useful information for root disease management and reduce fruit yield loss.

Huanglongbing (HLB), caused by the phloem-limited bacterium Candidatus Liberibacter asiaticus (Las) (Jagoueix et al., 1994; Teixeira et al., 2005), is the most devastating disease of citrus worldwide (Bové, 2006). The most interesting finding from previous study of the root status of Las-infected seedlings is that Las not only causes root damage, but also increases root production at certain times, which led to further investigation of the mechanisms for these responses and the consequence of reduced canopy development.
Linkages of root traits to root function, and leaf traits to leaf function have previously been used to predict plant adaptation to environmental conditions (Eissenstat et al., 2000; Eissenstat and Achor, 1999). Selection of the appropriate root and leaf traits for evaluation is critical to obtain a reliable estimate of host response to pathogen infection. Root length and root color provide the most current information about root growth and viability (Fitter, 2002). These measures reveal how the pathogen reduces root health, either by inhibiting new root growth, facilitating root collapse, or both. To measure the influence of root damage on canopy development, characters like total leaf area and specific leaf area (SLA) are used to estimate photosynthetic capacity and leaf morphology response. SLA is defined as the leaf area divided by dry biomass (Wilson et al., 1999). SLA represents a plant strategy to modulate the investment in leaf area per unit biomass under different conditions (Evans and Poorter, 2001; Wilson et al., 1999). Higher SLA represents greater leaf area per unit biomass to capture more light for photosynthesis.

Based on Chapter 2 results, we hypothesize that: 1) Las damages citrus root by increasing carbohydrate allocation which induces faster root growth and turnover; 2) _P. n._ damages fibrous roots by causing more rapid root collapse immediately after infection, accelerating collapse compared to roots infected with Las; 3) canopy development will be inhibited after root function is limited by both pathogens. To test these hypotheses, we measured total root length, fibrous root length by diameter (<2mm), new root length, fibrous root sucrose, total leaf area and specific leaf area after inoculation with Las or _P. n._ The overall goal is to understand root disease development.
from the whole plant perspective to improve management of root health on HLB-affected trees.

3.2 Materials and Methods

3.2.1 Plant Materials and Inoculation with Candidatus Liberibacter asiaticus

Seeds of Cleopatra mandarin (C. reticulata) were sown in Metro Mix 500 (Hummert International, MO, USA) in 120 cm³ containers in the greenhouse. Seedlings were fertilized with Jack’s Pro 20-20-20 (Liquid) (A.M. Leonard, Inc., OH, USA) and Harrell’s 18-5-10 (Harrell’s, FL, USA) every other week and every 6 mo, respectively, and watered 3 times per week. One hundred and twenty-six mo-old seedlings were graft inoculated with budwood from Las-infected trees. The budwood was from greenhouse grown ‘Madam Vinous’ sweet orange (Citrus sinensis) trees infected with Las isolate FC6 as previously described (Folimonova et al., 2009). Six months after inoculation (April 8, 2013; May 13, 2014), 60 Las-infected Cleopatra mandarin seedlings (PCR confirmed) and 60 non-inoculated seedlings were selected for uniform size, and transferred into autoclaved Candler fine sand in 120 cm³ containers for four treatments P.n. +/- and Las+/-(10 replicates for each treatment) and 3 harvest times. In 2014, this protocol was repeated for Swingle citrumelo (August 12, 2014) and X639 (August 5, 2014).

3.2.2 Phytophthora nicotianae – Candidatus Liberibacter asiaticus interaction

P. nicotianae (P.n. 198) isolated from declining Cleopatra mandarin in the greenhouse in 1999 was maintained on clarified V8 juice agar. Chlamydospores were produced for inoculum of P.n. by the method of Tsao (1971). For inoculum preparation, chlamydospores were blended, added to Candler fine sand and mixed manually. Soil inoculum was diluted 1:10 in water agar, incubated at room temperature in the dark for
2 days on pimaricin-ampicillin-rifampcin-pentachloronitrobenzene-hymexazol (PARPH) semi-selective agar medium for assay of propagule density. An inoculum density of 20 propagules/cm³ of soil was obtained by mixing the inoculum concentrate with autoclaved Candler fine sand. Seedling roots were pruned to a uniform length (8cm) to induce root growth. 30 Las-infected and 30 non-inoculated seedlings were transplanted into soil infested with *P. n.* and 30 Las-infected and 30 non-infected seedlings were transplanted into autoclaved soil. Seedlings infected and non-infected with *P. n.* were located on different benches in greenhouse (27°C-32°C), watered 3 times a week and fertilized with Jack’s Pro 20-20-20 every other week.

At 5, 8 and 11 weeks post inoculation (wpi) with *P. n.*, seedlings roots were thoroughly watered and harvested 24 h after irrigation. During harvest, roots were washed and 20 fibrous root tips were randomly selected from *P. n.* infected/non infected seedlings and plated on PARPH medium. Plated root tips were incubated in dark for 3-4 d, and the number of *P. n.* positive tips of *P. n.* infected seedlings was recorded and contamination was checked for non-inoculated seedlings. Roots and leaves detached from the seedlings were evenly spread on the blue background of an Epson 37 scanner (scale: 100%, dpi: 600, and Bit depth: 24) to obtain digital color photographs (TIFF image) for each sample. Fibrous roots (diameter ≤ 2mm) and leaves were separated, dried and weighed (70°C for 48 h).

**3.2.3 Root and Leaf Morphology Analysis**

The photographs were analyzed by WinRhizo Pro (Regent Instrument Inc, Canada) to measure root length, root diameter and leaf area. Root diameter classes were as follows: 0-0.2 mm, 0.2-0.4 mm, 0.4-0.6 mm, 0.6-0.8 mm, 0.8-1.0 mm, 1.0-1.2 mm, 1.2-1.4 mm, 1.4-1.6 mm, 1.6-1.8 mm and 1.8-2.0 mm. Color classes were set as
follows: white for new roots, green for leaves, and blue for background. Root length and leaf area were calculated based on the sum of the pixels of each color. Specific leaf area was expressed as the total leaf area divided by dry leaf weight.

3.2.4 *Candidatus Liberibacter asiaticus* Detection

To detect Las in leaves, midribs were chopped into cubic segments, placed in 2 mL screw cap tubes with one 5-mm stainless steel bead (Qiagen, Valencia, CA, USA). The tubes were stored at -80°C for at least 2 h and after removal from the freezer immediately ground twice at 30 revolutions per sec in a Tissuelyzer II (Qiagen, Valencia, CA, USA). Ground midrib tissue was then processed for DNA extraction using the Qiagen (Valencia, CA, USA) DNeasy Plant Mini kit according to the manufacturer’s instructions, except that DNA was eluted twice by 50 μl AE buffer instead of once by 100 μl at last step (Johnson et al., 2014). Randomly selected fibrous roots were cut and ground as described above for the midrib tissue. Root DNA was extracted using the Mo Bio PowerSoil DNA Isolation kit (Mo. Bio. Laboratories) as previously described (Johnson et al., 2014). The first step of protocol was modified as initial buffer, garnet and solution C1 was added into 2 ml screw cap tubes which were used for grinding and then vortexed for 5 sec instead of 10 min (Johnson et al., 2014). Purified DNA was stored at -20°C for later analysis.

qPCR for Las detection. qPCR was carried out using primers and probes as previously described (Wang et al., 2006). qPCR reaction was run on 7500 Fast Real-Time PCR System. The master mix included Qiagen HotStar Taq, ROX passive reference dye (Bio-Rad), primers, probe and buffer in a concentration ratio as described by the manufacturer’s protocol. 4 μL of template DNA and 16 μL master mix were loaded into each well of a 96 micro-well plate, and each sample was replicated.
Samples were run in triplicate if significant variation was found between 2 replicates in the initial assay. The standard curve included concentrations of las plasmids from $1 \times 10^1$ to $1 \times 10^6$ copies.

### 3.2.5 Quantification of Sucrose in Fibrous Roots

A 25 mg subsample of dried fibrous root was randomly selected for sucrose analysis, and processed by a modified method previously described (Rosales and Burns, 2011). Fibrous roots were cut and ground in 2 ml tubes as described above for DNA extraction. A 900 μl aliquot of DI water was mixed with the root powder. The solution of ground roots or standard was vortexed and boiled for 10 min. Samples were centrifuged for 2 min at 2500 x G. A 300 μl aliquot of supernatant was stored at -20°C for sucrose assay. A 4μl aliquot of supernatant from each sample was processed according to the manufacturer’s protocol for the Glucose and Sucrose Colorimetric/Fluormetric Assay (Sigma-Aldrich). Sucrose concentration in fibrous roots was tested with the same kit.

### 3.2.6 Statistical Analysis

Total root length, root length by diameter, new root length, root sucrose, total leaf area and SLA were analyzed by one-way ANOVA using PROC GLM (SAS v. 9.4). The interaction between Las and *P.n.* was analyzed by two-way ANOVA using PROC GLM (SAS v. 9.4). The significance level was set at $P \leq 0.05$.

