INCIDENCE, DISTRIBUTION, AND EPIDEMIOLOGY OF *PHYTOPHTHORA* DISEASES OF CITRUS IN SOUTH TEXAS AND THEIR INTERACTION WITH HUANGLONGBING

A Dissertation

by

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ABSTRACT

Foot rot, gummosis and root rot disease caused by *Phytophthora* are major constraints for commercial citrus production in the Lower Rio Grande Valley (LRGV), Texas. Not much is known about the extent of foot rot and gummosis incidence, severity, and characteristics of the *Phytophthora* species found in the commercial citrus orchards in the LRGV. To further elucidate the epidemiology of *Phytophthora*-caused foot rot and gummosis in the commercial citrus orchards of Texas, a survey of 30 orchards was conducted in 2015 and 2017. Foot rot lesions were detected in 33% of the trees surveyed and disease severities measured in 2015 and 2017 were 14.2 and 15.9% respectively. *P. nicotianae* was found to be the most prevalent species as all 89 *Phytophthora* isolates obtained from plant and soil samples collected from citrus orchards were identified as *P. nicotianae*.

Twenty-nine percent and 71% of these isolates were determined to be A1 and A2 mating types, respectively and the presence of both mating types was confirmed in three orchards. Among the tested isolates from the LRGV, none were found to be mefenoxam resistant, but the EC50 value for one isolate from Corpus Christi measured 143 µg/ml. The other objective was to study the effect of the interaction of foot rot and gummosis disease in *Candidatus* Liberibacter asiaticus (*C*Las) positive trees on the root health, nutrient status and canopy thinning. To evaluate this, 46 *C*Las positive and negative trees with and without foot rot from four grapefruit orchards in Donna, Texas in 2014 and 2016 were studied.

The presence of *C*Las and foot rot disease was associated with significantly altered levels of sodium, copper, and sulfur in the symptomatic leaves and greater canopy thinning. Foot rot significant effect was observed on lowering root density, while *C*Las was

associated with significant increases in starch, sucrose, and glucose, and significantly reduced phosphorus and magnesium in the symptomatic leaves.

The existence of a mefenoxam resistant isolate in the Corpus Christi and the identification of both mating types in the two orchards in the LRGV means that new strains could arise and present control challenges. The presence of both *C*Las and foot rot resulting in canopy thinning and potential tree decline emphasizes the need for continued control of *Phytophthora.*

DEDICATION

This dissertation is dedicated to my late mother (Maya Ganguly), my father (Sukdeb Ganguly), my loving husband Raj, and my children Anirudh and Chhavi.

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NOMENCLATURE

BrCA	Brown citrus aphid
CTV	*Citrus tristeza virus*
CTLV	Citrus tatter leaf virus
KLA	Key lime anthracnose
CLas	*Candidatus* Liberibacter asiaticus
DSI	Disease severity index
CV8A	Clarified V8 agar
EC50	Effective concentration 50
MT	Mating type
FRD	Fibrous root density
RLD	Root length density
TPDW	Total plant dry weight
LMA	Leaf mass per area
HLB	Huanglongbing
LRGV	Lower Rio Grande Valley

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Citrus originated in northeastern India and southern China (Moore 2001) but now is grown throughout the tropical and subtropical parts of the world. The top citrus producing countries in the world are China, Brazil, India, and the USA, where the 2016 citrus production was 32,705, 16,555, 9,755 and 7,829.0 thousand metric tons respectively (FAO 2016). Other major producing countries are Mexico, South Africa, Argentina and the Mediterranean basin region (FAO 2016). In the US, California is the leading citrus producing state (51%) followed by Florida (45%) and Texas and Arizona (4%) (USDA 2017).

Commercial citrus orchards in Texas are concentrated in three counties located in the Lower Rio Grande Valley (LRGV), Hidalgo, Cameron, and Willacy. The varietal composition of the industry includes grapefruit (*Citrus* x *paradisi* Macf.) (70%) and sweet orange (C. *sinensis* (L) Osbeck) (30%) (Citrus Fruits 2017 Summary, USDA, National Agricultural Statistics Service 2016). Sour orange (*C. aurantium* L.) is the standard rootstock used in most of the commercial orchards in the LRGV, as it is generally adapted to different soil types, shows tolerance to alkalinity, salinity, less than optimal soil drainage and cold temperatures (Sauls 2008)

This dissertation follows the format and style of the Journal of Phytopathology

1.1 Citriculture

Citriculture dates to at least 2100 BC in China when a description in the book "Yu King" mentions "small oranges and pumeloes" (Schora 1975). In India, the oldest reference to citrus is mentioned in Sanskrit literature, dating back to 800 B.C (Scora 1975). It is challenging to ascertain its origin due to natural hybridization, and the presence of few citrus in the wild. Citrus probably originated in an area comprising present-day East Asia possibly north-east India, north Burma and southwest China (Moore 2001). Its exact route of dispersion is not clearly known. Columbus and Ponce de Leon brought citrus to New World around the early 1500s (Moore 2001). Citriculture proliferated in Florida in the late 1700s, and at around the same time, it was introduced into California.

The first record of citrus planting in Texas was in Brazoria County in 1848 (Waibel 1953). The earliest record of a citrus tree planted in the Lower Rio Grande Valley (LRGV) was a seedling orange tree in 1882 (Sauls 2008). By 1940 Texas's reputation for quality red flesh grapefruit was established. Changes in the Texas citriculture has been mostly driven by the severe freezes it experienced in 1940, 1951, 1962, 1983 and 1989 (Sauls 2008). Texas citrus industry is a fresh fruit industry and is based on its red fleshed, high-quality grapefruit and some orange varieties.

Ninety-five percent of Valley's citrus is presumably grown on sour orange rootstock (Sauls, 2008b). It is moderately tolerant to cold, salinity and alkalinity and relatively tolerant to *Phytophthora*. Grapefruit and sweet orange yields on sour orange rootstock are moderate and of good quality (Sauls, 2008b). Sour orange is highly susceptible to *Citrus tristeza virus* (CTV) and the Texas citrus industry can incur heavy losses if the virulent strain of CTV spreads to the LRGV. Several other rootstocks like Cleopatra mandarin,

Swingle citrumelo, Carrizo and Troyer citrange has their strengths as rootstocks, but sour orange is still the most widely used rootstock in the LRGV. C22, another promising rootstock that was obtained from a cross between Sunki mandarin and Swingle trifoliate orange has good yield potentials in high pH and calcareous soils and is also resistant to CTV (Louzada et al. 2008).

The most common scion variety grown in LRGV commercial orchards is grapefruit. Grapefruit trees account for 70% of the citrus grown in Texas with 75% of it being the deep red 'Rio Red' grapefruit (USDA, National Agricultural Statistics Service, 2016; Sauls, 2008b). The region is known for its high-quality grapefruits with red flesh, high sugar, and low acidity. Twenty-five percent of grapefruits grown are Ruby-Sweet varieties that include Ruby Red and Ray Ruby. Ray Ruby has more red rind than Ruby Red. Both are of excellent quality and very suitable as a gift, fresh or processed fruit market. The other 75% of the grapefruit varieties grown here are deep red-flesh Rio Star Grapefruit consisting of Rio Red and Star Ruby. Hardly any Star Ruby orchards exist now, so most of the Rio Star grapefruit grown is Rio Red variety. It has good color retention until the late season (Sauls 2008). Oranges are grown on about 30% of the total citrus acreage in the LRGV. Marrs is the primary early season with some navels, while Pineapple and Jaffa are mid-season varieties, and Valencia's are late season oranges.

1.2 Citrus diseases in Texas

Citrus production and quality are affected by many biotic and abiotic factors. Several diseases caused by viruses, viroids, bacteria, nematodes, and fungi are present in Texas. The main viral pathogens recognized as a threat to the Texas citrus or potentially can affect are *Citrus psorosis virus* (CPsV), *Citrus tristeza virus* (CTV), and citrus tatter leaf virus

(CTLV) (Amador et al. 1981). CTV can potentially be a serious threat to the LRGV citrus industry since sour orange is the dominant rootstock which is highly sensitive to CTV. It causes two main diseases in citrus, a quick decline of scions on sour orange rootstock, and stem pitting in grapefruit and Mexican lime. In a survey conducted from 1991 to 2000, in which 11,000 citrus trees were evaluated from LRGV and other places from Texas for the presence of CTV, 1.1% of the trees from the LRGV were infected (Gracia et al. 2001). The absence of the very efficient CTV aphid vector, the brown citrus aphid (BrCA) *Toxoptera citricida* Kirkaldy, is attributed as the reason for the lower incidence of CTV in the LRGV (Herron et al. 2005). *T. citricida* is present in Mexico (Michaud and Alvarez 2000), and Florida (Halbert and Brown 1996) which may be a risk factor for the citrus industry in Texas.

CPsV was reported in Texas orchards in the 1940's (Fawcett 1948). The incidence of CPsV in nucellar trees and the psorosis-free Rio-Red grapefruit trees suggest virus spreading by natural means in the orchards (Gottwald et al. 2005). A root infecting fungus called *Olpidium* has been confirmed to transmit CPsV from infected trees to healthy trees in Texas (Palle et al. 2005). It is hypothesized that if not for four significant tree-killing freezes that occurred between 1951 and 1989, and the use of CPsV free budwood, the incidence of psorosis disease would have been worse in the LRGV orchards. CTLV was shown by Timmer (1975) and da Graça and Skaria (1996) to be present in Meyer lemon, a symptomless host. Seed transmission of CTLV was also confirmed in Eureka lemon (Tanner et al. 2011). CTLV causes bud union crease and subsequent decline of trees on trifoliate rootstocks (Miyakawa and Tsuji, 1988) but these rootstocks are not commonly used in Texas.

Several viroid species have been found infecting citrus in Texas. Viroids are small infectious agents consisting of non-capsulated, non-translated, single-stranded and circular RNA (Flores et al. 2005; Olson 1952) demonstrated via biological indexing the presence of the agents of exocortis and cachexia, but the diseases are not widespread because the commonly used sour orange rootstock in Texas is tolerant to these viroids. Exocortis is caused by citrus exocortis viroid (CEVd), and cachexia by hop stunt viroid (HSVd). Most old-line grapefruit (*C.* x *paradisi* Macf.) from the LRGV have some cachexia, and sweet orange has both cachexia and exocortis viroids (Olson 1952; Olson and Shull 1955; Olson et al. 1958). More recently, Kunta et al. (2007) demonstrated the symptomless presence of CEVd, HSVd, Citrus bark cracking viroid and Citrus dwarfing viroid in Texas orchards.

Two important bacterial diseases of citrus are citrus canker and Huanglongbing (HLB). Citrus canker is a serious disease of citrus affecting most commercial citrus cultivars. It is caused by the bacterium *Xanthomonas citri* subsp. *citri* was previously known as *Xanthomonas axonopodis* subsp. *citri* (Xac) (Gottwald et al. 2002). The disease is characterized by an erumpent lesion on leaf, stem, and fruits of susceptible cultivars. Foliar symptoms of citrus canker appear as raised lesions with a yellow halo around it. Raised and corky lesions may also be observed on twigs and fruits surrounded by oil margins. No chlorosis is observed on twigs but may be present on the fruits. In severe cases, defoliation, fruit drop and dieback of the citrus tree is observed (Gottwald et al. 2002). Marketability of fruits is the most affected due to the presence of blemishes caused by canker. Citrus canker was introduced into the United States with the import of trifoliate orange and satsuma seedlings from Japan in the early 1900s (Skaria and da Graça 2012). It was successfully

eradicated from Florida (1994), Louisiana (1940), and Texas (1943), though it re-emerged in Florida in 1986 and 1995. Since then, it became endemic to Florida.

Until recent years, the last reported canker case was in Corpus Christi, Texas in 1943 (Skaria and da Graça 2012). Continuous surveillance of citrus trees is still required in Texas as grapefruit, the dominant citrus variety in the state, is highly susceptible to citrus canker. Recently, a Mexican lime specific strain was found in the LRGV (Kunta et al. 2016) and the Asian strain has been found near Houston (USDA reports 2016). Another bacterial disease threatening to severely affect the Texas citrus industry is HLB. HLB is associated with three different Gram-negative, phloem-inhabiting alpha-proteobacteria species, *Candidatus Liberibacter* asiaticus (s), *Ca.* L. africanus (*C*Laf) and *Ca.* L. americanus (*C*Lam). The main vectors of *C*Las and *C*Laf are the Asian citrus psyllid (*Diaphorina citri* Kuwayama) and African citrus triozid (*Trioza erytreae* Del Guercio), respectively (Bové 2006). No resistant citrus scion-rootstock combination has been found for HLB (da Graca et al. 2015). Some of the challenges in controlling this devastating disease are long incubation period in which trees can remain asymptomatic but serve as a source of bacterial inocula for efficient vector spread. So far, *C*Las has not been successfully isolated in axenic culture (Bové et al. 2010).

HLB was first detected in 2012in Texas (da Graça et al. 2015), and since then the number of trees affected with it is growing. However, HLB-affected trees in Texas are yet to show signs of significant decline. It could be possibly due to aggressive psyllid management programs, few flush cycles, prolonged hot and dry summer weather, or other unknown factors (da Graça et al. 2017). It remains to be seen how HLB will affect the Texas

citrus industry in the coming years. More on HLB is discussed in Chapter 4 where its interaction with the oomycete pathogen *Phytophthora* is elaborated.