### 3.3 Results

#### 3.3.1 Total Root Length

Cleopatra mandarin rootstock. A significant interaction between Las and *P.n.* for total root length occurred at 5 and 8 wpi ($P \leq 0.05$). Total root length of non-inoculated Cleopatra mandarin roots increased from 681 to 982 cm between 5 and 11 wpi (Fig 3-
For Las-inoculated seedlings, total root length decreased 41% from 886 to 520 cm between 5 and 11 wpi. At 5 wpi, P.n.-inoculated seedlings with or without Las had lower total root length ($P \leq 0.05$) than seedlings inoculated with Las only. Total root length for Las-inoculated seedlings was 30% higher ($P \leq 0.05$) than the non-inoculated controls at 5 wpi, but by 11 wpi total root length was 47% lower ($P \leq 0.05$) than the non-inoculated controls. Total root length of P.n.-inoculated seedlings by 11 wpi was lower ($P \leq 0.05$) compared to non-inoculated controls. At 11 wpi, P.n.-inoculated seedlings were higher ($P \leq 0.05$) in total root length than Las-inoculated seedlings. Total root length of seedlings inoculated with both Las and P.n. was lower compared to non-inoculated seedlings at 11 wpi ($P \leq 0.05$).

Swingle citrumelo rootstock. A significant interaction between Las and P.n. for total root length occurred at 8 and 11 wpi ($P \leq 0.05$). Total root length of non-inoculated Swingle citrumelo roots increased from 345 to 608 cm between 5 and 11 wpi (Figure 3-1B). For Las-inoculated seedlings total root length decreased 26% from 461 to 341 cm between 5 and 11 wpi. Total root length of P.n.-infected seedlings increased 18% between 5 and 11 wpi. Total root length for P.n.-inoculated seedlings with or without Las infection was lower ($P \leq 0.05$) than for seedlings inoculated with Las from 5 to 11 wpi. Las-inoculated seedlings were 34% higher ($P \leq 0.05$) in total root length than the controls at 5 wpi, but 44% lower ($P \leq 0.05$) by 11 wpi. The total root length of seedlings inoculated with both Las and P.n. was comparable to seedlings inoculated with P.n., but lower ($P \leq 0.05$) than for Las-inoculated seedlings.

X639 rootstock. A significant interaction between Las and P.n. for total root length occurred at 8 wpi ($P \leq 0.05$). Total root length of non-inoculated X639 roots
increased from 617 to 921 cm between 5 and 11 wpi (Figure 3-1C). Total root length for 
P.n.-inoculated seedlings was significantly ($P \leq 0.05$) lower than for seedlings 
inoculated with Las at all harvests. Total root length of Las-inoculated seedlings was 
22% and 13% lower than non-inoculated seedlings at 8 ($P \leq 0.05$) and 11 wpi ($P \leq 
0.05$), respectively. The total root length of seedlings inoculated with both Las and 
P.n. was significantly lower ($P \leq 0.05$) at 11 wpi compared to seedlings inoculated with Las.

3.3.2 Root Length and Diameter Class

Cleopatra mandarin rootstock. The greatest root length fell into 0.6-0.8 mm 
diameter class for all treatments (Figure 3-2A, B, C). Root length in all diameter classes 
for non-inoculated seedlings increased from 5 to 11 wpi. Root length in 0.4-1.2 mm 
diameter size class comprised 63%, 65% and 51% of the total fibrous root length at 5, 8 
and 11 wpi, respectively. For Las-inoculated seedlings, length of roots for all diameter 
classes was higher compared to non-inoculated seedlings at 5 wpi (significantly higher 
for the 0.8-1.4 and 1.8-2 mm diameter size class) (Figure 3-2A) and lower at 8 
(significantly lower for the 0.4-0.6mm diameter size class) (Figure 3-2B) and 11 
(significantly lower for the 0.4-2 mm diameter size class) wpi (Figure 3-2C). Length of 
roots for all diameter classes of P.n.-inoculated seedlings was lower than the non-
inoculated and Las-inoculated seedlings at 5 wpi (significantly lower in 0.2-1.0 mm 
diameter size class) (Figure 3-2 A). By 11 wpi (Figure 3-2 C), non-inoculated seedlings 
had the highest length of roots for all diameter classes followed by P.n.-inoculated 
seedlings and Las-inoculated seedlings. Seedlings inoculated with both Las and P.n. 
compared with seedlings inoculated with each pathogen alone had lower length of roots 
in 0.8-2 mm (significant for the 0.8-1.0 and 1.2 -2mm diameter size classes) compared 
to other treatments at 5 (Figure 3-2 A) and 11 wpi (Figure 3-2 C), respectively.
Swingle citrumelo rootstock. The greatest root length fell into the 0.4-0.6 and 0.6-0.8 mm diameter classes for all treatments (Figure 3-3). Root length for non-inoculated seedlings in the 0.2-1.4 mm diameter classes increased up to 11 wpi (Figure 3-3A, B, C). Root length in the 0.4-0.8 mm diameter class for non-inoculated seedlings represented almost 60% of total fibrous root length at 5, 8 and 11 wpi (Figure 3-3A, B, C). Las-inoculated seedlings had a higher ($P \leq 0.05$) and lower ($P \leq 0.05$) root length for all diameter classes than non-inoculated seedlings at 5 (Figure 3-3A) and 8 wpi respectively (non significant lower root length in 1.2-1.4 mm diameter size class) (Figure 3-3B), respectively. *P.n.*-inoculated seedlings had a lower root length ($P \leq 0.05$) of roots for all diameter classes than non-inoculated seedlings for all harvest times (Figure 3-3A, B, C). Seedlings inoculated with both Las and *P.n.* had a lower root length ($P \leq 0.05$) for all diameter classes compared with non-inoculated seedlings and seedlings inoculated with Las at all three harvests. The difference in root length for all diameter classes between co-inoculated seedlings and *P.n.*-inoculated seedlings was not significant.

X639 rootstock. The highest root length for X639 seedlings fell into the 0.4-0.6 and 0.6-0.8 mm diameter classes in all treatments (Figure 3-4). The length of roots in the 0.4-0.6 mm diameter class for non-inoculated seedlings increased up to 11 wpi. *P.n.* inoculation reduced root length ($P \leq 0.05$) for all diameter classes compared to non-inoculated and Las-inoculated seedlings (Figure 3-4A, B, C). Seedlings inoculated with both Las and *P.n.* had a similar root length for all diameter classes compared to seedlings inoculated with *P.n.* alone.

### 3.3.3 New Root Length

Cleopatra mandarin rootstock. A significant interaction between Las and *P.n.* for new root length occurred at 11 wpi ($P \leq 0.05$). New root length for non-inoculated
seedlings of Cleopatra mandarin increased from 187 to 295 cm between 5 and 11 wpi (Figure 3-5A). Las-inoculated seedlings produced more new root length ($P \leq 0.05$) than the non-inoculated controls at 5 wpi (Figure 3-5A). *P.n.*-inoculated seedlings produced less ($P \leq 0.05$) new root length compared to the non-inoculated controls at 8 and 11 wpi. Seedlings inoculated with both pathogens had similar new root length of *P.n.*-inoculated seedlings at all three harvests.

Swingle citrumelo rootstock. A significant interaction between Las and *P.n.* for new root length occurred at 8 and 11 wpi ($P \leq 0.05$). New root length for non-inoculated seedlings of Swingle citrumelo increased from 186 to 296 cm between 5 and 11 wpi (Figure 3-5B). Las-inoculated seedlings produced less ($P \leq 0.05$) new root length compared to non-inoculated seedlings at 8 and 11 wpi. *P.n.*-inoculated seedlings produced less new root length ($P \leq 0.05$) compared to non-inoculated seedlings at all three harvests. Seedlings inoculated with both pathogens had similar new root length of *P.n.*-inoculated seedlings.

X639 rootstock. A significant interaction between Las and *P.n.* for new root length occurred at 5 and 8 wpi ($P \leq 0.05$). New root length for non-inoculated seedlings of X639 increased from 228 to 305 cm between 5 and 11 wpi (Figure 3-5C). Las-inoculated seedlings produced more ($P \leq 0.05$) new root length than the non-inoculated controls at 5 wpi. *P.n.*-inoculated seedlings had less new root length ($P \leq 0.05$) than Las-inoculated at all three harvests. Seedlings inoculated with both pathogens had similar new root length of *P.n.*-inoculated seedlings.

**3.3.4 Fibrous Root Sucrose**

Cleopatra mandarin rootstock. A significant interaction between Las and *P.n.* for fibrous root sucrose occurred at 5 and 8 wpi ($P \leq 0.05$). Fibrous root sucrose of non-
inoculated Cleopatra mandarin ranged from 12 to 20 mg/g (Figure 3-6A). Las-infected roots had lower sucrose ($P \leq 0.05$) than non-inoculated roots at 5 and 8 wpi. *P.n.*-infected roots had lower sucrose ($P \leq 0.05$) than non-inoculated roots at 5 and 8 wpi. Roots infected with Las and *P.n.* had comparable sucrose to roots infected with Las and *P.n.* alone at all three harvests.

Swingle citrumelo rootstock. A significant interaction between Las and *P.n.* for fibrous root sucrose occurred at 8 wpi ($P \leq 0.05$). Fibrous root sucrose of non-inoculated Swingle citrumelo ranged from 18 to 23 mg/g (Figure 3-6B). Las-infected roots had lower sucrose ($P \leq 0.05$) than non-inoculated roots at 8 wpi. *P.n.*-infected roots had higher sucrose ($P \leq 0.05$) than Las-infected roots at 8 wpi.

X639 rootstock. Fibrous root sucrose of non-inoculated X639 ranged from 14 to 20 mg/g (Figure 3-6C). Las-infected roots had higher sucrose ($P \leq 0.05$) than non-inoculated roots at 8 wpi. Fibrous root sucrose in *P.n.*-infected roots was lower ($P \leq 0.05$) than in non-inoculated roots at 5 and 11 wpi. Roots infected with Las and *P.n.* had lower ($P \leq 0.05$) fibrous root sucrose compared to Las-inoculated and non-inoculated roots at 11 wpi.

### 3.3.5 Total Leaf Area

Cleopatra mandarin rootstock. Total leaf area for non-inoculated seedlings of Cleopatra mandarin increased from 519 to 568 cm$^2$ between 5 to 11 wpi (Figure 3-7A). Total leaf area of Las-inoculated seedlings increased from 462 to 550 cm$^2$ and *P.n.*-inoculated seedlings from 460 to 550 cm$^2$ between 5 to 11 wpi. The difference of total leaf area between Las-inoculated, *P.n.*-inoculated or co-inoculated seedlings was not significant.
Swingle citrumelo rootstock. Total leaf area for non-inoculated seedlings of Swingle citrumelo increased from 246 to 272 cm$^2$ between 5 and 11 wpi (Figure 3-7B). Total leaf area for Las-inoculated seedlings decreased from 254 to 216 cm$^2$ and $P.n.$ inoculated seedlings decreased from 228 to 159 cm$^2$ between 5 and 11 wpi. Seedlings inoculated with both pathogens declined in total leaf area from 263 to 183 cm$^2$ between 5 and 11 wpi. At 11 wpi, seedlings inoculated with each pathogen had lower total leaf area ($P \leq 0.05$) compared to non-inoculated seedlings. By 11 wpi, $P.n.$-inoculated seedlings with and without Las had less total leaf area ($P \leq 0.05$) than Las-inoculated seedlings.

X639 rootstock. A significant interaction between Las and $P.n.$ for total leaf area occurred at 8 wpi ($P \leq 0.05$). Total leaf area for non-inoculated seedlings of X639 increased from 273 to 351 cm$^2$ between 5 and 11 wpi (Figure 3-7C). Total leaf area for Las-inoculated seedlings decreased from 280 to 161 cm$^2$ and $P.n.$-inoculated seedlings decreased from 248 to 186 cm$^2$ between 5 and 11 wpi. Seedlings inoculated with each pathogen had lower total leaf area ($P \leq 0.05$) compared to non-inoculated seedlings at 11 wpi. Seedlings inoculated with both pathogens had the lowest total leaf area compared to all other treatments at 5 ($P \leq 0.05$) and 8 wpi (not significant compared to Las-inoculated seedlings).

3.3.6 Specific Leaf Area

Cleopatra mandarin rootstock. SLA for non-inoculated seedlings of Cleopatra mandarin increased from 109 to 122 cm$^2$/g (Figure 3-8A). SLA of Las-inoculated seedlings increased from 110 to 118 cm$^2$/g. SLA of $P.n.$-inoculated seedlings increased from 107 to 109 cm$^2$/g. Seedlings inoculated with either pathogen had a lower SLA compared to non-inoculated seedlings at 8 wpi ($P \leq 0.05$). The difference of SLA
between the non-inoculated control and Las-inoculated, \textit{P.n.-}inoculated seedlings or co-inoculated seedlings was not significant at 11 wpi.

Swingle citrumelo rootstock. SLA for non-inoculated seedlings of Swingle citrumelo increased from 148 to 159 cm$^2$/g (Figure 3-8B). SLA for Las-inoculated seedlings increased from 134 to 152 cm$^2$/g and for \textit{P.n.-}inoculated seedlings increased from 144 to 157 cm$^2$/g. Seedlings inoculated with Las with or without \textit{P.n.} infection had a lower SLA ($P \leq 0.05$) compared to non-inoculated controls at 5 wpi. Seedlings inoculated with both pathogens had the least SLA compared to all other treatments at 5 ($P \leq 0.05$) wpi.

X639 rootstock. SLA of non-inoculated seedlings of X639 increased from 127 to 136 cm$^2$/g (Figure 3-8C). SLA for Las-inoculated seedlings increased from 124 to 135 cm$^2$/g and for \textit{P.n.-}inoculated seedlings from 115 to 120 cm$^2$/g. Las and \textit{P.n.} inoculation did not significantly affect SLA compared to control seedlings.

3.4 Discussion

Kosola et al. (1999) used minirhizotrons to observe that \textit{P.n.} caused higher root mortality and reduced lifespan of fibrous roots within 6 weeks after \textit{P.n.} was present. In this study, we confirmed that \textit{P.n.} damaged fibrous roots within 5 weeks. In contrast with \textit{P.n.}, Las increased fibrous root length that was rapidly followed by root collapse. For example at 5 wpi, Las induced greater total root length in Cleopatra mandarin and Swingle citrumelo (Figure 3-1), indicating that Las increased carbohydrate allocation to fibrous roots. However by 8 and 11 wpi, Las infection reduced total root length of these two rootstocks compared to the control seedlings. \textit{P.n.} did not increase root length. The greater root growth, also called root replacement, was induced by \textit{P.n.} at low (1-2 propagules/cm$^3$) inoculum density, which is an explanation for tolerance of some
rootstocks (Graham, 1995). In this study, the lack of observation of *P. n.* induced root replacement is probably because we waited too long to harvest the plant to detect stimulation by *P. n.* or due to the rapid root decline caused by *P. n.* inhibited root replacement.

To determine if the effect on root length caused by Las and *P. n.* is specific for certain classes of root diameter size, we quantified root length by diameter class. Higher root length for Las-inoculated seedlings than for non-inoculated controls was observed for all root diameter classes for Cleopatra mandarin, Swingle citrumelo and X639. This indicates that Las infection increased root length irrespective of diameter class and rootstock. By comparison, *P. n.* with or without Las reduced length of all root diameter classes for these rootstocks at 5 wpi, which confirms that *P. n.* damages all size roots more rapidly and progressively than Las at this inoculum density (20 propagules/cm$^3$). Lower total root length of Las seedlings compared to *P. n.* seedlings of Cleopatra mandarin at 11 wpi suggests that Las caused more root damage than *P. n.* by 11 wpi for this rootstock. Greater total root length for Las-inoculated seedlings of X639 than for *P. n.* inoculated seedlings by 11 wpi indicates slower development of root damage by Las than by *P. n.* for this rootstock compared to Cleopatra mandarin.

New (white) root length of the three rootstocks seedlings was higher for Las-infected roots and lower for *P. n.*-infected roots than for non-inoculated seedlings at 5 wpi. This finding further confirms that Las initially increased new root accumulation and collapsed roots thereafter. Stimulation of root replacement by *P. n.* was not detected.

Sucrose was measured to evaluate the potential for Las to disrupt the phloem. Fluctuation of sucrose levels between 5 and 11 wpi for Cleopatra mandarin, Swingle
citrumelo and X639 confirms that phloem was functional during the experimental period in Las-infected seedlings. Lower sucrose content was detected in Cleopatra mandarin and Swingle citrumelo seedlings inoculated with Las than in non-inoculated controls at 5 wpi when significantly greater root length was observed in Las-infected seedlings than controls. This finding indicates that the bacterium interferes with sucrose metabolism of these rootstocks either by consuming sucrose, stimulating sugar consumption for plant defense, and/or increasing loss as root exudates.

Carbohydrate metabolism related gene expression in leaves, fruits, stems and roots at advanced stage of disease development has been reported (Albrecht and Bowman, 2008; Aritua et al., 2013; Kim et al., 2009; Martinelli et al., 2012). A large number of genes’ expression was significantly influenced in leaves by Las infection, indicating sugar metabolism was altered by Las (Kim et al. 2009). Different hypotheses for disease causation were proposed concerning starch accumulation (Kim et al., 2009; Liao and Burns, 2012). Liao and Burns (2012) suggested that development of HLB symptoms may directly result from the host response rather than as a consequence of carbohydrate starvation, by comparing the gene expression of HLB infected fruit and girdled fruit. Unfortunately, not much information on gene expression at early stage of HLB in roots is available. In this study, Las altered carbohydrate allocation to roots as sucrose content fluctuated over time. Investigation of sucrose synthesis and transportation and starch metabolism in roots at an early stage of HLB is required to understand Las-induced root replacement and the competition between Las, root and canopy for photoassimilates.
To assess the influence of root damage on canopy development, total leaf area and specific leaf area were measured. For Swingle citrumelo and X639, significantly less total leaf area compared to non-inoculated seedlings was detected at 11 wpi. This showed that canopy development was reduced by root damage caused by Las, *P.n.*, and the combination of both pathogens. Interestingly, less total leaf area caused by Las, *P.n.* and both was not detected for Cleopatra mandarin. SLA in Cleopatra mandarin seedlings was increased by the different pathogen treatments at 8 wpi, but not by 11 wpi. This indicated that leaf morphology was altered by the interaction between pathogens and roots but more study is needed to elucidate the mechanisms. The non-significant reduction in SLA of Swingle citrumelo and X639 suggest that more time may be needed to detect changes in leaf traits after root damage.