Several fungal pathogens have been identified in citrus orchards in Texas. Greasy spot and greasy spot rind blotch (GRSB) are caused by *Mycosphaerella citri*. Its primary effect is defoliation leading to the reduction of yield and fruit size. Fruit reductions of 25% and 45% in sweet orange and grapefruit, respectively were observed in Florida (Timmer 2000). Honeydew secretion by rust mite increases greasy spot severity by promoting the epiphytic growth of *M. citri* (Whiteside 1974). Control of rust mite populations and use of fungicide helps in reduction of the greasy spot (Timmer 1980). Melanose caused by *Diaporthe citri* Wolf (syn. *D. medusa* Nitschke; anamorph *Phomopsis citri* Fawc) produces pustules on fruits, leaves, and stems. Grapefruit is very sensitive to melanose. The melanose fungus can also cause a serious post-harvest disease known as stem end rot in which infected tissue shrinks at the stem end part (Anciso et al. 2002). Dry root rot causes sudden death of citrus trees (Timmer et al. 2000).

The disease presents characteristically blackened, rotted and discolored roots and is associated with the presence of *Fusarium* spp. including *F. solani* and *F. oxysporum*. Dry root rot is often associated with poor drainage, heavy soil, poor aeration and possible mechanical injury to the roots (Timmer et al. 2000). The first report of dry root rot of grapefruit and on sour orange rootstock in Texas was made in 2015 (Kunta et al. 2015). Another fungal pathogen that is known to cause root rot is *Ganoderma lucidum*, a wood-decaying basidiomycete. In Texas, it was reported in young grapefruit trees on sour orange rootstock planted next to dead tree stump. *G. lucidum* growth on and around the dead stump increased propagule pressure for the young trees and caused the death of several 4-year old

Rio Red grapefruit and Marrs early orange trees (Skaria 1990). *Ganoderma* isolates from Texas were found to be a different taxon within the *G. lucidum* species-complex based on a molecular and phylogenetic study of the ITS region (Kunta et al. 2010).

Lime anthracnose caused by *Colletotrichum gloeosporioides* Penz is an economically important fruit and foliar disease of citrus. It affects flowers, young leaves, shoots, and fruits (Timmer et al 2000). It grows well and produces many spores in acervuli which can be spread by rainwater. Ethylene, used during de-greening, is known to break dormancy and activate the spores (Anciso et al. 2002). Key lime anthracnose (KLA) caused by *C. accutatum* J. H. Simmonds seriously affects Mexican (Key) lime. KLA may not be a serious threat to the LRGV commercial citrus industry as Mexican lime is grown in the residential areas. Recently, molecular characterization of KLA isolates collected from leaf, twig, and fruits of symptomatic Mexican lime trees from three residential areas in Brownsville confirmed *C. acutatum* as the causative pathogen (Ruiz et al., 2014).

1.3 *Phytophthora* diseases of citrus and their management

Phytophthora can cause diseases of both rootstock and scion, and foot and root rot are two of the most important *Phytophthora* diseases affecting Texas citrus (Timmer 1973). *Phytophthora* species are alga-like Oomycetes that superficially resemble filamentous fungi but have a close relationship to diatoms and brown algae in the Kingdom Stramenopila (Gunderson et al. 1987). *Phytophthora* is prevalent in citrus orchard soils worldwide causing serious losses. Some of the diseases caused by *Phytophthora* in citrus are damping off of seedlings, root rot, brown rot of fruit, foot rot and gummosis (Timmer et al. 2000).

Under suitable conditions, *Phytophthora* spp. can infect many parts of the citrus tree. Foot rot occurs when a scion is infected near ground level. In resistant rootstocks, the

infection may progress up to the bud union whereas on susceptible rootstocks the infection may progress below the bud union and cause crown rot (Graham 1995). The most obvious symptoms of foot rot or gummosis is an amber color gummy exudate, yellowing in the cambium region and vertical cracking of the stem (Fawcett 1923; Timmer 1972). The cambium and inner bark are damaged but the outer bark remains firm with cracks oozing amber exudate (Timmer et al. 2000).

Lesions may sometimes extend around the circumference of the tree and partially girdle it. Such trees will have canopy thinning due to defoliation accompanied with twig dieback. In affected trees, the downward movement of photosynthates is affected (Futch and Graham 2009). In young trees, foot rot on trunks can completely girdle the trunk and kill the trees in 2 to 3 months. Chlorosis, twig dieback, and weak flushes are some of the visible damages seen in the canopy of older trees with partially girdled trunks (Graham and Feichtenberger 2015). *Phytophthora* spp. cause decay of fibrous roots. The cortex turns soft, becomes discolored, and appears water soaked. The cortex sloughs off leaving behind the white stele (vascular tissue) (Timmer et al. 2000). On susceptible rootstocks, lesions may occur on the structural roots below the soil line leading to canopy decline before any obvious symptoms (Timmer et al. 2000). Translocation of water and minerals are affected causing loss of vigor (Sandler et al. 1989). Fibrous root loss can be severe in susceptible rootstocks killing seedlings in a nursery bed and reducing yield in mature trees (Timmer et al. 2000).

Phytophthora infection of fruit is called brown rot. The affected area of the rind appears light brown and leathery. White mycelia form on the rind surface under humid conditions. Fruits become infected when they are hanging close to the ground or come in contact

with propagules through rain or irrigation water splash. Brown rot of fruit occurs when rain coincides with an early maturity of fruits (Timmer et al. 2000). Under favorable conditions, the fruits infection can takes place, and the infected fruit will drop. Infected fruit starts to show symptoms after harvest later during storage.

Severely affected trees progressively show a decline in fruit yield and canopy decline causing the death of the tree. The disease cycle begins with the production of sporangia which release large numbers of zoospores. Infection occurs when zoospores are splashed on tree trunks above the bud union (Graham 1995). Under unfavorable conditions, *Phytophthora* species also produce abundant chlamydospores in the soil (Tsao 1971). Use of resistant rootstocks, chemical control, and orchard management are still the most effective techniques to control *Phytophthora* diseases. *Phytophthora* spp. can cause severe damage to citrus plants in conjunction with some biotic and abiotic factors.

Interaction with other serious plant pests or pathogens with *Phytophthora* is reported in citrus. Root system injured due to feeding by *Diaprepes abbreviates* larvae have significantly increased the root rot infection by *Phytophthora* in susceptible rootstocks causing rapid tree decline (Graham et al. 1996). The so-called *Diaprepes-Phytophthora* disease complex has been found in Florida and California and was reported from Texas in 2001 (Skaria and French 2001). Similarly, studies have also described the possible interaction of *C*Las with *Phytophthora* species which causes pre-symptomatic fibrous root decline in citrus trees (Graham et al. 2013).

Flood irrigation is the common means of irrigation in the commercial orchards and can lead to a buildup of *Phytophthora* propagules. Alternative planting techniques such as raised beds (Maloney et al. 1997) may contribute to lower *Phytophthora* disease incidence.

Fosetyl-Al and metalaxyl are active fungicide ingredients with proven efficacies against *Phytophthora* disease. Overuse of one class of fungicide can lead to resistance (Timmer et al. 1997). *Phytopthora* caused diseases are better managed when along with chemical control, cultural practices are followed that reduce excessive moisture in the soil.

1.4 *Phytophthora* species pathogenic to citrus

The most common *Phytophthora* species associated with *Phytophthora* diseases of citrus are *Phytophthora nicotianae* Breda de Haan (Syn *P. parasitica* Dastur), *Phytophthora palmivora* (E. J. Butler) and *Phytophthora citrophthora* (R. E. Sm. & E. H. Sm.) Leonian. (Erwin and Ribeiro, 1996) Due to their different optimum growth temperatures, *P. nicotianae* is more active in subtropical parts of the world while *P. citrophthora* is more common in the Mediterranean climate (Alvarez et al. 2011). *P. nicotianae* (syn. *P. parasitica* Dastur) is increasingly reported as the predominant species in the citrus rhizosphere from many parts of the world (Hung et al. 2015; Mekonen et al. 2015; Álvarez et al. 2007; Das et al. 2016; Bright et al. 2004).

Temperature is a critical factor for the occurrence of these three species since each has a different temperature range of tolerance. *P. nicotianae* is the only species that can tolerate above 35^0C, whereas *P. citrophthora* and *P. palmivora* will only proliferate in locations with less than 35^0C (Gallegly and Hong 2008). The presence of *P. palmivora* in Texas orchards is not confirmed by previous investigators (Timmer 1973; Kunta et al. 2007). Recently, *P.citrophthora* presence was reported by molecular testing in some sites in LRGV commercial orchards (RoyChowdhury et al. 2016). However this study did not report cultural isolation or biological characteristics of the identified *P.citrophthora* isolates. Compared to *P. nicotianae*, *P. citrophthora* is more aggressive and attacks aerial

parts of the citrus tree. (Timmer et al. 2000). *P. palmivora* can be very damaging to roots under certain conditions and also causes brown rot epidemics of fruit. *P. palmivora* was first reported in orchards near Fort Pierce, Florida in 1991 (Zitko et al. 1991). Both *P. palmivora* and *P. nicotianae* were found associated with the brown rot of fruit epidemics in Florida from 1994 to 1997 (Graham et al. 1998).

P. nicotianae is considered the eighth most important pathogenic oomycete in plants (Kamoun et al. 2015). *P. nicotianae* has a high-temperature optimum (30 to 31^0C) surviving well at high temperatures (higher range is 37^0 C) compared to other *Phytophthora* species affecting citrus (Erwin and Ribeiro 1996). Under unfavorable conditions, *P. nicotianae* also produces abundant chlamydospores in the soil (Tsao 1971). Chlamydospores release zoospores within a short time of wetness; zoospores remain viable up to 20 h in irrigation water (Thomson and Allen 1976). *P. nicotianae* has a wide host range and causes disease in a wide variety of crops of economic importance, though it also shows some degree of host specialization (Erwin and Ribeiro 1996).

Genetic diversity analysis of a global collection of *P. nicotianae* isolates, including those collected from citrus rhizospheres in different geographical regions, found that majority of citrus isolates are results of asexually propagated heterozygote clones specialized to a specific host (Mammella et al. 2013). Compared to homothallic species, there is greater genetic uniformity amongst heterothallic species like *P. nicotianae* (Mammella et al. 2013).

In heterothallic species like *P. nicotianae*, both mating types need to be present for it to reproduce sexually. It should be noted though that occurrence of both mating types A1 and A2 have been reported from LRGV orchard soils, although not from the same orchard

(Timmer 1973). It will be important to know if both mating types occur in the same orchard and if isolates from a citrus orchard in the LRGV are genetically similar. Also, re-evaluation of species types in the LRGV citrus orchard soils is required to devise effective *Phytophthora* disease management.

The use of resistant rootstocks is the most effective control method for *Phytophthora* disease. However, there are some limitations to using *Phytophthora* resistant rootstocks because of their susceptibility to some other diseases, poor fruit yield and quality, and lack of adaptation to the calcareous, high pH and poorly drained soils (Graham and Feichtenberger 2015). Effective steps should be taken to raise disease-free plants at the nursery level. Use of disease-free plants is important, not only to control the introduction of *Phytophthora* into the orchards, but other serious disease caused by viruses, viroids, and bacteria. A virus-free budwood program has been in effect in Texas since 1997 that ensures disease-free certified buds are supplied to the nurseries (Skaria et al. 1997; Kahlke et al. 2005).

1.5 Huanglongbing (HLB) interaction with *Phytophthora*

Huanglongbing (HLB) or citrus greening is the most dreaded disease of citrus known today that causes citrus decline (Gottwald 2010). Characteristic symptoms of HLB are blotchy mottling with green islands on the leaves giving rise to a yellow shoot (Bové 2006). Tree growth is stunted and fruits become misshapen, green in color with a bitter taste. Of the three species, *Candidatus* Liberibacter asiaticus (*C*Las) is commonly reported form the Asian countries and is recently introduced into the Americas. *Candidatus* L. africanus(*C*Laf) and *Candidatus* L. americanus (*C*Lam) occur occur in Africa and Brazil respectively (Gottwald 2010).

HLB was first reported from Florida in 2005 (Halbert 2005), and *D. citri* was reported from the state in 1998 (Knapp et al. 1998). Since then it has drastically affected Florida's citrus industry. The economic impact of HLB on Florida's orange production from 2006/07 to 2013/14 was reported to be $7.80 billion (Hodges et al. 2014). In Texas, ACP was detected in 2001 (French et al. 2001). HLB was first reported in Texas in 2012 (Kunta et al., 2012; da Graça et al. 2015b) and has since extended to several citrus orchards in all three counties in the (LRGV) and counties in and around Houston (http://www.citrusalert.com/south-texas-quarantine-map).

*C*Las infects all parts of the citrus tree (Tatineni et al. 2008), and cause massive pre-symptomatic loss of fibrous roots (Johnson et al. 2014). A study has shown that fibrous roots are pre-disposed to *Phytophthora* damage when the tree is infected with *C*Las (Ann et al. 2004). It is possible that *Phytophthora* is attracted to roots damaged by *C*Las infection even before the appearance of foliar HLB symptoms (Graham et al. 2013). Another study showed that *C*Las after infecting leaf tissue might move to the roots (Johnson et al. 2014). The authors speculated that it is not phloem plugging of leaf tissue but the multiplication of *C*Las in the fibrous roots that causes root damage leading to tree decline (Johnson et al. 2014). Studies conducted to quantify the spatial distribution of *C*Las in roots measured higher bacterial titer in the horizontal roots just below the soil surface when compared to the deeper vertically growing roots (Louzada et al. 2015).

The overall objectives of this study were as follows:

1. To quantify foot rot and gummosis incidence and severity in the commercial citrus orchards in the LRGV.

2. To determine the prevalent species of *Phytophthora* occurring in the citrus rhizosphere and investigate diversity in biological characteristics of the isolates in the LRGV.
3. To determine the mating type and test the mefenoxam sensitivity of *Phytophthora* isolates in the LRGV.
4. To determine effects of the presence of both *C*Las and foot rot and gummosis disease on nutrient status, root health, and canopy thinning of the grapefruit trees.