Similar interaction between Las and *P.n.* as found in Chapter 2 confirmed that *P.n.* causes root damage faster than Las, and that each pathogen reduces root length but no more reduction occurs after inoculation with both pathogens. Based on these findings, it is crucial to maintain root function for nutrient and water uptake and minimize stress from pathogen interactions and bicarbonates in soil (Graham et al., 2014). If damaging populations of *Phytophthora* are detected in groves, fungicide application may be considered to reduce *P.n.* infection. However, since the root damage is also caused by Las directly, reducing *P.n.* infection with fungicides may provide only marginal benefit for maintaining fibrous root health.
Figure 3-1. Total root length of A) Cleopatra mandarin, B) Swingle citrumelo and C) X639 seedlings at 5, 8 and 11 weeks post inoculation (wpi). Treatments: inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). Different letters denote significant difference at $P \leq 0.05$. * Denotes significant interaction at $P \leq 0.05$. Bars represent the means of 5-8 replicates.
Figure 3-2. Root length by diameter size class of Cleopatra mandarin at A) 5, B) 8 and C) 11 weeks post inoculation (wpi). Treatments: inoculation with *Candidatus Liberibacter asiaticus* (Las) (Las+Pn-), inoculation with *Phytophthora nicotianae* (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). Bars represent standard error of the mean of 5-9 replicates.
Figure 3-3. Root length by diameter size class of Swingle citrumelo at A) 5, B) 8 and C) 11 weeks post inoculation (wpi). Treatments: inoculation with *Candidatus Liberibacter asiaticus* (Las) (Las+Pn-), inoculation with *Phytophthora nicotianae* (P.n.) (Las-Pn+); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). Bars represent standard error of the mean of 5-9 replicates.
Figure 3-4. Root length by diameter size class of X639 at A) 5, B) 8 and C) 11 weeks post inoculation (wpi). Treatments: inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn+); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). Bars represent standard error the mean of 5-9 replicates.
Figure 3-5. New root length of A) Cleopatra mandarin, B) Swingle citrumelo and C) X639 seedlings at 5, 8 and 11 weeks post inoculation (wpi). Treatments: inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn -). Different letters denote significant difference at $P \leq 0.05$. * Denotes significant interaction at $P \leq 0.05$. Bars represent the means of 5-9 replicates.
Figure 3-6. Fibrous root sucrose of A) Cleopatra mandarin, B) Swingle citrumelo and C) X639 seedlings at 5, 8 and 11 weeks post inoculation (wpi). Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). Different letters denote significant difference at $P \leq 0.05$. * Denotes significant interaction at $P \leq 0.05$. Bars represent the means of 5-8 replicates.
Figure 3-7. Total leaf area of A) Cleopatra mandarin, B) Swingle citrumelo and C) X639 seedlings at 5, 8 and 11 weeks post inoculation (wpi). Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn+); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). Different letters denote significant difference at $P \leq 0.05$. * Denotes significant interaction at $P \leq 0.05$. Bars represent the means of 5-8 replicates.
Figure 3-8. Specific leaf area of A) Cleopatra mandarin, B) Swingle citrumelo and C) X639 seedlings at 5, 8 and 11 weeks post inoculation (wpi). Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). Different letters denote significant difference at $P \leq 0.05$. Bars represent the means of 5-8 replicates.
4.1 Introduction

Plants must maintain carbon assimilation, storage and usage to adjust to different developmental and environmental conditions (Smith and Stitt, 2007). The relationship of carbon assimilate, storage and usage has been studied in terms of a source (i.e. storage organs and source leaves) - sink (i.e. fruits, roots, shoots, new leaves, wounds and so on). Over allocation of carbon to one sink organ can reduce other sink organ’s growth (Eissenstat, 2000; Goren et al., 2010). Sink strength is believed to be the main factor that regulates carbon distribution within the plant (Kühn et al., 1999). Carbon distribution involves phloem loading, sap movement and phloem unloading (Koch, 2004). Citrus, like most other higher plants, uses sucrose as the main sugar form for carbon transport due to sucrose's stability and the few enzymes for degradation (Sauer, 2007; Zimmermann and Ziegler, 1975). Therefore, sucrose metabolism in sink organs is central to understanding carbohydrate transport and utilization processes. Sucrose can exit from sieve element-companion cell complex to nearby sink cells (phloem unloading) through symplastic (through plasmodesmata) and apoplastic pathways (through cell wall space). Many genes and enzymes are involved in this process (Koch, 2004; Sturm and Tang, 1999). In sink cells with intact plasmodesmata, symplastic flow is predominant (Koch, 2004). Through the cell wall space, sucrose may be transported into cytoplasm by sucrose transporters or cleaved by invertase to hexoses and then exported by hexose transporters (Sturm and Tang, 1999). Furthermore, sucrose cleavage by invertase (catalyzes the reaction:}
sucrose+$\text{H}_2\text{O} \rightarrow \text{fructose} + \text{glucose}$) and sucrose synthase (catalyzes the reverse reaction: sucrose +$\text{H}_2\text{O} \leftrightarrow \text{fructose} + \text{UDP-glucose}$) play pivotal roles in carbon partitioning rate and plant growth as different sugar concentration creates an osmotic pressure that further creates turgor pressure to drive phloem flow (Koch, 2004; Sturm and Tang, 1999). Cleavage of sucrose by invertase (vacuole, cytoplasm and cell wall invertase) is reported to be correlated with sink strength, growth and cell expansion as two fold more hexoses are produced than are produced by sucrose synthase with the product as glucose rather than UDP-glucose (Koch, 2004). Cleavage of sucrose by sucrose synthase is more related with tissue maturation as one of its products, UDP-glucose, is the precursor of starch and callose (Koch, 2004). Sucrose cleavage is not only important in carbon partitioning, but also is vital in sugar sensing systems, which alter plant development and stress acclimation (Koch, 2004; Sturm and Tang, 1999). Pathogen infection is one such stress factor. Sucrose concentration is balanced by sucrose cleavage and synthesis. Sucrose-phosphate-synthase which catalyzes the reaction of UDP-glucose + Fru-6-P $\leftrightarrow$ sucrose-6'-P + UDP + $\text{H}^+$, is thought to mediate plant growth, sugar storage and transport of carbon to other sink cells (Huber and Huber, 1996).

Huanglongbing (HLB) (Jagoueix et al., 1994; Teixeira et al., 2005), is the most devastating disease of citrus worldwide (Bové, 2006). Typical symptoms of HLB are blotchy mottle on leaves, small leaves, yellow shoots, and reduced root density (Johnson et al., 2014). Fibrous root loss has been considered a consequence of phloem blockage and reduced carbohydrate allocation from leaves to roots (Etxeberria et al., 2009). Johnson et al. (2014) recently discovered that root decline occurs before phloem
blockage and starch loss (Johnson et al., 2014). Since no toxins or specialized secretion systems have been found in the Las genome, pathogen interruption of host metabolic pathways is believed to cause HLB symptoms (Duan et al., 2009). So far, it has been reported that carbohydrate metabolism genes are largely altered in leaves and fruit of Las-infected trees (Albrecht and Bowman, 2008; Aritua et al., 2013; Fan et al., 2010; Liao and Burns, 2012; Rosales and Burns, 2011). However, an extensive analysis of carbohydrate status and related gene expression in roots at the early stage of Las infection is still lacking. In Chapter 3, we found that root growth is stimulated by Las and the sucrose content of Cleopatra mandarin and Swingle citrumelo fibrous roots is differentially altered. The limitation on canopy development after root growth is disrupted suggests that carbon partitioning is altered by Las before or at the same time as phloem blockage occurs in the shoot, which is believed to cause the drop in root sugar content.

*Phytophthora nicotianae* (P. n.) incites root rot which reduces water and nutrient uptake and depletes carbohydrate reserves in citrus fibrous roots (Graham, 1995; Graham and Feichtenberger, 2015). In Chapter 2, we found the higher root exudation of sucrose in Las positive seedlings. To better understand the plant response to *P. n.* and Las infection, the role of sucrose and gene expression of key enzymes involved in sucrose metabolism at the early stage of disease development is useful to evaluate.