CHAPTER II

INCIDENCE AND SEVERITY OF *PHYTOPHTHORA* FOOT ROT AND GUMMOSIS OF CITRUS IN SOUTH TEXAS

2.1 Introduction

Texas ranks third for citrus production in the United States after Florida and California (USDA, National Agricultural Statistics Service 2016). Citrus industry in Texas is mainly concentrated in the Lower Rio Grande Valley (LRGV) and is worth about $86.5 million annually (USDA, National Agricultural Statistics Service 2017). Bearing acreage of citrus production in Texas is estimated to be 24,500 with 85% of the commercial citrus orchards in Hidalgo County, 14% in Cameron County and approximately1% in Willacy County.

Foot and root rot caused by *Phytophthora* spp. have been present in most of the commercial orchards of the LRGV since the introduction of citrus into the region (Timmer 1972). *Phytophthora* constitutes an important and damaging pathogen to citrus tree health besides affecting the yield. Highly calcareous soils, high soil salinity, and poorly drained soil in the LRGV support increased *Phytophthora* propagules in the soils. Heavy rains and high temperatures in early fall are very conducive to *Phytophthora* infections and cause most outbreaks of foot rot during this time of the year in the LRGV (Timmer 1972). *Phytophthora* diseases are highly favored in conditions of high soil moisture and salinity. Most land in LRGV is reported to have high salinity due to saltwater intrusion and use of highly saline groundwater deemed unfit for irrigation (Carter and Wiegend 1965). Water from the Rio Grande River used for irrigation has moderate salinity, which increases downstream in the Mercedes irrigation district (Enciso et al. 2008).

Soil salinity can also increase in-between irrigation episodes due to evaporation and poor drainage. Soil salinity has been proven to cause stress to the roots by affecting the permeability of the plasma membrane of the root cells and increase root infection by *P. nicotianae* (Blaker and MacDonald 1986; MacDonald 1981). Besides *Phytophthora,* several other biotic and abiotic factors can cause rots and gumming of the trunks and branches. Physical damage due to wind, water or frost, lead to wood rot caused by injuries invaded by pathogens, but it normally affects xylem and is not accompanied by gumming (Fawcett 1923). Bark scaling and gummosis symptoms are also observed in certain viral and viroid diseases like cachexia, psorosis and exocortis (Timmer et al. 2000).

A disease that is reported to present characteristic gummosis symptom is Rio Grande gummosis (RGG). RGG affects wood trunk and large limbs of mature citrus trees. The disease also induces production of pale yellow gum release through narrow cracks (Childs 1950). The etiology of RGG is uncertain, and a complex of fungi has been associated with the gumming lesions (Timmer et al. 2000). A psorosis-like agent was proposed to be a possible causative agent of RGG since symptoms caused by RGG closely resembles bark scaling caused by CPsVCPsV (Lee 1994). However, psorosis hardly produce any gumming symptom whereas RGG induces heavy gumming and cycles of drying and gumming of the lesions. Psorosis also present formation of callus beneath the bark whereas RGG has characteristic gum pockets formed beneath the bark. Therefore, psorosis and RGG are symptomatically distinct and are caused by different pathogens. Initial expression of RGG may look like foot rot, except the cambial surface of the wood beneath the bark lacks brownish-yellow stain characteristic of foot rot disease (Childs 1950). In RGG the wound may dry and thin outer scaly bark may slough off. In foot rot lesion bark is killed

down to the wood and sloughs off as a slab. Healing of foot rot lesion occurs only at the margin. Therefore, RGG and foot rot lesion on the trunk may present similar symptoms although after careful inspection can be distinguished from each other.

Productivity in citrus is affected by root rot and gummosis. Yield losses due to gummosis and root rot are hard to co-relate since the two are not strictly proportional to each other (Graham and Kosola 2000). In Florida, yield losses due to root and foot rot without any chemical control have been estimated to be between 3 to 6% per year (*i.e.,* 30 to 60 million US dollars) (Graham and Menge 1999), although these estimates may not apply to Texas due to differences in the edaphic, horticultural and climate factors etc. Fungicide use is successful in controlling the disease but may not always be economical (Smith et al. 1989). An assessment of *Phytophthora* soil propagule counts in 61 citrus orchards across the LRGV reported that 33% of orchards had significantly higher than 20 cfu/cm^3 (RoyChowdhury et al. 2016). This study has some limitation as only one tree per orchard was selected for soil sampling in each of the evaluated orchards. To achieve representative results it is recommended that between 20 to 40 samples from different part of the orchard should be selected randomly to represent the number of soil propagules accurately (Timmer et al. 1988). Unfortunately, the RoyChowdhury et al. (2016) study did not address the association of *Phytophthora* propagules with foot rot incidence and severity, an important aspect for epidemiological studies.

Foot rot and gummosis incidence and severity data are very scant in the LRGV, with only anecdotal evidence on their common occurrence in many orchards. In order to achieve effective management of *Phytophthora* disease in this region, a comprehensive study is required.

Applying study results available from Florida and California to the LRGV situation is not feasible due to wide differences in soil types and climatic conditions. As earlier work done in this region did not study foot rot and gummosis incidence, but reported high *Phytophthora* propagule counts, it is hypothesized that very high incidence of foot rot and gummosis disease is affecting the citrus orchards in the region. Therefore, I undertook the question and investigated it in my study. Actual counts of foot rot and gummosis affected trees in the orchards, can explain the implication of ubiquitous and high propagule counts in the soil. Actual counts of foot rot and gummosis affected trees in the orchards, can explain the implication of ubiquitous and high propagule counts in the soil.

The main objective of this study were (1) to estimate the foot rot and gummosis incidence and severity in the commercial citrus orchards in the LRGV, region of South Texas; (2) to quantify *Phytophthora* propagule count in the soil and correlate the counts to foot rot and gummosis incidence and severity; (3) and to evaluate the effect of age, and cultivar type on disease incidence and severity in the region; (4) To evaluate the site of infection in scion starting below or above the bud union. It is hypothesized that this study will record a very high incidence of foot rot and gummosis disease in the citrus orchards in the region.

2.2 Materials and Methods

2.2.1 Disease surveys

A region-wide survey was conducted across the three-county of citrus growing area of the LRGV region from February to June of 2015 and 2017 (Figure 1). Thirty orchards from the original 61 orchards (RoyChowdhury et al. 2016) were selected as the site to

study *Phytophthora* propagule count and its association with foot rot incidence and severity (RoyChowdhury et al. 2016). Twenty-four grapefruit, two tangerine and four sweet orange orchards (10 to 20 acres), all on sour orange rootstock, were selected for the study. Records of orchard age, location, cultivar, and type of irrigation were taken (A-1**,** A- 4). In the 2017 survey, only orchards under flood irrigation were surveyed. Orchards irrigated by methods other than flood irrigation forms a small pool and statistical comparison could not be made. Location of lesion below or above bud union was also noted. Lesion was distinguished on branch or on trunk of the tree. The trunk lesion was distinguished as present below or above the bud union. Grapefruit orchards more than 20 years of age were categorized as old and less than 20 years as young.

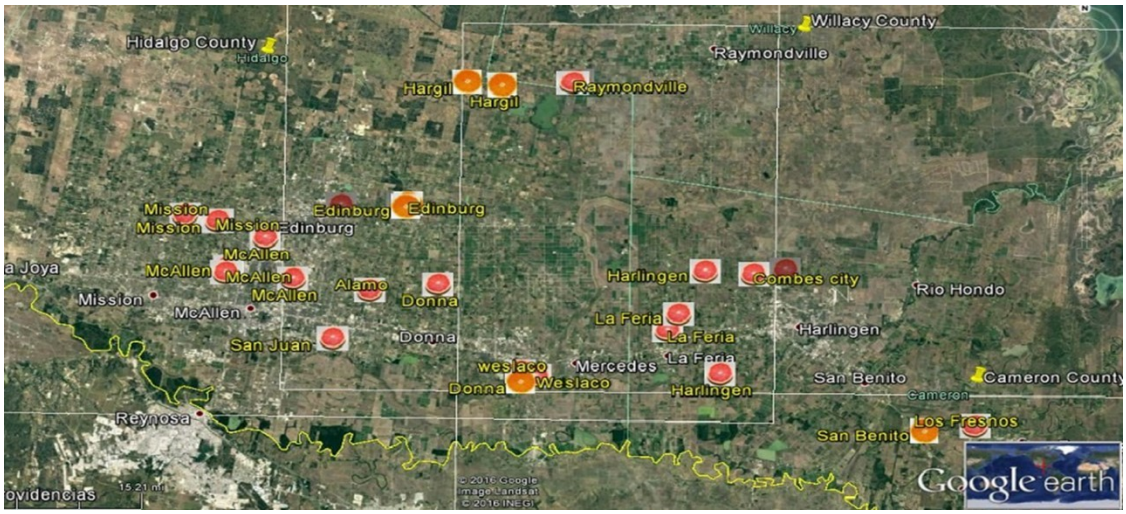

Figure 1. **Survey sites**. Location of commercial citrus orchards evaluated for foot rot incidence and disease severity in the Lower Rio Grande Valley, Texas. Red color and orange color legend represent grapefruit and orange varieties orchards respectively.

2.2.2 Disease assessment

The incidence of lesions and gummosis on the trunk of the twenty trees from each orchard was rated. Trees were sampled as a diagonal transect through the orchard. Foot rot and gummosis on tree trunks were rated on a scale of 0 to 5 (Little 1978) (Figure 2**,** Figure 3**).**

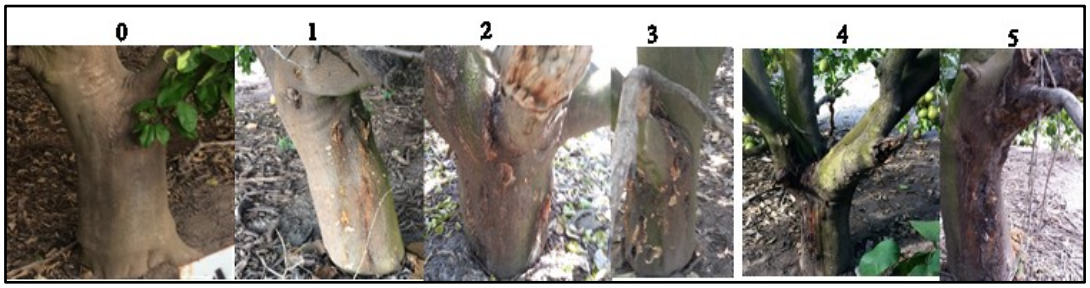

Figure 2. Visual rating scale for rating foot rot and gummosis disease severity of citrus trees. Trees were rated from 0 to 5 based on the severity of foot rot and gummosis symptoms on the tree trunk. A rating scale of "0" represents lesion-free trunk. One is < 10% of the trunk covered with the lesion and has callus formation around the lesion, no vertical extension. 2 is 11 to 35 % of the trunk covered with lesions, wound closed with some callus formation. 3 is 65% of trunk covered with lesions, may have callus formation. Lesions vertically extended. Some gummosis. 4 is 65% of trunk covered with lesions, may have callus formation. Lesions vertically extended associated with gummosis. 5 is more than 90% of the trunk covered with lesions, showing extensive gummosis and extending to major branch.

Incidences of foot rot and gummosis were determined as the proportion of trees showing gummosis symptoms, expressed as a percentage of the total number of trees assessed. The value of the empirical scale of foot rot disease rating was utilized to calculate the McKinney index (McKinney 1923) or disease severity percent using the formula:

$Mi = [\sum(d.f) \div (Tn.D)] \times 100$

Where d is the degree of foot rot intensity rated on an empirical scale and f is the frequency of trees with a rating. Tn is the total number of trees assessed in the orchard and D is the highest degree of disease intensity on an empirical scale. The values are multiplied with

100 to express it as a percentage. The index represents a weighted average expressed as an actual percentage concerning the maximum disease possible (100%).

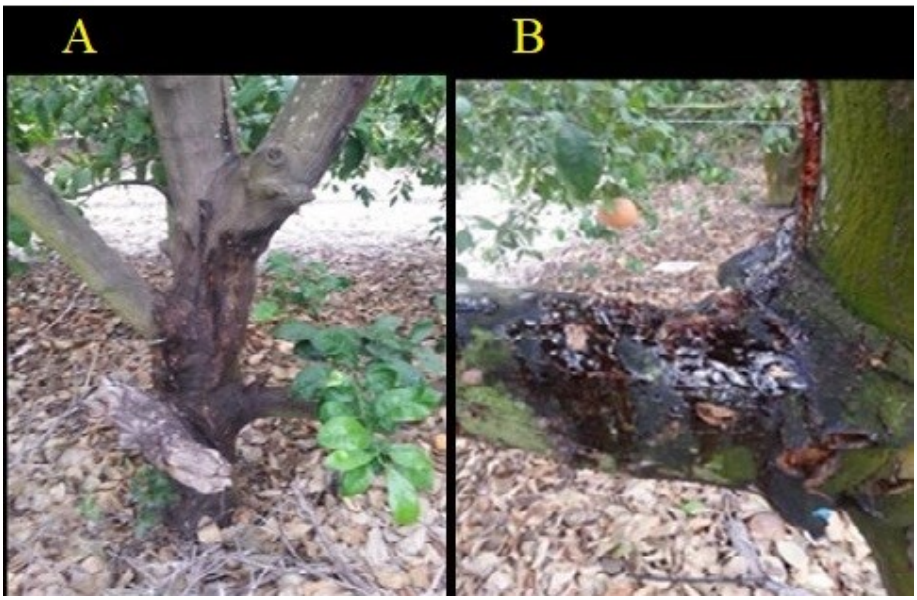

Figure 3. Grapefruit tree showing foot rot and gummosis symptoms. (A) Foot rot lesion on trunk extending to primary branch. (B) Primary branch is showing active gummosis.