Albrecht et al. (2012) reported that Cleopatra mandarin is more susceptible to Las compared to trifoliate hybrid rootstocks. Likewise, Cleopatra mandarin and Swingle citrumelo are considered as susceptible and tolerant to *Phytophthora nicotianae,* respectively, based on the rate of root replacement to maintain tree health (Graham,
1995). The objective of this study was to investigate sucrose metabolism related gene expression in roots to Las, P.n. and the combination of both pathogens to explore disease development from the perspective of root carbohydrate metabolism. The response of Cleopatra mandarin and Swingle citrumelo will be compared by evaluating fibrous root sucrose and glucose, and gene expression of acid invertase (βFruct1 and βFruct2), netural/alkaline invertase (CitCNV1), sucrose transporter (SUST1), sucrose synthase (CitSUSA, CirSUS1) and sucrose-phosphate-synthase (SPS).

4.2 Materials and Methods

4.2.1 Plant Materials

Seeds of Cleopatra mandarin (C. reticulata) were sown in Metro Mix 500 (Hummert international, MO, USA) in 120 cm³ containers in the greenhouse. Seedlings were fertilized with Jack’s Pro 20-20-20 (A.M. Leonard, Inc., OH, USA) and Harrell’s 18-5-10 (Harrell’s, FL, USA) every other week and every 6 mo, respectively, and watered 3 times per week. Twenty 6-mo-old seedlings were graft inoculated with budwood from Las-infected trees. The budwood was from greenhouse grown ‘Madam Vinous’ sweet orange (Citrus sinensis) trees infected with Las isolate FC6 as previously described (Folimonova et al., 2009). Six months after inoculation (May 13, 2014), 10 Las-infected Cleopatra mandarin seedlings (PCR confirmed) and 10 non-inoculated seedlings were selected for uniform size, and transferred into autoclaved Candler fine sand in 120 cm³ containers for four treatments P.n. +/- and Las+/- (10 replicates for each treatment). This protocol was repeated for Swingle citrumelo (August 12, 2014).

4.2.2. Phytophthora nicotianae – Candidatus Liberibacter asiaticus Interaction

P. nicotianae (P.n. 198) isolated from declining Cleopatra mandarin in the greenhouse in 1999 was maintained on clarified V8 juice agar. Chlamydomspores were
produced by the method of Tsao (1971) for inoculum. Chlamydospores were blended, added to Candler fine sand and mixed manually for inoculum preparation. Soil inoculum was diluted 1:10 in water agar, incubated at room temperature in the dark for 2 days on pimaricin-ampicillin-rifampcin-pentachloronitrobenzene-hymexazol (PARPH) semi-selective agar medium for assay of propagule density. An inoculum density of 20 propagules/cm$^3$ of soil (threshold of density that causes root damage) was obtained by mixing the inoculum concentrate with autoclaved Candler fine sand. Seedling roots were pruned to a uniform length (8cm) to induce root growth. 5 Las-infected and 5 non-infected seedlings were transplanted into soil infested with P.n. and 5 Las-infected and 5 non-infected seedlings were transplanted into autoclaved soil. Seedlings infected and non-infected with P.n. were located on different benches in greenhouse (27°C-32°C), watered 3 times a week and fertilized with Jack’s Pro 20-20-20 every other week.

At 5 weeks post inoculation (wpi) with P.n., seedling roots were thoroughly watered and harvested 24 h after irrigation. During harvest, roots were washed and 20 fibrous root tips were randomly selected from P.n. infected/non infected seedlings and plated on PARPH medium. Plated root tips were incubated in dark for 3-4 d, and the number of P.n. positive tips of P.n. infected seedlings was recorded and contamination was checked for non-inoculated seedlings. Meanwhile, 0.1 gram of new white roots (for Las-inoculated seedlings, Las was confirmed in roots by PCR) were sampled and put into liquid nitrogen immediately. These roots were stored at -80°C for RNA extraction.

4.2.3. Candidatus Liberibacter asiaticus Detection

DNA extraction. To detect Las in roots, randomly selected fibrous root were chopped into cubic segments, placed in 2 mL screw cap tubes with one 5-mm stainless steel bead (Qiagen, Valencia, CA, USA). The tubes were stored at -80°C for at least 2 h
and after removal from the freezer immediately ground twice at 30 revolutions per sec in a Tissuelyzer II (Qiagen, Valencia, CA, USA). Ground root tissue was then processed for DNA extraction using the Mo Bio PowerSoil DNA Isolation kit (Mo. Bio. Laboratories) as previously described (Johnson et al., 2014). The first step of protocol was modified as initial buffer, garnet and solution C1 was added into 2 ml screw cap tubes which were used for grinding and then vortexed for 5 sec instead of 10 min (Johnson et al., 2014). Purified DNA was stored at -20°C for later analysis.

qPCR for Las detection. qPCR was carried out using primers and probes as previously described (Wang et al., 2006). qPCR reaction was run on 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA.). The master mix included Qiagen HotStar Taq, ROX passive reference dye (Bio-Rad), primers, probe and buffer in a concentration ratio as described by the manufacturer’s protocol. 4 μL of template DNA and 16 μL master mix were loaded into each well of a 96 micro-well plate, and each sample was replicated. Samples were run in triplicate if significant variation was found between 2 replicates in the initial assay. The standard curve included concentrations of Las plasmids from $1 \times 10^1$ to $1 \times 10^6$ copies.

4.2.4 Quantification of Sucrose and Glucose in Fibrous Roots Tissue

A 25 mg subsample of dried new fibrous root was randomly selected for sucrose analysis, and processed by a modified method previously described (Rosales & Burns, 2011). Fibrous roots were cut and ground in 2 ml tubes as described above for DNA extraction. A 900 μl aliquot of DI water was mixed with the root powder. The solution of ground roots or standard was vortexed and boiled for 10 min. Samples were centrifuged for 2 min at 2500 x G. A 300 μl aliquot of supernatant was stored at -20°C for sucrose assay. A 4μl aliquot of supernatant from each sample was processed according to the
manufacturer’s protocol for the Glucose and Sucrose Colorimetric/Fluormetric Assay (Sigma-Aldrich). Sucrose and glucose concentration in fibrous roots was tested with the same kit.

4.2.5 RNA Extraction and qRT-PCR

RNA was extracted from frozen roots of Cleopatra and Swingle citrumelo. The frozen root tissue was ground as described above for DNA extraction, and RNA was extracted using RNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer’s instructions. The RNA concentration was obtained using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA.). 1 µg of total RNA was used for cDNA synthesis using QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). qRT-PCR amplification was performed on 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA.) using QuantiTech SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) in 20-µl reactions according to the manufacturer’s instruction. The reaction mix included 10 µl of 2x QuantiTech SYBR Green PCR Master Mix, 2 µl of forward primer (0.5 µM), 2 µl of reverse primer (0.5 µM), 5 µl of RNase-free water and 1 µl of diluted cDNA (1:10) template. 18S and GAPDH were tested and used as the reference genes. Each sample was replicated and was run in triplicate if significant variation was found between 2 replicates in the initial assay. Sequences of the primer sets are listed in Table 4-1. The primer amplification efficiency was tested and relative gene expression was calculated by $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Katz et al., 2011; Nebauer et al., 2010).

4.2.6 Statistical Analysis

Fibrous root sucrose and glucose content were analyzed by one-way ANOVA using PROC GLM (SAS v. 9.4). The interaction between Las and $P.n.$ was analyzed by
two-way ANOVA using PROC GLM (SAS v. 9.4). The significance level was set at $P \leq 0.05$.

4.3 Results

4.3.1 Fibrous Root Glucose Content

Cleopatra mandarin rootstock. A significant interaction between Las and $P. n.$ for fibrous root glucose was detected ($P \leq 0.05$). The glucose content of new roots of non-inoculated, Las-infected, $P. n.$-inoculated and co-inoculated Cleopatra mandarin seedlings was 4.15, 0.63, 0.92 and 2.52 mg/g, respectively (Figure 4-1). For Las-infected seedlings, glucose content was 85% lower ($P \leq 0.05$) than non-inoculated seedlings. For $P. n.$-inoculated seedlings, glucose content was 78% lower ($P \leq 0.05$) than non-inoculated seedlings. For seedlings inoculated with both Las and $P. n.$, there was no significant difference in glucose content compared to non-inoculated, Las-infected and $P. n.$-inoculated seedlings.

Swingle citrumelo rootstock. The glucose content of new roots of non-inoculated, Las-infected, $P. n.$-inoculated and co-inoculated Swingle citrumelo seedlings was 3.19, 8.42, 2.22 and 4.3 mg/g respectively (Figure 4-1). For Las-infected seedlings, glucose content was 62% ($P \leq 0.05$), 76% ($P \leq 0.05$) and 49% ($P \leq 0.05$) higher than non-inoculated, $P. n.$-inoculated and co-inoculated seedlings respectively.

4.3.2 Fibrous Root Sucrose Content

Cleopatra mandarin rootstock. A significant interaction between Las and $P. n.$ for fibrous root sucrose was detected ($P \leq 0.05$). The sucrose content of new roots of non-inoculated, Las-infected, $P. n.$-inoculated and co-inoculated Cleopatra mandarin seedlings was 18.1, 3.6, 7.3 and 13.9 mg/g respectively (Figure 4-2). For Las-infected seedlings, sucrose content was 80% lower ($P \leq 0.05$) than non-inoculated seedlings. For
P.n.-inoculated seedlings, sucrose content was 60% lower \((P \leq 0.05)\) than non-inoculated seedlings. For seedlings inoculated with both Las and P.n., sucrose content was 23% lower (not significant) than non-inoculated seedlings, but 74% and 45% \((P \leq 0.05)\) higher than Las-infected and P.n.-inoculated seedlings, respectively.