2.2.3 *Phytophthora* propagule quantification

A single soil core was collected from the 20 randomly selected trees evaluated for foot rot and gummosis per orchard. The soil sample was collected from under the canopy facing the irrigation border. The soil was transported to the lab in Ziploc® bags, and one composite sample was developed by combining 100 cm^3 from each of 20 soil samples per orchard. From each composite soil sample, 100 cm^3 of soil was soaked in a foam cup with a drainage hole at the bottom, placed in another foam cup, and allowed to drain for 48 hours. The soil slurry (10cm^3) was taken from this sample and diluted with 90 mL of double distilled sterilized water. One milliliter (mL) each of diluted soil slurry was plated onto

five plates containing 17 g/L corn meal agar amended with 10 µg/ml Rifamycin-SV(sodium salt), 71.4 µg/ml Tachigaren (70% Hymexazol), 10 µg/ml Delvocid (50% Pimaricin), 66.7µg /ml of 75% PCNB, 250 µg/ml of Ampicillin (PARPH CMA) (Jeffers 2006). *Phytophthora* colonies were counted after five days of incubation of the plates in the dark. Propagule counts were reported in cfu/cm^3.

2.2.4 Isolation from infected bark and roots

Phytophthora was isolated from trunk lesions by excising tissue from the margin of the lesion with the help of a sharp knife. The tissues were surface sterilized with 70% ethanol for 10 seconds and immersed in 10% bleach solution for 10 minutes. Following surface sterilization trunk tissues were rinsed with sterile water 3-4 times before plating. The tissue taken from lesions and roots were plated on PARPH- CMA (Jeffers 2006). Plates were incubated in the dark and checked for colonies after 5 days. Colonies were visually inspected and the *Phytophthora*-like colony was subcultured on 10% Clarified V8 agar (CV8A) (Jeffers 2006). *Phytophthora* species was identified through biological and molecular characterization (Chapter 3).

2.25 Statistical Analysis

Statistical analysis was done on transformed data to take care of data not normally distributed as evident from the box plots graphs. Percent data were arcsine transformed to analyze the effect of, age, and cultivar type on disease incidence and severity in the orchards. Propagule means were log transformed for analysis. Data were analyzed with PROC GLM (SAS version 9.4 for Windows, SAS Institute, Cary, NC.) to test significance. Mean separations were made by the Student-Newman-Keuls multiple range test if "f" value was found significant at $P < 0.05$.

2.3 Results

2.3.1 Foot rot incidence and disease severity

Survey of 30 citrus orchards in the three different commercial citrus growing counties in the Lower Rio Grande Valley of Texas showed that foot rot is prevalent in 97% of those orchards. Out of the 600 trees evaluated, 33% had some level of foot rot. Orchards ranged between 5% to 90% in foot rot and gummosis incidence. Only one out of 30 orchards (Edinburg#25) had no foot rot during the 2015 survey, but the same orchard measured 15% foot rot incidence when evaluated in 2017. The disease severity index of foot rot ranged from 5% to 57%, with average orchard disease severity of 14.2%. Only flood-irrigated orchards were surveyed in 2017 as most orchards in the LRGV are flood irrigated. Only four orchards surveyed in 2015 were not flood irrigated and hence data could not be statistically compared to other orchards. Results for foot rot severity and incidence in the commercial orchards did not vary significantly between 2015 and 2017 except in sites Harlingen#56 and Combes#53 (Table 1, Figure 4). Most orchards surveyed in 2017 reported an increase in disease severity rating over the 2015 data.

Table 1. Foot rot incidence, disease severity index, and propagule counts in the commercial citrus orchards surveyed in the spring of 2015 and 2017.

	2015			2017		
	No.	Average (%)	Range	No.	Average (%)	Range
Percent foot rot and gummosis (NS)	30	33.7±4.2	0–90%	20	32.5±4.3	5-70%
Disease severity index (NS)	30	14.2±2.2	1-57%	20	15.9 ± 3.0	2-42%
Soil Propagule level (cfu/cm^3) *	30	28.5±4.2	0-118	20	9.1±2.3	0-34

(NS) No significant difference in mean at $p < 0.05$, *Mean was significantly different between years 2015 and 2017; df = 43, t = 2.72, P = 0.0094.

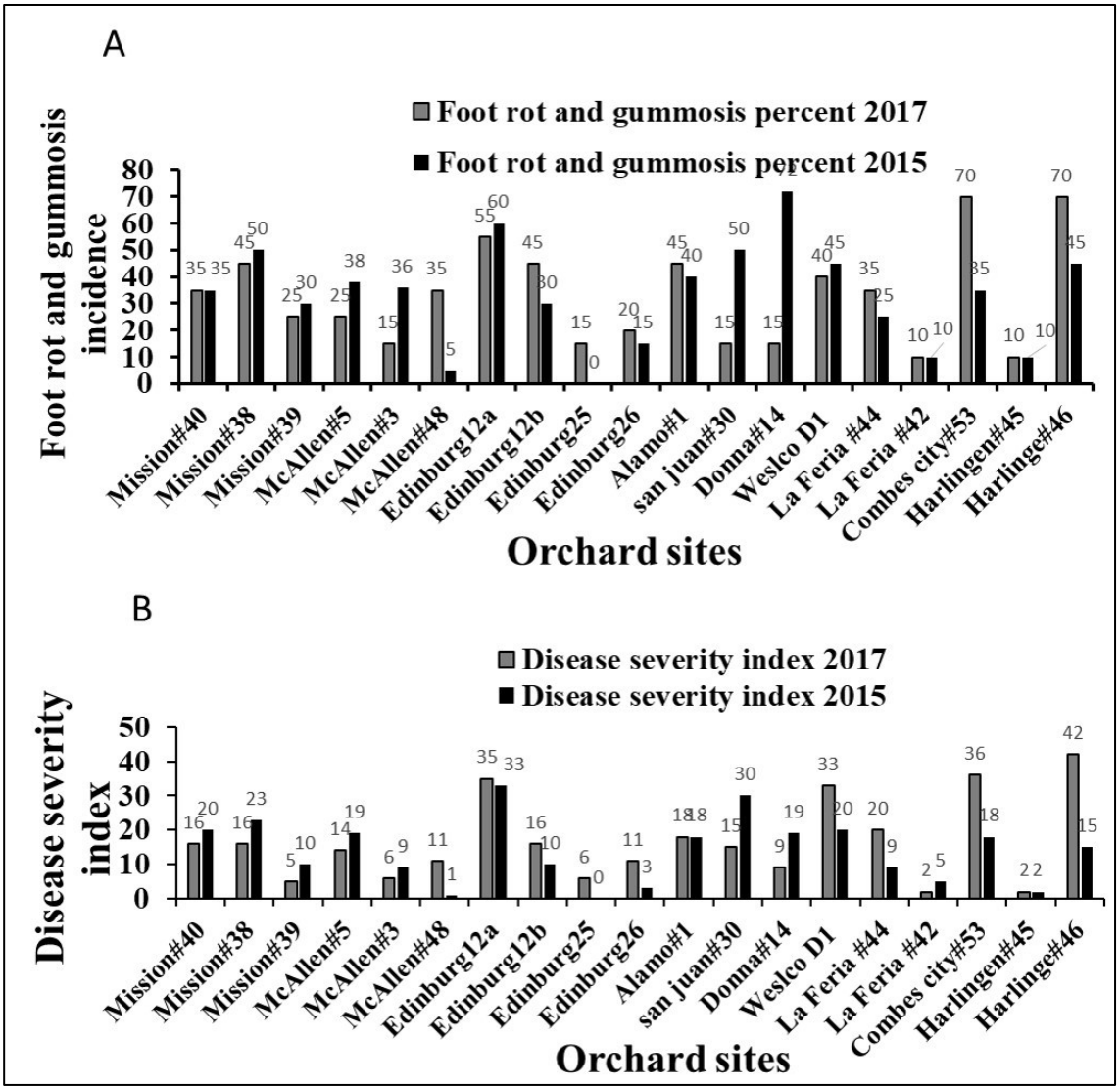

Figure 4. Foot rot and gummosis incidence and disease severity index of citrus orchards in the Lower Rio Grande Valley, Texas. Comparative analysis of (A) foot rot and gummosis incidence and (B) disease severity index of 19 orchard sites in the Lower Rio Grande Valley, Texas-based on surveys conducted in 2015 and 2017. Although 30 sites were studied in 2015, 10 sites were not studied in 2017 and cannot be compared.

2.3.2 *Phytophthora* propagule counts

Phytophthora propagule counts in orchard soils during the 2015 survey ranged from 4 to 118 cfu/cm^3, with 77% (23 out of 30) of the orchard soils measuring more than 10 cfu/cm^3 which is the experimentally established threshold for damaging levels of *Phytophthora* (Timmer et al. 1988). The highest number of propagules were recorded in Weslaco# D1 orchard (Figure 5). Propagule counts for the 2017 season were significantly lower and ranged between 5 to 34 cfu/cm$^{3.}$ During 2017 measurements, only 8 out of 20 orchards studied measured more than the threshold level of 10 cfu/ ml (Figure 5). Nevertheless, during both the year studies propagule counts were not correlated with either foot rot incidence ($P = 0.74$) or disease severity ($P = 0.41$).

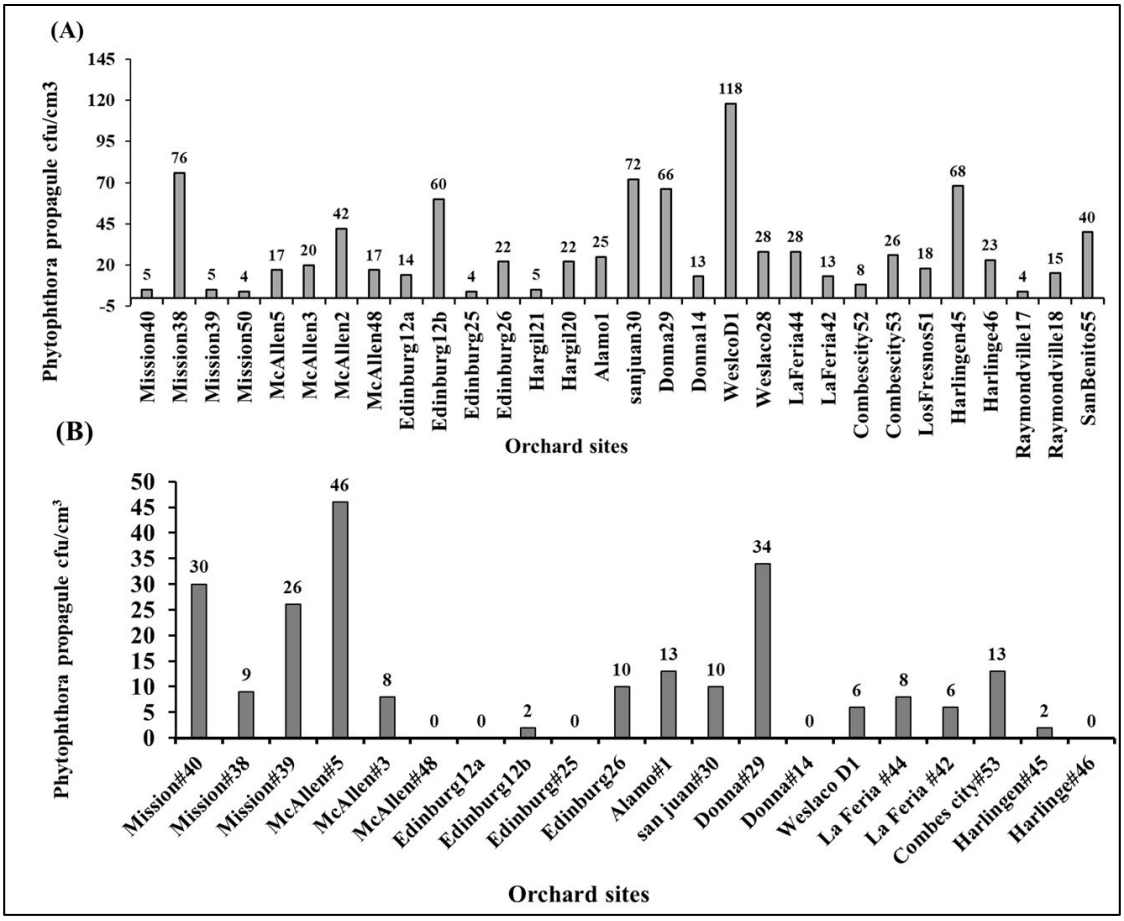

Figure 5. *Phytophthora* propagule counts (cfu/cm^3) in commercial orchard soils of the LRGV, Texas. The soil was assayed for *Phytophthora* propagule counts from February to May 2015 (N=30) (A) and 2017 (N=20**)** (B). Actual site-specific propagule counts are shown above each bar.

2.3.3 Effect of citrus cultivar type, location, and age of the orchard on foot rot and gummosis incidence, severity and soil propagule level of *Phytophthora*

Only flood-irrigated orchards were included to analyze the effect of age of the orchard on disease incidence, severity and *Phytophthora* propagules in the soil. Orchards not flood irrigated formed a very small sample size and could not be statistically compared. Four out of 30 orchards studied were not flood irrigated. Effect of irrigation on disease incidence and severity could not be analyzed due to small sample size. Older orchards had

significantly higher ($P < 0.05$) foot rot and gummosis incidence disease severity compared to younger orchards. *Phytophthora* propagule counts, though higher in older orchards, did not significantly differ from younger orchards (Figure 6). The orange and tangerine cultivars were pooled as no significant difference was observed in disease incidence between them and compared with grapefruit. Both foot rot incidence and disease severity index were significantly higher in grapefruit orchards compared to tangerine/orange orchards (Figure 6)

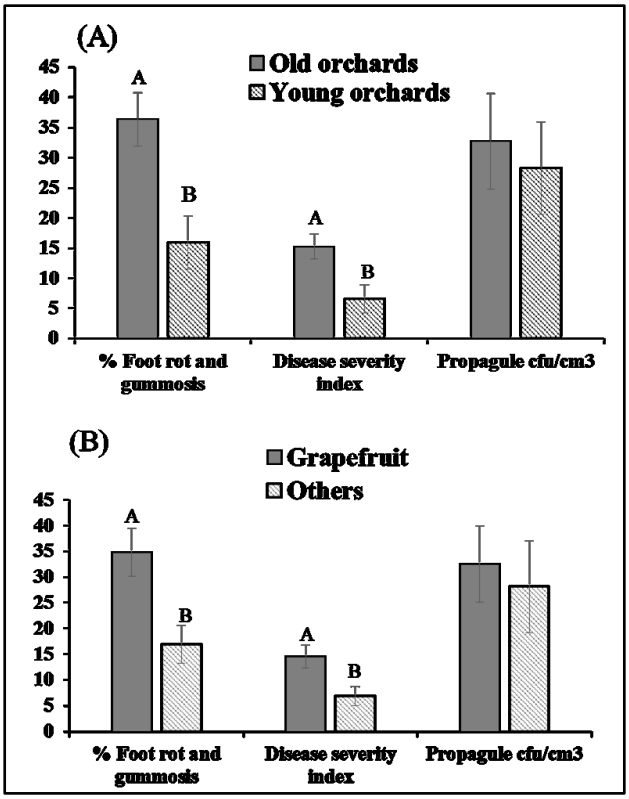

Figure 6. Comparison of *Phytophthora* propagule counts in the soil, foot rot incidence, and disease severity index based on tree age, and cultivar type. (A) Age (old orchards <20 years of age and young orchards>20 years of age) and (B) cultivar type (others include varieties of orange and tangerine) on foot rot incidence percent, disease severity index and soil propagule count of *Phytophthora.* Error bars with different letters represent a significant difference in the means at ($P<0.05$).

2.3.4 Tree parts affected and source of lesion

Primary part of the tree affected by gummosis was studied only in the 2017 survey. The presence of lesions on the branch was observed in 54% (214/400) of trees, whereas lesions on the trunk were observed in 34% (135/400) of surveyed trees (Figure 7). Lesions on the trunk extending from below the bud union were observed in 21% (29/135) and from above the bud union were observed in 79% (106/135) of the trees (Figure 7). Branch lesions originating from the trunk and extending to the primary branch, as well as lesions developed on the branch were observed at 15% (32/214) and 85% (182/214), respectively. Branch lesions originating in branches were significantly higher ($p>0.05$) than those originating in the trunk and extending to the branch (Figure 7).

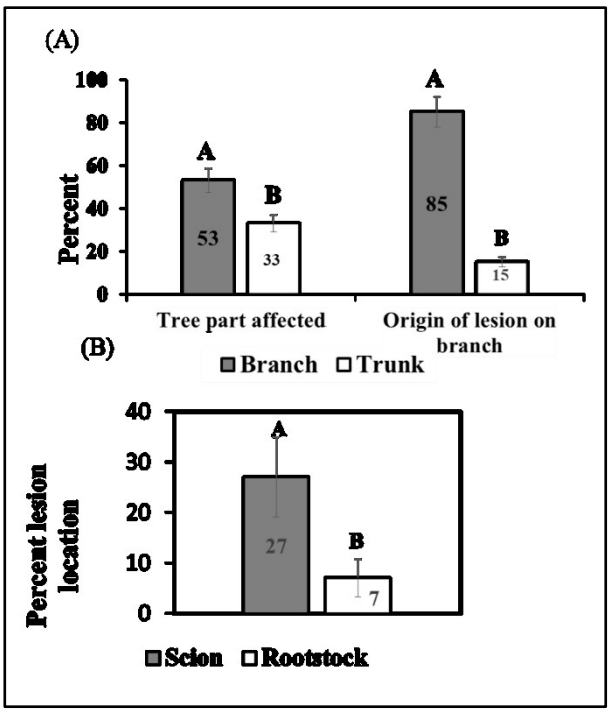

Figure 7 Location of the lesion. (A) Comparative analysis of part of the tree affected with gummosis and lesions on the branch as percent extending from the trunk or formed on branch itself. (B) The graph represents data showing the percent of the location of the lesion detected on rootstock or trunk. Error bars with different letter represent significant difference at *P>0.05*.

2.4 Discussion

Foot rot and gummosis are ubiquitous diseases in citrus orchards of LRGV region in South Texas. Their occurrence was reported as erratic and less common in the past, though outbreaks of foot rot and gummosis occurred after Hurricane Beulah (category 5) hit LRGV in the year 1967 (Timmer 1972). This work is the first comprehensive study that covers the entire Rio Grande Valley region and has attempted to quantify foot rot incidence and severity. Ninety-six percent of orchards surveyed in 2015 had 33% average foot rot and gummosis incidence with an average 14% (n=30) disease severity. High incidence of foot rot and gummosis have been reported from other citrus growing regions of the world

such as Kenya (79% disease incidence) (Mounde et al. 2009), South Africa (Schutte and Botha 2010), Spain (Alvarez-Rodriguez et al. 2016), Brazil (Urashima et al. 2016), and Ethiopia (90% disease incidence) (Mekonen et al. 2015). About 73% of orchards had more than 10 cfu/cm^3 of *Phytophthora* propagules in the soil, which is considered as the treatment threshold level for fruit-bearing orchards and has been experimentally calculated (Timmer et al. 1988). Comparatively, in 2017, *Phytophthora* propagule counts were significantly lower in the soil. This difference may be due to the more than average rainfall in the Spring of 2015 (http://www. us climate data. com) (A- 3)

The average rainfall and temperature significantly differed between 2015 and 2017 spring. More than average rainfall in spring of 2015, might have favored higher numbers of *Phytophthora* propagules in the soil. *Phytophthora* propagule count was higher in flood-irrigated orchards compared to orchards irrigated through drip and micro-sprinklers. This observation is anecdotal as statistical tests could not be done due to the small sample size of drip and micro-sprinkler types of irrigation.

Propagule counts of orchards in this study did not correlate with foot rot incidence and severity. Infection of tree trunks may have occurred in the past and the lesions develop over the years due to the invasion of the healthier tissue by the pathogen. Foot rot and gummosis is a chronic disease and once a scion is infected the severity can increase over time depending on environmental factors. Besides, foot rot does not add propagules to the soil. It may be one reason why foot rot and gummosis incidence and severity did not correlate to the fluctuating propagule levels in the soil. Therefore, the time of sampling of propagule and infection of trunk do not overlap in case of an endemic pathogen and perennial host.

The age of the orchard proved a critical factor since flood irrigated orchards older than 20 years had significantly higher disease incidence and severity compared to orchards below 20 years of age under similar irrigation regimes. These results confirm some earlier findings on the effect of the age of an orchard on foot rot incidence (Dhakad et al. 2015). Some of the present old orchards in the LRGV are the survivors of 1983 and 1989 severe freeze events. It may also be one of the leading causes of severe and high incidence of foot rot and gummosis observed in the older orchards. Therefore, results from both cultivar type were combined and were compared with grapefruit. Foot rot incidence and severity were highest in grapefruit orchards, compared to orange and tangerine orchards, confirming a higher susceptibility of grapefruit compared to orange in earlier studies (Klotz and Fawcett 1930).

The lesions were observed in significantly higher percentages (54%) on branches compared to trunks, and a significant percentage of the lesions on branches originated on the branch compared to an extension of infection from the trunk lesion. Similar findings have been observed in orchards in Spain about branch cankers caused by *P. citrophthora* (Alvarez et al. 2008). The mechanism of dispersal to branches is not clear as rain or irrigation water splashing could be the cause of dissemination of the propagule to the branches, especially those located within 1m off the ground. Hedging of the branches can also cause branch infection if hedging equipment is contaminated with soil containing *Phytophthora* propagules. Other sources of inoculation could be tools used during harvesting. A significant percent (79%) of foot rot and gummosis on the trunk were observed above the bud union, though some percentage (21%) originated from below the bud union and extended to

the trunk above. This finding confirms some degree of susceptibility on the part of sour orange rootstock to *Phytophthora* though the scion too could play a role in the susceptibility of sour orange (Ippolito et al. 1997).

This study determined thirty-three percent of the surveyed trees in the 30 commercial citrus orchards in the LRGV studied to be infected with foot rot. The finding of this study therefore has implications for the production of citrus and the lifespan of the infected trees. The current study expanded on the work of RoyChowdhury et al. (2016) where *Phytophthora* populations were quantified and identified. However, this current work surveyed orchards for foot rot and gummosis incidence and severity and correlated it to the soil propagule counts. Future work should include determining the pathway of *Phytophthora* sp. into orchards.

Phytophthora propagule numbers fluctuate in the orchard soil, depending on biotic and abiotic factors. Besides the presence of *Phytophthora* in the soil, several other factors like soil moisture, tree age, and injury to the bark are factors that will influence infection. *Phytophthora* can reach damaging levels if soil moisture is excessive. Since foot rot can be chronic, once a scion is infected with *Phytophthora* the lesion can spread slowly over the years. Foot rot does not necessarily add *Phytophthora* propagules to the soil, which explains the absence of correlation found in this study between soil propagule level and foot rot incidence and severity. An important factor in disease control will be soil moisture management and reducing the risk of contact between tree trunk or branches and soil containing *Phytophthora* propagules. Also, cultural practices that increase prolonged wetting of trunks or location of the bud union close to the ground need to be avoided.

CHAPTER III

IDENTIFICATION AND CHARACTERIZATION OF *PHYTOPHTHORA* ISOLATES FROM TEXAS CITRUS ORCHARDS

3.1 Introduction

Identification of *Phytopthora* species causing foot rot and root rot is an essential aspect of an epidemiological study on *Phytophthora* diseases. More than one species of *Phytophthora* can cause disease in citrus. Three earlier studies reported the presence of *P. nicotianae* Breda de Haan (synonymous with *P. parasitica* Dast.) in commercial citrus orchard soils in Texas (Timmer 1973; Kunta et al. 2007; RoyChowdhury et al (2016). These reports were based on biological and molecular characterization of field isolates *Phytophthora* obtained from different orchards. Also, RoyChowdhury et al. (2016) reported the identification of *P. citrophthora* (Sm. & Sm.) Leonian, in rhizosphere soils of commercial citrus orchards in the LRGV based on molecular testing. However, the *P. citrophthora* isolate was neither cultured nor morphologically identified. Isolation and confirmation through cultural methods is an essential step for a definitive confirmation of specific *Phytophthora* species sampled from the soil or plant tissue.

P. nicotianae primarily causes foot and root rot, but occasionally attacks aerial parts of the tree and causes brown rot of the fruit (Graham and Timmer 1995). *P. nicotianae* has been listed as one of the eighth most important oomycete pathogens (Kamoun et al. 2015) in the list of the top ten most economically important oomycete pathogens in the world. This species is tolerant to high temperature and infects a wide range of horticultural crops besides citrus (Panabieres et al. 2016; Kamoun et al. 2015; Erwin and Ribeiro 1996). It infects several ornamental and horticultural crops and as a result its global spread is facilitated through

potted plant and nursery ornamentals trading (Panabieres et al. 2016). The optimum temperature for growth is a few degrees higher in *P. nicotianae* compared to many other *Phytophthora* species (Erwin and Ribeiro 1996). *P. nicotianae* is a heterothallic species and will form oospores when mated with the opposite mating type. The occurrence of both mating types was confirmed in the LRGV soil (Timmer 1973). Therefore, it is predicted that *P. nicotianae* populations will exhibit diversity regarding pathogenicity and morphological characteristics.

The LRGV is an agriculturally productive region, and horticultural and agronomic crops of economic importance are grown there (Aggie-horticulture.tamu.edu, 2017). *P. nicotianae* has a wide host range and causes several economically important diseases. Rain and irrigation water can easily disperse *Phytophthora* propagules from the surrounding agricultural field plots into citrus orchards. Therefore, investigations into potential cross-pathogenicity of *Phytophthora* isolates between citrus and non-citrus hosts will increase our understanding of disease management for susceptible crops like tomato, bean, and squash that are grown in the region.

Control strategies against foot and root rot, such as resistant rootstocks, fungicides, and proper orchard management may not always be effective (Menge and Nemec 1997). Most of the citrus in South Texas is grown on sour orange rootstock which is considered moderately tolerant to root rot caused by *Phytophthora* spp., although susceptibility of scions can result in foot rot and gummosis and can affect aerial parts of the tree (Timmer 1972; Furr and Carpenter 1961). *Phytophthora* diseases in citrus can be minimized by choosing resistant rootstocks, planting seedlings with bud unions above the soil level, and planting disease-free trees. Fungicide treatment in young orchards is advised based on

rootstock susceptibility (Graham et al. 2014). Mefenoxam and Fosetyl-Al as systemic fungicides have been proven effective in controlling soil propagules of *P. nicotianae* and proven effective in improving tree health (Sandler et al. 1987).

Metalaxyl was introduced in 1977 to control Oomycete pathogens in many crops, and its isomer mefenoxam is widely used today to control *Phytophthora* related diseases (Erwin and Ribeiro 1996). Mefenoxam-based fungicides are one of the primary disease control chemicals for *Phytophthora* diseases, but due to its excessive use in nurseries, resistant isolates of *P. nicotianae* have been reported in ornamentals grown in nurseries (Hu et al. 2008; Olson and Benson 2011; Patel et al. 2016). Mefenoxam-resistant *P. nicotianae* isolates from citrus nurseries, and young orchards have been reported in Florida (Timmer et al. 1998; Donahoo et al. 2013).

The objective of this study was to identify the prevalent species of *Phytophthora* in the commercial citrus orchards in LRGV, Texas. This study was also undertaken to provide further information about the efficacy of mefenoxam as a fungicide and if any resistant *P. nicotianae* isolates occur in the citrus rhizosphere soil in the region. Another objective was also to test diversity amongst the *Phytophthora* isolates in LRGV and determine their pathogenicity and biological characteristics. I hypothesized that *P. nicotianae* would be the prevalent species and isolates will show differences in biological characteristics.