Swingle citrumelo rootstock. The sucrose content of new roots of non-inoculated, Las-infected, P.n.-inoculated and co-inoculated Swingle citrumelo seedlings was 19.7, 26.5, 19.4 and 23.5 mg/g, respectively (Figure 4-2). There is no significant difference in fibrous root sucrose between different treatments.

4.3.3 Relative Gene Expression of Sucrose Transporter 1 (SUT1)

Cleopatra mandarin rootstock. A significant interaction between Las and P.n. for expression of SUT1 gene was detected \((P \leq 0.05)\). The expression of SUT1, which is involved in the apoplastic sucrose transport pathway was significantly changed in roots of Las-infected, P.n.-inoculated and co-inoculated Cleopatra mandarin seedlings compared to non-inoculated seedlings \((P \leq 0.05)\) (Figure 4-3). The transcription of SUT1 was increased 6 fold by Las, 10 fold by P.n. and 4 fold by the combination of Las and P.n. There were no significant differences for transcription of SUT1 in Cleopatra mandarin roots with different pathogen treatments.

Swingle citrumelo rootstock. A significant interaction between Las and P.n. for expression of SUT1 gene was detected \((P \leq 0.05)\). The expression of SUT1 was only significantly \((P \leq 0.05)\) changed in roots of Las-infected seedlings compared to non-inoculated seedlings (Figure 4-3). The transcription of SUT1 was increased 7 fold by Las. A greater differential SUT1 expression in fibrous roots between Cleopatra mandarin and Swingle citrumelo seedlings was caused by P.n.
4.3.4 Relative Gene Expression of Acid Invertase- βFruct1

Cleopatra mandarin rootstock. The expression of βFruct1, which is involved in sucrose cleavage pathway in the vacuole, was significantly ($P \leq 0.05$) changed in roots of Las-infected seedlings with and without $P.n.$ compared to non-inoculated Cleopatra mandarin seedlings (Figure 4-4). The transcription of βFruct1 was increased 18.5 fold by Las, and 18.1 fold by the combination of Las and $P.n.$

Swingle citrumelo rootstock. A significant interaction between Las and $P.n.$ for expression of βFruct1 gene was detected ($P \leq 0.05$). The expression of βFruct1 was only significantly ($P \leq 0.05$) changed in roots of Las-infected seedlings compared to non-inoculated Swingle citrumelo seedlings (Figure 4-4). The transcription of βFruct1 was increased 4.1 fold by Las (much less than 18 fold in Cleopatra mandarin). The transcription of βFruct1 was repressed 9.6 fold by the combination of Las and $P.n.$

4.3.5 Relative Gene Expression of Acid Invertase-βFruct2

Cleopatra mandarin rootstock. A significant interaction between Las and $P.n.$ for expression of βFruct2 gene was detected ($P \leq 0.05$). The expression of βFruct2, another isoform of vacuolar invertase, which is involved in sucrose cleavage pathway, was significantly ($P \leq 0.05$) changed in roots of Las-infected and $P.n.$-inoculated Cleopatra mandarin seedlings compared to non-inoculated seedlings (Figure 4-5). The transcription of βFruct2 was increased 14.6 fold by Las, and 33.9 fold by $P.n.$ There were no significant differences for expression of βFruct2 between seedlings with different pathogen treatments.

Swingle citrumelo rootstock. There was no significant change of βFruct2 expression among the different pathogen treatments. A marked difference for βFruct2 expression in fibrous roots between Cleopatra mandarin and Swingle citrumelo
seedlings was detected in all inoculated seedlings compared to non-inoculated seedlings. Both Las and \( P.n. \) significantly \((P \leq 0.05)\) increased expression of \( \beta \text{Fruct2} \) in Cleopatra mandarin seedlings. Neither Las nor \( P.n. \) infection significantly altered \( \beta \text{Fruct2} \) expression in fibrous roots of Swingle citrumelo seedlings, though expression was slightly repressed.

### 4.3.6 Relative Gene Expression of Neutral/alkaline Invertase-CitCNV1

Cleopatra mandarin rootstock. A significant interaction between Las and \( P.n. \) for expression of CitCNV1 gene was detected \((P \leq 0.05)\). The expression of CitCNV1, which is involved in sucrose cleavage pathway in cytoplasm, was significantly \((P \leq 0.05)\) changed in roots of Las-infected, \( P.n.- \)inoculated and co-inoculated Cleopatra mandarin seedlings compared to non-inoculated seedlings (Figure 4-6). The transcription of CitCNV1 was increased 5.5 fold by Las, 4.5 fold by \( P.n. \) and 5.6 fold by the combination of Las and \( P.n. \). There was no significant difference in expression of CitCNV1 for Cleopatra mandarin seedlings inoculated with the different pathogens.

Swingle citrumelo rootstock. None of the pathogen treatments significantly changed the expression of CitCNV1 in Swingle citrumelo (Figure 4-6).

### 4.3.7 Relative Gene Expression of Sucrose Synthase- CitSUSA

Cleopatra mandarin rootstock. The expression of CitSUSA, which is involved in the sucrose cleavage pathway in cytoplasm, was significantly \((P \leq 0.05)\) changed in roots of \( P.n.- \)inoculated Cleopatra mandarin seedlings with and without Las infection, compared to non-inoculated seedlings (Figure 4-7). The expression of CitSUSA was increased 4.8 fold by \( P.n. \) and 7.9 fold by the combination of Las and \( P.n. \). There was no significant difference for transcription of CitSUSA for Cleopatra mandarin seedlings inoculated with the different pathogens.
Swingle citrumelo rootstock. None of the pathogen treatments significantly altered the expression of CitCNV1 in Swingle citrumelo seedlings (Figure 4-7).

4.3.8 Relative Gene Expression of Sucrose Synthase- CitSUS1

Cleopatra mandarin rootstock. The expression of CitSUS1, which is involved in sucrose cleavage in cytoplasm, was significantly ($P \leq 0.05$) changed in roots of Las-infected Cleopatra mandarin seedlings with and without P.n. inoculation, compared to non-inoculated seedlings (Figure 4-8). The expression of CitSUS1 was increased 2.8 fold by Las, and 3 fold by the combination of Las and P.n. There was no significant difference for transcription of CitSUS1 in Cleopatra mandarin seedlings inoculated with the different pathogen treatments.

Swingle citrumelo rootstock. The expression of CitSUS1 was significantly ($P \leq 0.05$) changed in P.n.-inoculated Swingle citrumelo seedlings with and without Las infection, compared to non-inoculated seedlings (Figure 4-8). Las repressed CitSUS1 expression in Swingle citrumelo roots ($P \leq 0.05$). The different impact of Las on CitSUS1 expression between Cleopatra mandarin and Swingle citrumelo seedlings was that Las increased CitSUS1 expression in fibrous roots of Cleopatra mandarin and reduced CitSUS1 expression in fibrous roots of Swingle citrumelo.

4.3.9 Relative Gene Expression of Sucrose-phosphate-synthase (SPS)

Cleopatra mandarin rootstock. The expression of SPS, which is involved in sucrose synthesis pathway in cytoplasm, was significantly ($P \leq 0.05$) changed in roots of Las-infected Cleopatra mandarin seedlings with and without P.n. inoculation compared to non-inoculated seedlings (Figure 4-9). The transcription of SPS was increased 2.2 fold by Las and 2.9 fold by the combination of Las and P.n. There was no significant
difference in transcription of SPS for Cleopatra mandarin seedlings inoculated with the different pathogen treatments.

Swingle citrumelo rootstock. A significant interaction of Las and P. n. for expression of SPS gene was detected. The expression of SPS was only significantly ($P \leq 0.05$) changed in roots of Las-infected Swingle citrumelo seedlings compared to non-inoculated seedlings (Figure 4-9). The transcription of SPS was increased 3.4 fold by Las. There were no significant changes for SPS expression in Swingle citrumelo seedlings inoculated with both pathogens compared to non-inoculated seedlings. The differences in SPS expression in fibrous roots between Cleopatra mandarin and Swingle citrumelo seedlings were due to infection by Las and P. n.