3.2 Materials and Methods

3.21 Sampling sites

Soil, roots, and bark samples were collected during a region-wide survey of 30 commercial orchards in the Lower Rio Grande Valley (LRGV), and six cities located along the coastal bend of the Gulf of Mexico, (Galveston, Corpus Christi, Alvin, Palacios, Orange, and Rockport) from February to June in 2015 and 2017. Soil samples from six cities in Texas, located near the coastal bend were analyzed to compare the *Phytophthora* isolates present in them to those found in soils from the LRGV citrus orchards (Figure 8). The acreage of orchard sites located in the LRGV range from 0.04 to 0.08 Km^2.Sites from outside the LRGV region were mainly dooryard types of citrus cultivation or were grown on less than 0.012 Km^2 of land. *Citrus* spp. hosts included grapefruit, some oranges and tangerine trees.

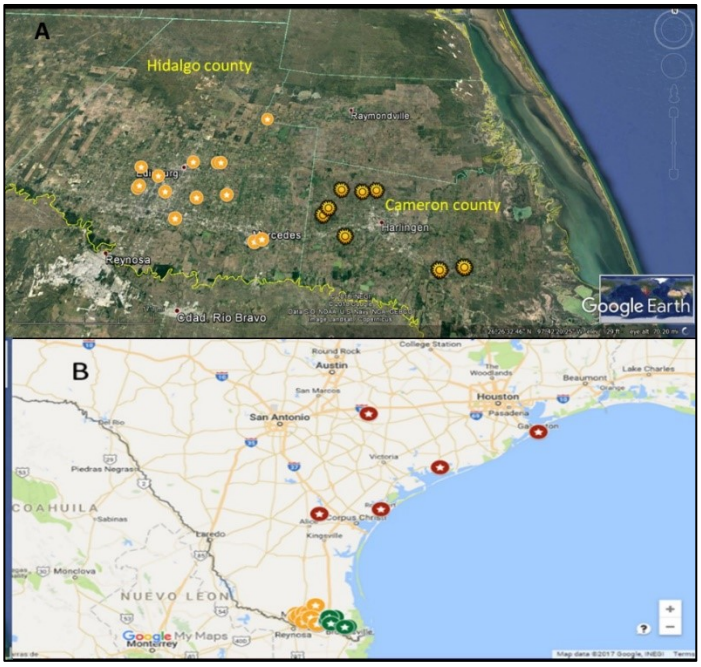

Figure 8. Orchard sampling sites for *Phytophthora* isolate collection. A) The orchards sites in the Hidalgo County (light orange) and Cameron County (dark orange) of Lower Rio Grande Valley (LRGV) of South Texas. (B) Citrus tree sites in counties located outside the LRGV region, along with the east coastal bend of the Gulf of Mexico. Light and dark orange legends in the map A, represent orchards surveyed in the Hidalgo and Cameron county respectively.

3.22 Sample collection

Tissue samples from trunks showing signs of gummosis were obtained from the advancing margin of the lesions. Bark showing gummosis was removed with the help of a sharp field knife, diseased along with some healthy tissue from the margin of each lesion was removed with the help of an Azpack™ carbon steel razor blade of dimensions 38.1 x 19mm (L x W). Soil containing fibrous roots were collected under the tree canopy, from the section facing the irrigation borders from a depth of 7 to 8 cm with the help of a garden spade. Both tissue and soil samples were transferred in the Ziploc® bags kept on ice in an ice chest. Roots showing symptoms of rot along with healthy looking roots were assayed for the presence of *Phytophthora*. Soil, root, and stem tissue samples collected from 37

sites were analyzed for the presence of *Phytophthora*. Stem and root tissues were washed in running water for 10 min and dried. The samples were surface sterilized with 70% ethanol for 30 seconds, rinsed and treated with 10% household bleach for 1 minute, then rinsed again with sterilized distilled water (Drenth and Barbara 2001). The root segments were cut into 1 cm pieces and transferred to selective media PARPH-CMA containing 17 g/L corn meal agar (CMA) amended with 10 µg/ mL Rifamycin-SV(sodium salt), 71.4 µg/mL Tachigaren (70% Hymexazol), 10 µg/ mLDelvocid (50% Pimaricin), 66.7µg /mL of 75% PCNB, 250 µg / mL of Ampicillin) (Jeffers, S. N. 2006) (Drenth and Barbara 2001). The plates were incubated at 23 ± 2^{0}C for 4 to 5 days, in the dark.

Two methods were used to isolate *Phytophthora* from soil samples. In the first technique, *Phytophthora* was baited using the leaf disk technique. Leaf disks (5mm) were cut from grapefruit leaves, washed in tap water, and surface sterilized with 70% ethanol for 30 seconds and rinsed with sterilized water. A soil sample of volume 100 cm^3 was placed in a foam cup and was topped with enough water to form 3 to 5 cm of the free layer of water above the soil surface. Leaf disks baits (5mm) were floated on the top and cups were left in the dark to incubate for 48 hrs. Leaf disks were dried with a sterilized paper towel and transferred on to PARPH-CMA. After 2 to 3 days of incubation in the dark, plates were inspected for *Phytophthora* colonies and checked for non-septate mycelium under 10X objective with the help of an Olympus CK2 inverted phase contrast microscope ULWCD -.30 (New York Microscope Company Inc., Hicksville, NY). In the second method, *Phytophthora* colonies from soil samples were obtained by directly plating diluted soil slurry on PARPH-CMA media. After an incubation period of 4 to 5 days in dark, *Phy-*

tophthora colonies were isolated and subcultured (Jeffers et al. 2001). Single zoospore colonies were obtained from the isolates to get single genotype isolates for species identification purposes.

Based on colony characteristics, mycelial morphology or presence of sporangia, *Phytophthora*-like colonies were identified. Agar plugs were removed and subcultured on PARPH-CMA plates (Erwin and Ribeiro 1996). Working cultures were maintained on 10% clarified V8 agar (100 ml Campbell vegetable juice V8 amended, 1 g $CaCO_{3,}$ 900 ml of distilled water, 15 g agar) (Jeffers 2006).

3.23 Identification and phylogenetic analysis

Total genomic DNA was extracted from lyophilized mycelium of 93 isolates collected from citrus orchards. Isolates were grown in 15 mL of 10% clarified V8 broth (100 ml Campbell vegetable juice V8 amended, 1 g $CaCO_{3,}$ 900 ml distilled water) at 22^0 C for 7 to 10 days. DNA was extracted with DNeasy Plant Mini Kit (Qiagen Inc, Valencia CA) according to manufacturer's instructions. The nuclear rDNA internal transcribed spacer (ITS) region including the 5.8S was amplified using the primer pair ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') (Cooke et al. 2000; White et al. 1990).

The PCR reaction mixture consisted of 10 µl of 10X buffer, 4 µl of 2 mM of $MgCl_2$, 1 µl of 0.2Mm dNTP, 1 µl each of 0.2µm forward and reverse primer, 0.25 µl 1.25 U Taq polymerase enzyme mixed with nuclease-free water to make a 50 µl total volume. Amplification was carried out in MyCycler^{TM} Bio-Rad Laboratories Inc, thermal cycler with the condition of pre-denaturation at 94^0C for 3 min, followed by 35 cycles at 94^0C for

1 min, 55^0C for 1 min, and 72^0C for 1 min. A 900 to 1000 bp length amplicon was generated, which was checked by running 5 µl of amplicon and 1 µl of loading dye on a 1% agarose gel in the 1X Tris-acetate (TAE) buffer at 100 A, for 45 minutes (Grunwald et al. 2011). The PCR product with the commercial kit QIAquick PCR purification from QIAGEN® and submitted to McLAB DNA Sequencing Services (San Francisco, CA, USA) for sequencing. The in-house developed Python script (Dr. Kranti Mandadi lab, Texas A&M University, AgriLife Experiment Station, Weslaco) was used to generate the consensus sequences based on the BLAST (Altschul et al. 1997) sequence similarity. The sequences were searched for similarities at National Center for Biotechnology Information's (NCBI) GeneBank using BLASTn program.

Eighty-one out of 93 isolates' consensus sequences were further used to generate a phylogenetic tree. The phylogenetic tree was inferred using the distance-based Neighbor-Joining method with 100 bootstrap replicates (Saitou et al. 1987, Felsenstein et al. 1985). The evolutionary analysis was performed using the MEGA7software (Kumar et al. 2016).

3.24 Morphological characterization

Thirty-four of 89 *Phytophthora* isolates were chosen for further biological characterization (A- 5). The isolates selected for further study were chosen to represent different orchard sites. Length and breadth measurement of sporangia and diameter of chlamydospore, oogonia, antheridium, and oospore were taken. Sporangia formation was induced by transferring agar plugs from actively growing part of the 7-day old culture on 10% CV8 agar to Petri dishes containing 1% sterile soil solution. Plates were left under fluorescent light for 24 hours and inspected with a stereomicroscope under 100X magnification. If sporangia were detected, then the plugs were mounted on a slide with 1-2 drops

of 0.1% Lacto-Fuschin stain (0.1g acid fuschin and 100.0 mL 85% lactic acid) and incubated overnight at room temperature for the agar to melt. Sporangia were viewed under 400X magnification with a Leica microscope. The length and breadth of 10 sporangia were measured with the microscope image analysis software program (Leica Microsystem Version 4.7.1. Switzerland Ltd). Chlamydospores were observed in 10 to 15-day old cultures growing on 10% CV8A. The diameters of 10 chlamydospores were measured as above. The length to breadth ratio of sporangia was calculated.

The mating type (MT) was identified by pairing unknown isolates with the A1 and A2 known testers for *Phytophthora capsici* (Dr. Veronica Ancona Plant pathology lab, Texas A&M Kingsville, Citrus Center, Weslaco TX). The *P. nicotianae* isolates PhH66 and PhE48 of mating type A1 and A2 were used to test mating type of *P. nicotianae* isolates of unknown mating type. Both mating type testers were paired with each other to test if they are not self-fertile. A 5 mm plug from unknown and tester isolates for A1 or A2 were placed on two opposite ends of 10% clarified V8, agar plates (Figure 9) (Erwin and Ribeiro 1996). All plates were incubated at 23 ± 2^{0}C in the dark for one week. The plates were checked for the presence of oospores in the zone where the mycelia of both isolates meet. The presence of oospores was used to confirm the mating type. Agar plugs with oospore were mounted on a slide and stained with lactofuschin. Oospores were viewed under 400X with Leica ICC50 W microscope and photographed with integrated Wi-Fi 5-megapixel camera. The antheridium width, and the oogonium, and oospore diameters were measured with the Leica Microsystem image analysis software.

Colony morphology was measured based on growth rate and growth pattern on CV8A and potato dextrose agar (PDA) plates. Each isolate was plated in triplicates. The

growth rate was determined as the average daily increase in diameter over a 5-day period expressed as mm day^{-1}. Growth measurements were also recorded after 24 and 48 hours. Colony patterns were determined for 7-day-old colonies on CV8A and were classified into slight chrysanthemum, slight stellate, slight rosaceous to diffuse, radiate and chrysanthemum (Figure 13) (Erwin and Ribeiro 1996).

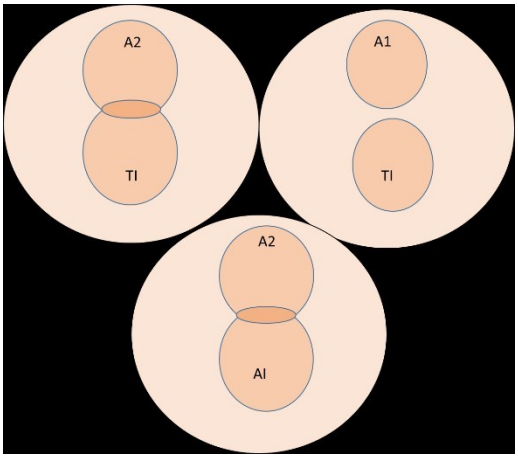

Figure 9 Mating type crosses made for *Phytophthora nicotianae*. Representation of arrangements for mating type testing of isolates of the unknown mating type with known A1 and A2 mating type of reference isolates of *Phytophthora nicotianae* growing on 10% CV8 agar plates. Shaded area between two isolates indicates compatibility and TI represents unknown mating type isolates.

3.25 Mefenoxam sensitivity test

Sensitivity to the fungicide mefenoxam was tested in 34 *P. nicotianae* isolates at 0.1, 1, 10 µg/ mL concentrations. Mefenoxam (Ridomil Gold SL EC. 45.3% a.i., Syngenta Crop Protection, Greensboro, NC) was suspended in sterile distilled water and added to autoclaved CV8 agar cooled at 50^0 C. Five-mm plugs were removed from the actively growing region of a 5-day old culture of *P. nicotianae* colonies maintained on 10% CV8A

plates using a flamed cork-borer. The culture was transferred to 90mm agar plates having 15ml 10% CV8 juice agar amended with mefenoxam. All isolates were plated in triplicates and allowed to incubate in the dark for 5 days at 22±3^{0}C. The radial growth of *P. nicotianae* was measured when the control plates reached the edge of the plate. Average radial growth was calculated for each isolate at different concentration. Percent growth was calculated by dividing the radial growth on amended media by growth on non-amended media (Timmer et al.1998).

The experiment was repeated twice and data were pooled from both trials. Percent growth inhibition was determined and converted into probability (probit) scale using the excel formula "NORM.S.INV (probability)." Percent values were plotted as probits versus $\log_{10}$ for the different fungicide concentrations (Finney 1962). The regression equation was used to calculate EC_{50} and EC_{90} values, which will provide 50 and 90% inhibition in the presence of mefenoxam. Isolates which grew as well at 10 µg/ mL, were tested for sensitivity at 100 and 1000 µg/ mL concentrations.

3.26 Pathogenicity test

Pathogenicity of *P. nicotianae* isolates obtained from the citrus rhizosphere were tested on non-citrus hosts such as tomato (*Solanum lycopersicum* L.) (23 isolates), garden bean (*Phaseolus vulgaris*) (25 isolates), early golden crookneck summer squash (*Cucurbita pepo*) (26 isolates), Russell hybrid lupin (*Lupinus polyphyllus*) (19 isolates), and tobacco (*Nicotiana tabacum*) (13 isolates). These isolates chosen are from 34 isolate pool for further biological characterization. Not all the 34 isolates were tested for all the hosts for pathogenicity test. Each plant was chosen based on their susceptibility to a wide range of *Phytophthora* species. *Lupinus* was chosen for its high susceptibility to many *Phytophthora*

species to check pathogenicity of the isolates. Tobacco was chosen as it is considered a sensitive host to *P. nicotianae*. Germination of seeds was accelerated by placing the seeds inside the wet sterilized paper towels and then placing inside sterilized Petri dishes which were left in the dark for 48 hours. After 1-week, seedlings were removed and inoculated with zoospore suspensions for each isolate tested. The propagule was prepared by first inducing sporangia formation. This was done by transferring 5 mm plugs from 5-day old culture into 15ml of the sterile soil solution. After 24 hours of incubation under fluorescent light, zoospore release was initiated by chilling the plugs in sterile soil solution at 4^0C (refrigerating the plates for 3 hours). Plates were removed and left in the dark for 30 min at room temperature.

Zoospore release was confirmed by viewing under the dissecting microscope. Zoospore suspension was quantified with a hemocytometer slide. The final volume of propagule was adjusted to 10^4 zoospores/ml with the addition of sterile distilled water. Thirty-five ml of zoospore suspension were transferred into a sterilized beaker covered with aluminum foil on all sides. For each tested host (tomato, bean, and squash), bare roots of three-seedlings per isolate were incubated in 35 ml of zoospore propagules for 8 to 10 hour (Matheron 1998). Due to the small size of *Lupinus* and tobacco seedlings, they were incubated in 15 ml of zoospore suspension taken in Petri plates. Plates holding the inoculum were covered with sterilized aluminum foil with 5 mm holes on it. Seedlings radicle were inserted through the holes to keep them in place. Inoculated seedlings were transplanted into a separate plastic pot filled with potting soil mix and were maintained under greenhouse conditions. This setup was repeated for each host plant and isolate combination. Control plants from each host were immersed in 15 mL of sterilized distilled water.

Plants were watered daily; mortality was counted on day 3 and at the end of one week when the experiment was terminated. As the radicle began to emerge in germinating seeds, they were planted in plastic pots filled with Sungrow® potting soil and kept under green-house conditions with average highest and lowest temperature was recorded as 91^0 F and 66^0 F respectively. Light duration ranged from 12:55 Hours to 13:05 Hours.

Seedlings were noted for mortality and the presence of lesions on the roots, stems, and leaves. The entire seedling was cut into segments and plated on selective media PARPH-CMA and incubated in the dark for 4 to 5 days. *Phytophthora* colonies were checked on a segment of plated root, stem and leaves. The number of segments giving rise to *Phytophthora* colonies was noted. Based on the lesion, percent plant part infected, and mortality, isolates were grouped into three categories. The isolates were rated as highly pathogenic (+++) on the host if all seedlings (3/3) died and had more than 90% lesion and 100% of segments of seedling planted is positive for *Phytophthora* subculture. Isolates were noted as moderately virulent (++) if mortality was recorded in 2 out of 3 seedlings, 40 to 90% lesion covered the plant and the same percent of segment results in positive subcultures. Low virulence was rated (+) when only 1 out of 3 seedlings died, 1 to 40% of the plant parts had lesions, and the same percent of segments formed positive subcultures. Isolates were considered not pathogenic (negative) if they caused no mortality, formed no lesion, and no plated segment was positive for *Phytophthora* colonies.

Pathogenicity of *Phytophthora* isolates was tested on 6-week old sour orange seedlings using the inoculation method similar to that used for testing non-citrus hosts. Thirty-five mL of zoospore suspension at a concentration of 10^4/mL was placed in a sterilized glass bottle for each isolate. Free root ends of nine sour orange seedlings for each isolate

tested were immersed in the zoospore inoculum overnight in the dark. The inoculated seedlings were planted into the plastic pots filled with Sungrow® potting soil mix. The pots were maintained under greenhouse conditions. Each isolate was tested by replicating three times using three plants per replicate (Figure 10). Control plants were inoculated with the same volume of sterilized distilled water. Zoospore suspension was obtained in a similar manner used to test non-citrus host and all plants were watered every day until the experiment was terminated. Plants were harvested after 10 weeks and 30 root segments with visible lesions were plated on selective media PARPH-CMA. Plates were incubated in the dark for 4 to 5 days, the number of root segments that resulted in *Phytophthora* colonies was counted and the percentage of successful *P. nicotianae* isolation was calculated by dividing the number of positive root segments by the number of root segments plated. Pathogenicity on grapefruit twigs was tested for 10 isolates. These isolates were chosen based on the virulence results on sour orange.

Three isolates from each category of no virulence, low, medium, and high pathogenicity were taken. Green twigs of 25 cm length and 1 cm width were taken from 12-year-old grapefruit trees from the orchard. Twigs were inoculated with *Phytophthora* isolates by puncturing the twigs with a sharp and sterilized 5 mm diameter cork-borer. The bark was flipped with the help of a sterilized scalpel, and a 5 mm agar plug from 5-day old *Phytophthora* culture growing on 10% CV8 agar was placed along with 2-3 drops of sterilized distilled water. The bark flap was placed over it, and the whole site was covered with parafilm. The setup was repeated for each isolate, and each isolate was replicated five times. Inoculated twigs were wrapped in moist sterilized paper towels and aluminum foil

and placed in the dark for 10 days (Afek and Sztejnberg 1990). The lesion length was measured from the site of inoculation to the end of the lesion.

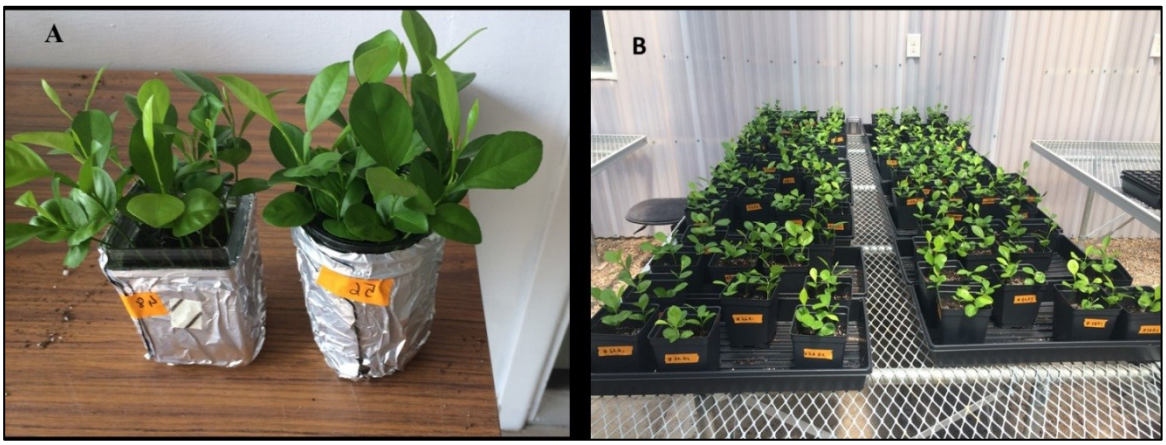

Figure 10 Greenhouse experiment for *P. nicotianae* isolates pathogenicity test in sour orange. (A) Six-week-old sour orange seedlings with roots immersed in a *Phytophthora nicotianae* zoospore suspension at a concentration of 10^4 zoospores per mL (B) Greenhouse trial of pathogenicity test of *P. nicotianae* isolates in sour orange. Each isolate was tested in three replicates with three seedlings per replicates planted in each pot.

3.27 Data analysis

Data were analyzed with PROC GLM (SAS version 9.4 for Windows, SAS Institute, Cary, NC.) to test significance. When "f" value for the Model was found significant, mean separations test was performed with Student-Newman-Keuls multiple range test at alpha value, $P<0$ .05.

3.3 Results

3.31 Isolate collection

Ninety-three isolates were obtained from root and soil samples obtained from 27 orchard sites from the LRGV and five sites located along the east coastal bend of the Gulf of Mexico (Figure 8, Table2) species identification and characterization. Out of the total number of isolates characterized, 74 isolates were obtained from soil, and 19 came from the roots.

Table2 Description of the surveyed sites of citrus orchards for *Phytophthora* isolation**.** Sites with an asterisk are located along the gulf coast of South Texas. Other sites are in the LRGV region of South Texas**.**

County	City	# of orchard sites	# of Isolates [a]	Host
Hidalgo	Mission	3	3	Grapefruit
Hidalgo	McAllen	4	13	Grapefruit, orange
Hidalgo	Edinburg	4	12	Tangerine, Grapefruit
Hidalgo	Hargill	2	3	Orange
Hidalgo	Alamo	1	2	Grapefruit
Hidalgo	San Juan	1	4	Grapefruit
Hidalgo	Donna	2	9	Orange, grapefruit
Hidalgo	Weslaco	2	5	Grapefruit
Cameron	Harlingen	2	5	Grapefruit
Cameron	La Feria	2	7	Grapefruit
Cameron	Combes	2	10	Grapefruit
Cameron	Los Fresnos	1	3	Orange
Cameron	San Benito	1	6	Grapefruit
Nueces	Corpus Christi*	1	2	Orange
Jim Well	Orange Grove*	1	2	Orange
Aransas	Rockport*	1	4	Orange
Galveston	Galveston*	1	2	Orange
Matagorda	Palacios*	1	1	Orange
Total	18	32	93	

[a]Details of the *Phytophthora* isolates obtained from the type of plant tissue type is given in the **A- 5**

3.32 Isolate identification

The primer pair ITS4/ITS6 resulted in amplification of a 900bp DNA fragment spanning an 18S RNA partial sequence, the complete sequence of ITS1, 5.8S and ITS2 and a 28S ribosomal RNA partial sequence (Figure 11 A**)**. The gel bands for four isolates were distinctly different from those obtained for 89 other isolates, ranging in sizes from 900 to 1000bp (Figure 11 B). In Blastn analysis, 82 isolates showed 99% of 100% similarity with those of *P. parasitica* and *P. nicotianae* with Accession numbers KR827692 and KJ494902 respectively (A- 5). Seven isolates showed 97% similarity with *P. nicotianae* (accession no. KF147901). Based on Blastn sequence comparison four isolates were identified as *Pythium ultimum* (PYE2& PYE3), *P. nodosum* (PyE1) and *Phytopythium vexans* (PyMi5) (Figure 11 B).

GeneBank and detail information on identified isolates are given in A- 5. Morphological characteristics and growth at 37^0C° also confirmed the identity of the isolates to be *P. nicotianae. Pythium* species were not characterized further though sporangia structure, homothallic nature, and oogonia structure were confirmed from preliminary observations.

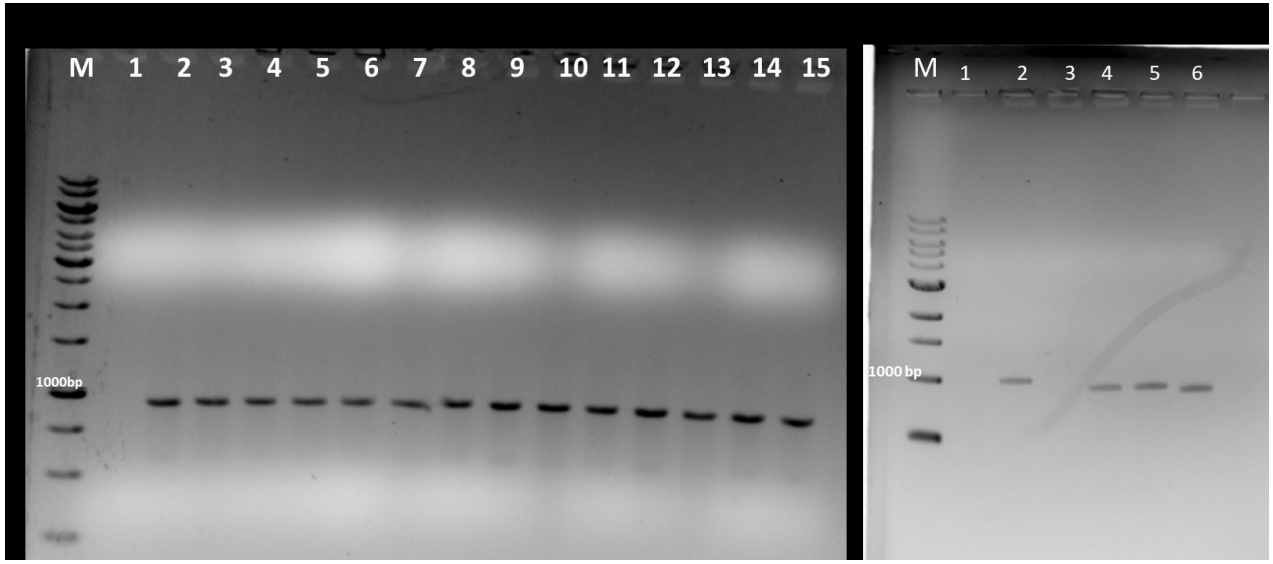

Figure 11 Agarose gel showing amplified PCR products with primer pair ITS4 and ITS6. Lane M is a molecular marker (GelPilot 1kb, QIAGEN®). (A) Lane 1 and 2 represent negative and positive controls, and lane 3 to 15 contain *Phytophthora* samples respectively. (B) Lane 1 has control; Lane 2 is for isolate (PyE1), lane 3& 4 for isolate (PyE2& PyE3) and band in lane 6 is for isolate (PyE4) was confirmed as Pythium species.