4.4 Discussion

Lower sucrose and glucose was found in Las, P. n. and Las and P. n. co-inoculated fibrous roots of Cleopatra mandarin seedlings compared to non-inoculated seedlings. Reduced sucrose and glucose could result from lower carbohydrate availability in roots caused by partially blocked phloem in the shoot or higher carbohydrate consumption by roots and pathogen infection, or both. Combined with the results from Chapter 3 that root replacement was increased by Las, it is likely that more sucrose is allocated to roots due to higher carbohydrate consumption in Las-infected Cleopatra mandarin fibrous roots. For P. n.-infected Cleopatra mandarin seedlings, higher sugar consumption is also an explanation for low sucrose and glucose content in fibrous roots. Higher sucrose and glucose was found in Las-infected fibrous roots of Swingle citrumelo seedlings with and without P. n. infection, which indicates that Las induced more sugar allocation to roots than P. n. Furthermore, the significantly higher glucose in Las-infected Swingle citrumelo seedlings compared to non-infected seedlings
suggests that Las induces higher sucrose cleavage. The comparison of sugar content in fibrous root between Cleopatra mandarin and Swingle citrumelo seedlings indicates that sucrose metabolism was more disrupted by Las and P.n. in Cleopatra mandarin than Swingle citrumelo at 5 wk after inoculation.

As discussed above, sucrose partitioning within the plant is probably altered by both pathogens in roots. The sucrose import into roots occurs through symplasmic and apoplastic pathways. Plasmodesmatal movement is the predominant pathway for sucrose in roots, whereas in the zone of root that plasmodesmata does not develop, the apoplastic transfer of sucrose is the pathway (Achor, 1999; Eisenstat and Koch, 2004). Sucrose import into roots requires several isoforms of transporters, invertase, sucrose synthase and sucrose-phosphate-synthase that are localized in different subcellular compartments. Sucrose transporter 1 (SUT1) in sieve tubes of potato, tomato and tobacco (Kühn et al., 1997) maybe involved in the unloading of sucrose in sinks including actively growing roots. In this study, SUT1 expression was significantly up-regulated by Las infection in Cleopatra mandarin and Swingle citrumelo fibrous roots, which indicates there is greater unloading of sucrose from the phloem. Once sucrose is imported into sink cells, (through plasmodesmata, cell wall space or both), with the low activity of cytoplasmic invertase, sucrose is usually transported into vacuoles to be cleaved and the hexose transported back into cytoplasm for subsequent cytoplasmic metabolism (Koch, 2004; Winter and Huber, 2000). Vacuolar invertases generate abundant hexoses and hexose-based sugar signals (Herbers and Sonnewald, 1998; Sturm and Tang, 1999), and this process mediates sucrose partitioning (osmotic and turgor pressure) and signaling. In this study, gene expression of two isoforms of
vacuolar invertase, βFruct1 and βFruct2, were 18 and 15 fold up-regulated by Las in Cleopatra mandarin fibrous roots, respectively. These results are consistent with the reduced sucrose content in fibrous roots of Las-infected Cleopatra mandarin seedlings. Elevated expression of vacuolar invertase is an indication of greater sucrose allocation to roots and greater sucrose consumption for growth and defense against Las infection, and benefits the bacterium at the same time. For *P.n.*-infected Cleopatra mandarin seedlings, only βFruct2 (34 fold) was significantly up-regulated, indicating that Las and *P.n.* infection induce different isoforms of vacuolar invertase. In contrast, not much change of expression of vacuolar invertase was detected in Swingle citrumelo, except for the 4 fold up-regulation of βFruct1 expression in fibrous roots of Las-infected seedlings. This suggests that for Swingle citrumelo, sucrose metabolism and transportation was less disrupted by Las than for Cleopatra mandarin. Likewise, compared with 34 fold increased expression for Cleopatra mandarin, βFruct2 in Swingle citrumelo was slightly repressed by *P.n.* infection. Higher and lower upregulation of cytoplasmic invertase (CitCNV1) was detected in pathogen-infected Cleopatra mandarin and Swingle citrumelo seedling roots, respectively.

Different contributions of sucrose cleaving enzymes to sequential stages of sink development: initiation, expansion, and storage/maturation have been extensively examined (Koch, 2004; Sturm and Tang, 1999). Unlike invertase producing large amounts of hexose, which upregulates genes for early developmental, sucrose synthase is more important in the tissue maturation stage by directing UPD-glucose to starch accumulation (Sturm and Tang, 1999). In this study, the different expression of two isoforms of sucrose synthase was detected between Las and *P.n.*-infected
Cleopatra mandarin. Komatsu et al. (2002) reported different structure and physiological roles for CitSUS1 and CitSUSA in citrus fruit. In this study, we detected altered expression patterns of these two isoforms by Las in both rootstocks, which confirmed their different functions in sucrose metabolism pathways. Sucrose synthase in sink cells was not only found to mediate callose and starch and many other biosynthesis pathways to maintain a steep sucrose gradient across the plasma membrane cytoplasm, but also in sieve tubes to regulate phloem development (Winter and Huber, 2000). The lower response of sucrose synthase related gene expression compared to other gene expression in Las and P.n. infected fibrous roots of Cleopatra mandarin indicates less disruption of maturation and phloem development by Las and P.n. at 5 wk after inoculation. Finally, sucrose-phosphate-synthase (SPS) was upregulated by Las and P.n. in both Cleopatra mandarin and Swingle citrumelo fibrous roots. Additional sucrose production might be used for transport to nearby sink cells or account for the greater exudation of sucrose into the rhizosphere reported in Chapter 2.

Although reduced sucrose content in roots could be a consequence of dysfunctional sieve elements at the late stage of disease development, disruption of sucrose transport and cellular sucrose metabolic regulation could also contribute to a carbohydrate imbalance within the plant. Such disruption may account for reduced canopy and fruit growth and leading to small fruit size in HLB-affected trees (Liao and Burns, 2012).

In Chapter 2, we detected the different relationship between P.n. infection incidence and total sugar content, with and without Las, which suggested that Las-induced plant response to P.n. was mediated by sugar allocation and consumption in
roots. Two out of 7 genes (SUT1 and βFruct2) showed lesser gene expression in co-
inoculated Cleopatra mandarin seedlings than Las or P.n.-infected seedlings. Three out
of 7 genes (βFruct1, CitCVN1 and CitSUS1) showed similar gene expression in co-
inoculated Cleopatra mandarin seedlings as Las-infected seedlings than P.n.-infected
seedlings. Two out of 7 genes (CitSUSA and SPS) showed greater gene expression in
co-infected Cleopatra mandarin seedlings than Las or P.n.-infected seedlings. The
different gene expression of SUT1, βFruct2, CitSUSA and SPS in roots of co-inoculated
Cleopatra mandarin seedlings compared to P.n. and Las-infected seedlings is indicative
of a complex interaction between Las and P.n. at the molecular level.

Taken together, we detected significant expression changes induced by Las and
P.n. The much higher rate of sucrose cleavage related gene expression compared to
sucrose transporter and sucrose phosphate-synthase related gene expression is
consistent with the lower sucrose and glucose content detected in fibrous roots, and
indicates greater sucrose allocation for root growth than for canopy growth. Higher
sucrose cleavage through the invertase pathway rather than the sucrose synthase
pathway suggests that a hexose-based signal might be initiated in response to
pathogen infection and root replacement. Swingle citrumelo was not influenced by Las
and P.n. as severely as in Cleopatra mandarin at least at this time point (5wk). This
might explain the different performance of these two rootstocks under field conditions
with the two pathogens (i.e. Lower P.n. infection incidence for Swingle citrumelo than
Cleopatra mandarin reported in Chapter 2).

A more in-depth investigation with labeled carbon of carbohydrate allocation in
response to pathogen infection is needed to precisely measure carbon partitioning to
different sinks. The analysis could provide better understanding of the basis for the interactions of Las and P.n. and different carbon utilization between plant and Las as the bacteria are restricted in phloem. Investigation of integrated gene expression, the allocation of post-transcription product and pathogen regulated gene function will help to elucidate disease causation mechanism and the physiology of this pathogen.
Figure 4-1. Fibrous root glucose of Cleopatra mandarin and Swingle citrumelo seedlings at 5 weeks post inoculation (wpi). Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). Different letters denote significant difference at $P \leq 0.05$. * Denotes significant interaction at $P \leq 0.05$. Bars represent the means of 3-5 replicates.

Figure 4-2. Fibrous root sucrose of Cleopatra mandarin and Swingle citrumelo seedlings at 5 weeks post inoculation (wpi). Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). Different letters denote significant difference at $P \leq 0.05$. * Denotes significant interaction at $P \leq 0.05$. Bars represent the means of 3-5 replicates.
Figure 4-3. Relative gene expression of Sucrose transporter 1 (SUT 1) of Cleopatra mandarin and Swingle citrumelo fibrous roots at 5 weeks post inoculation (wpi). Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). * Denotes significant interaction at $P \leq 0.05$. Bars represent standard error of 3-5 replicates.

Figure 4-4. Relative gene expression of acid invertase-βFruct1 of Cleopatra mandarin and Swingle citrumelo fibrous roots at 5 weeks post inoculation (wpi). Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). * Denotes significant interaction at $P \leq 0.05$. Bars represent standard error of 3-5 replicates.
Figure 4-5. Relative gene expression of acid invertase-βFruct2 of Cleopatra mandarin and Swingle citrumelo fibrous roots at 5 weeks post inoculation (wpi). Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). * Denotes significant interaction at $P \leq 0.05$. Bars represent standard error of 3-5 replicates.

Figure 4-6. Relative gene expression of Neutral/alkaline invertase-CitCNV1 of Cleopatra mandarin and Swingle citrumelo fibrous roots at 5 weeks post inoculation (wpi). Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). * Denotes significant interaction at $P \leq 0.05$. Bars represent standard error of 3-5 replicates.
Figure 4-7. Relative gene expression of sucrose synthase—CitSUSA of Cleopatra mandarin and Swingle citrumelo fibrous roots at 5 weeks post inoculation (wpi). Treatments: Inoculation with *Candidatus* Liberibacter asiaticus (Las) (Las+Pn-), inoculation with *Phytophthora nicotianae* (P.n.) (Las-Pn+); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). * Denotes significant interaction at $P \leq 0.05$. Bars represent standard error of 3-5 replicates.

Figure 4-8. Relative gene expression of sucrose synthase—CitSUS1 of Cleopatra mandarin and Swingle citrumelo fibrous roots at 5 weeks post inoculation (wpi). Treatments: Inoculation with *Candidatus* Liberibacter asiaticus (Las) (Las+Pn-), inoculation with *Phytophthora nicotianae* (P.n.) (Las-Pn+); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). * Denotes significant interaction at $P \leq 0.05$. Bars represent standard error of 3-5 replicates.
Figure 4-9. Relative gene expression of sucrose-phosphate-synthase (SPS) of Cleopatra mandarin and Swingle citrumelo fibrous roots at 5 weeks post inoculation (wpi). Treatments: Inoculation with Candidatus Liberibacter asiaticus (Las) (Las+Pn-), inoculation with Phytophthora nicotianae (P.n.) (Las-Pn +); co-inoculation of Las and P.n. (Las+Pn+); non-inoculated (Las-Pn-). * Denotes significant interaction at $P \leq 0.05$. Bars represent standard error of 3-5 replicates.
Table 4-1. Primer sets used to amplify specific regions of Citrus genes involved in sucrose metabolism (5’-3’)

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<th>Gene</th>
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<td>SUT1</td>
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<td>TTTTGTATCAAGCGCCACTG</td>
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<td>βFruct2</td>
<td>GACGGGTATTTCGCTTGTGT</td>
<td>CATTCCACATACCACGTACC</td>
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<tr>
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<td>CTGGCAGAGACACCTGAGT</td>
<td>CATTGAGCATCCATCAGCAC</td>
</tr>
<tr>
<td>CitSUSA</td>
<td>TTGTGGACTTCCGACATTCG</td>
<td>TGACGCACCATGCTGATAA</td>
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<td>TGAGCCATTCAATGCTCCTG</td>
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</tr>
<tr>
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<td>GTTAAGGCTCAAGCGACGAG</td>
<td>CCCTCAAGCTGCTTTTCTG</td>
</tr>
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<td>CGTCCCTCTGCAAGATGACTCT</td>
</tr>
<tr>
<td>18S</td>
<td>GTGACGGAGAATTAGGTTTG</td>
<td>CTGCCCTCTTGGATGTGGTA</td>
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CHAPTER 5
SUMMARY

Whole-plant carbohydrate partitioning is balanced by sink strength which is defined as the ability of the sink organ to compete for carbohydrate. A stronger sink can be created by increased sucrose usage in a sink organ affected by mechanical damage, environmental stress or pathogen infection. The premature fruit drop at the early stage of HLB symptom expression for trees in Florida citrus groves indicates that unobserved root damage is occurring belowground. As the hidden part of the tree, root damage is usually overlooked until significant symptoms are observed above ground. Root damage could contribute to the premature fruit drop of HLB symptom trees by disrupting carbohydrate availability and limiting water and nutrient supply required for healthy fruit growth and development.

To investigate the causal mechanism for premature fruit drop of HLB symptomless trees from carbohydrate partitioning perspective, experiments were carried out as follows: 1) examination of root damage caused by Las, the soil borne pathogen Phytophthora and the interaction of the two root pathogens; 2) investigation of the relationship between root damage and canopy reduction; 3) exploration of root damage’s contribution to premature fruit drop from the perspective of disruption of carbohydrate metabolism.

The results showed that roots of HLB symptomless seedlings were damaged by Las and the interaction of the two pathogens. The combination of Las and P.n. showed the trend to reduce the fibrous root biomass more than each root pathogen alone at some (not significant) but not all time periods after inoculation. P.n. infection incidence of Las-infected seedlings was increased as the zoospores were more attracted to root
surface and host tolerance was broken down by Las. Under optimal conditions for pathogen development in field, the increased *P.n.* infection incidence might play an even more important role than in the greenhouse by accelerating *P.n.* population development to the damaging level.

The further examination of total leaf area and sucrose metabolism related gene expression in roots confirmed why canopy development was reduced after root decline. This occurred because more carbohydrate was imported into roots as a result of greater sink strength created by up-regulation of sucrose cleavage. Higher sucrose consumption in Cleopatra mandarin could have resulted from the plant’s utilization for defense, the pathogens’ utilization for sustenance, or both host and pathogen factors.

Interestingly, we detected very different responses of the susceptible rootstock Cleopatra mandarin and less susceptible Swingle citrumelo to pathogen infection. Cleopatra mandarin showed a steeper slope of the linear regression between *P.n.* infection incidence and sugar concentration for seedlings with and without Las compared to Swingle citrumelo, which indicates that the pathogens are more responsive to sugar concentration in roots for Cleopatra mandarin than for Swingle citrumelo. This result is consistent with the finding that sucrose metabolism is more disrupted by Las and *P.n.* in Cleopatra mandarin than in Swingle citrumelo. This might explain the different susceptibility to these pathogens of trees on these two rootstocks under field conditions.

Although reduced sucrose content in Las-infected roots occurs as a consequence of dysfunctional sieve elements at the later stage of disease development, disruption of sucrose transport and cellular sucrose metabolic regulation could also
contribute to a carbohydrate imbalance within the plant. Such disruption may account for reduced canopy and fruit growth and leading to small fruit size in HLB-affected trees.

Based on these results, grove managers should consider practices that: 1) minimize root damage caused by the combination of Las and P.n. (e.g. use of soil fungicides); 2) reduce disruption of sucrose metabolism in rootstocks and scions through balanced use of water and fertilizers to promote regular cycles of root and shoot flushes and sustain fruit growth and maturation; 3) provide optimal growing conditions to maintain tree growth by minimizing the effects of abiotic (e.g., drought, freezes) and biotic stress (root pests and pathogens).
APPENDIX A
NEW ROOT LENGTH OF CLEOPATRA MANDARIN AT 6 MO POST INOCULATION

![Graph showing new root length in cm for different treatments.]

- Las-Pn-
- Las+Pn-
- Las-Pn+
- Las+Pn+
### Harrell's 18-5-10

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Percentage by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td></td>
</tr>
<tr>
<td>Nitrate Nitrogen</td>
<td>6.0500%</td>
</tr>
<tr>
<td>Ammonical Nitrogen</td>
<td>7.4000%</td>
</tr>
<tr>
<td>Urea Nitrogen</td>
<td>4.5500%</td>
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<tr>
<td>Phosphate</td>
<td>5.0000%</td>
</tr>
<tr>
<td>Potash</td>
<td>10.0000%</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1.1190%</td>
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<tr>
<td>Sulfur</td>
<td>0.2770%</td>
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<tr>
<td>Copper</td>
<td>0.0660%</td>
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<tr>
<td>Iron</td>
<td>0.2750%</td>
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<tr>
<td>Manganese</td>
<td>0.1120%</td>
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<tr>
<td>Molybdenum</td>
<td>0.0090%</td>
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<tr>
<td>Zinc</td>
<td>0.0660%</td>
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</table>

### Jack's Pro 20-20-20

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<th>Ingredients</th>
<th>Percentage by weight</th>
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<tbody>
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<tr>
<td>Nitrate Nitrogen</td>
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<tr>
<td>Ammonical Nitrogen</td>
<td>6.0700%</td>
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<tr>
<td>Urea Nitrogen</td>
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<tr>
<td>Phosphate(P₂O₅)</td>
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<tr>
<td>Potash</td>
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<tr>
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<tr>
<td>Boron</td>
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<tr>
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<tr>
<td>Molybdenum</td>
<td>0.0009%</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.0025%</td>
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</tbody>
</table>
LIST OF REFERENCES


Matheron, M.E., Porchas, M., 1996. Colonization of citrus roots by *Phytophthora citrophthora* and *Phytophthora parasitica* in daily soil temperature fluctuations between favorable and inhibitory levels. Plant disease. 80(10), 1135-1140.


Schneider, H., 1967. Phloem necrosis associated With the greening disease of sweet orange (*Citrus sinensis*). Phytopathology. 57, 829.


BIOGRAPHICAL SKETCH

Jian Wu was born and raised in Tongliao, Inner Mongolia, China. She received her bachelor’s degree majoring in forestry from Northeast Forestry University, Harbin, China. She received her master’s degree majoring in turfgrass science from Beijing Forestry University, Beijing, China. In 2011, Jian Wu joined the Department of Soil and Water Science at the University of Florida for her Ph.D. and completed her program in December 2015.