3.33 Phylogenetic analysis

ITS sequence of 81 out of 93 isolates was only used to compare diversity. A parsimoniously constructed phylogenetic graph from the consensus sequence of 81 isolates consisting of 77 *Phytophthora* and four *Pythium* isolates formed two different clusters. All *Pythium* species formed one cluster with PyE3& PyE4 forming one sub-cluster supported by 100% bootstrap value. Isolate C83-R formed some association with *Phytopythium* vexans, but it is not supported by a bootstrap value suggesting it could be a closer *Pythium* species. **(**Figure 12**).**

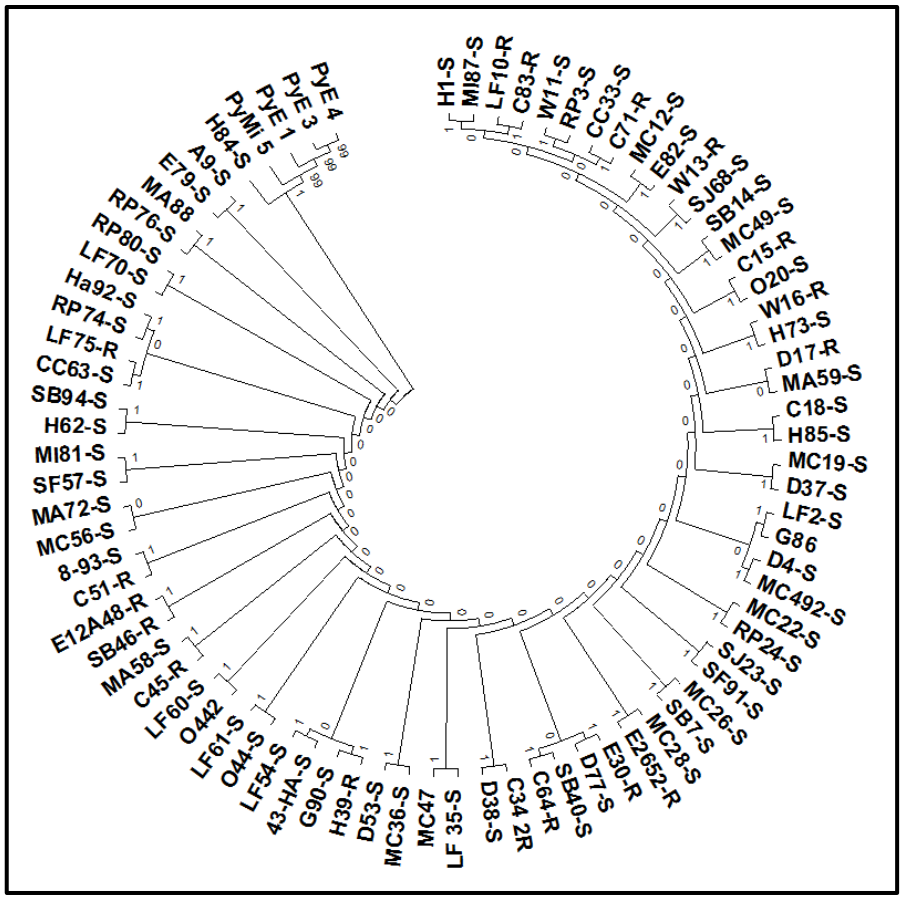

Figure 12. Phylogenetic tree of *Phytophthora nicotianae and Pythium* isolates from citrus orchards of the Lower Rio Grande Valley, Texas. Phylogenetic tree representing the relationship between the *Pythium* and *Phytophthora* isolates collected from the citrus rhizosphere in South Texas. The phylogenetic tree was inferred using the distance-based Neighbor-Joining method. The evolutionary analysis was performed using MEGA7. The number above the branches indicates the bootstrap percentage (1000 replicates).

3.34 Morphological characteristics

Out of the 89 *Phytophthora* isolates identified as *P. nicotianae*, 34 isolates representing each selected site across the LRGV commercial citrus orchards were chosen for further studies on growth and morphological characteristics. Colony morphology on 10% CV8 agar varied from chrysanthemum, radiate, stellate to non-defined. Certain cultures

looked slight chrysanthemum (Figure 13, A) or slight stellate (Figure 13, B). The most visually distinct colony was formed by isolate PhCC63 (Figure 13, C) from Corpus Christi soil which did not match any other isolate colony motif (Figure 13). Colony characteristics on PDA were less variable and were stoloniferous, or fluffy and stoloniferous.

Isolates varied significantly ($P<0.05$) with regards to growth rate. The highest rate of growth was recorded between 7.3±0.3 to 7.6±0.3 mm/day for PhRP74 and PhMC28. The lowest growth rate was recorded for isolates PhC15, PhCC63, PhD55, and PhRP24 in the range of 3.6±0.3 to 4.0±0.0 mm/day (Table 3, Table 4). No significant difference in growth rate was found between isolates from LRGV and outside the region. The rate of growth after day1 of incubation was significantly correlated to total growth and growth rate per day, meaning that isolates that grew faster within 24 hr. of incubation also showed the highest colony diameter (Table 4).

All isolates readily formed sporangia on corn meal agar and CV8 agar within 7 to 10 days of culturing in the dark. Sporangia were also readily induced in sterilized soil solution within 10 hours of incubation in the dark. More than one type of sporangium shape was noted within the same isolates. Ovoid, obryform and spherical with single papilla were the more common shapes, but spherical and bi-papillate sporangia were also observed (Figure 14, A to E). Some isolates had turbinate sporangia type with long papilla (Figure 13, B). Chlamydospores were also readily observed in all isolates within 8 to 10 days of culturing in the dark. Both terminal and intercalary position of chlamydospore was observed (Figure 14, F). Vegetative hyphae are the coralloid type with hyphal swellings (Figure 14, F).

Sexual reproductive structures were formed when isolates were paired with an opposite mating type of known A1 and A2 *P. nicotianae* and *P. capsici* testers. None of the isolates produced sexual reproductive structures when paired with themselves, indicating that they were not self-fertile. Antheridia and oogonia were observed within 10 days of pairing between the opposite mating types. Nine out of 34 isolates tested were A1 and 25 as A2 mating types. Both mating types were obtained from the 2 orchards located in the LRGV and 2 sites outside LRGV. Isolates PhE32 (A1) and PhE48 (A2) were collected from the same orchard, and both were obtained from the roots. Similarly isolates PhSB14 (A2) and PhSB94 (A1) were obtained from the soil of the same orchard at San Benito. Presence of both mating types at the same site was also found in the isolates sampled outside the LRGV region. Isolates pair PhO20 (A2), PhO44 (A1) and PhRP74 (A2), PhRP24 (A1) were obtained from orchards at Orange grove and Rockport, respectively.

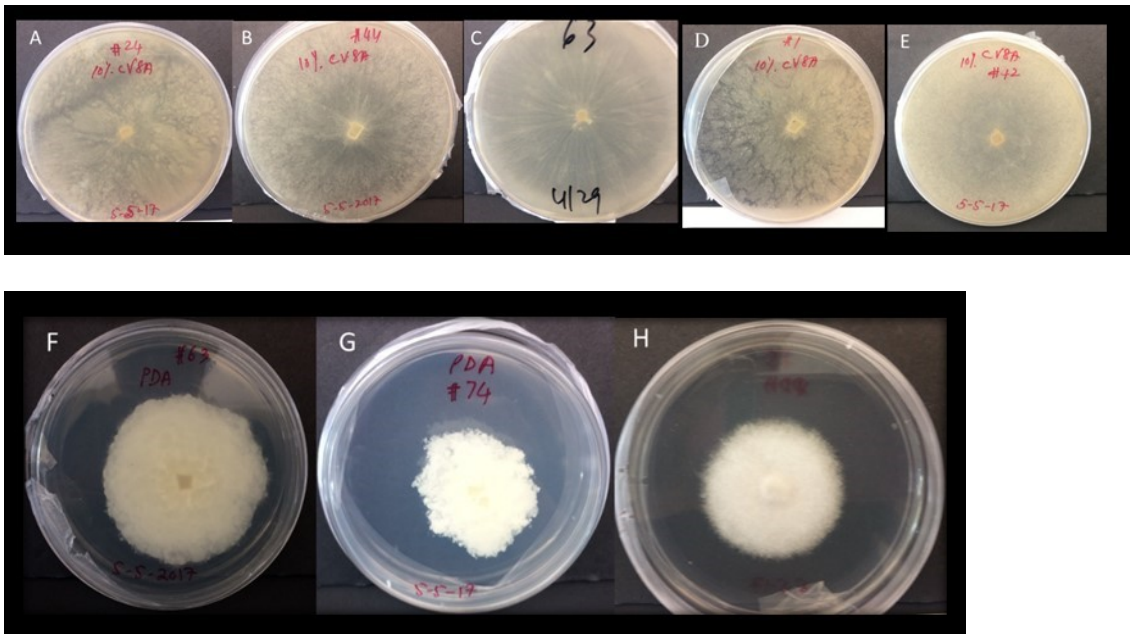

Figure 13. Colony pattern of *Phytophthora nicotiana*e. A to E is on 10% CV8A, and F to H is on PDA (potato dextrose agar). A: Slight chrysanthemum, B: Slight stellate. C: Stellate, D: slight radiate, E: non-defined, F slightly rosaceous, G: stoloniferous H: Stoloniferous and fluffy.

Table 3. Summary of morphological and mating type (MT) characteristics of the *Phytophthora nicotianae* isolates collected from citrus rhizosphere in South Texas.

Variable	N [a]	Mean	Std. Error	Minimum	Maximum
Number of isolates	34	--	--	--	--
Mating types A1	9	--	--	--	--
Mating type A2	25	--	--	--	--
Chlamydospore diameter (µm)	178	36.1	0.64	17.0	60.0
Sporangia length (L) (µm)	178	47.7	0.76	28.0	65.0
Sporangia breadth (B) (µm)	178	36.0	0.52	23.0	47.5
Length to breadth ratio	178	1.3:1	0.01	1.1	1.5
Antheridium (µm)	178	11.5	0.14	8.0	13.3
Oogonia diameter (µm)	178	24.9	0.16	22.6	27.0
Oospore diameter (µm)	178	20.3	0.17	17.5	22.8
Colony diameter (Day1)	105	3.9	0.13	1.0	6.0
Colony diameter (Day2)	105	5.2	0.13	1.0	9.0
Total growth (mm)	105	27.8	0.46	15.0	40.0
Growth per day (mm/day)	105	5.6	0.01	3.0	8.0

[a] N represents total number of observation made for 34 isolates
[b] Isolates growth at 37^0 C was checked.

Table 4. Colony motif, and growth rate and mating type of *Phytophthora nicotianae* isolate from citrus orchards of South Texas. Isolate characteristics labeled "NM" means not measured. Description of letters designated for colony morphology is described in Figure 13.

		Colony Morphology					
No.	Isolate ID	CV8A	PDA	Growth (Day 1)	Growth (Day2)	Growth rate average for 5 days (mm/day)	Mating type
1	PhH1	D	G	2.3±0.3	6.3±0.6	6.3±0.3	A2
2	PhLF2	D	G	4.3±0.3	4.6±0.8	6.0±0	A2
3	PhD4	E	NM	2.6±0.3	6±0	4.6±0.37	A2
4	PhC5	E	NM	4±0.0	4.3±0.3	5.3±0.3	A2
5	PhD6	E	NM	2.6±0.3	5±0	5.7±0.3	A2
6	PhW13	E	G	3.6±0.9	5.0±0	5.6±0.3	A2
7	PhSB14	NM	NM	5.0±0	6.0±1.0	6.6±0.6	A2
8	PhC15	D	H	2.3±.33	2.7±0.7	4±0	A1
9	PhO20	E	G	5.0±0	5.0±0.6	5.3±0.3	A2
10	PhA-21	A	G	3.3±0.3	4.3±0.9	5.0	A1
11	PhSJ23	D	H	3.6±0.6	7.6±0.6	5.3±0.3	A2
12	PhRP24	A	G	1.6±0.3	3.3±0.3	3.6±0.3	A1
13	PhLF25	D	F	2.6±0,3	6.3±0.3	5.0±0	A2
14	PhMC26	B	G	5.3±0.3	4.4±0.3	6.0±0	A2
15	PhMC28	E	G	6.0±0	6.0±1.2	7.3±0.3	A2
16	PhE32	E	G	4.0±0.6	6.0±0.6	5.6±0.3	A1
17	PhE41	E	G	3.60.2	5.6±0.3	6.0±0.9	A2
18	PhO44	B	H	3.3±0.3	6.6±0.3	6.6±0.4	A1
19	PhE48	B	H	3.6±0.3	5.0±0.6	5.0±0.9	A2
20	PhE52	B	H	1.0±0	6.3±0.3	5.0±0	A2
21	PhD53	A	NM	2.0	5.0	5.0	A2
22	PhD54	E	H	3.75±0.4	4.25±0,4	5.25±0.25	A2
23	PhMC56	E	G	NM	NM	NM	A2
24	PhMC59	D	H	3.0±0	6.3±0.3	5.6±0.3	A2

Table 4 Continued

No.	Isolate ID	Colony Morphology		Growth (Day 1)	Growth (Day2)	Growth rate average for 5 days (mm/day)	Mating type
		CV8A	PDA				
25	PhLF60	NM	G	4.0±0.6	4.5±0.5	5.3±0.3	A2
26	PhH62	D	H	4.6±0.9	6.0±0.6	5.6±0.3	A1
27	PhCC63	C	F	3.0±1	3.0±1	3.6±0.3	A2
28	PhC65	D	H	4.7±0.3	5.6±0.3	6.3±0.3	A2
29	PhH66	D	H	4.3±0.3	4.6±0.3	5.3±0.3	A1
30	PhMC72	NM	H	6.0±0	5.3±0.3	6.3±0.3	A2
31	PhRP74	E	G	5.6±0.3	7.0±0.6	7.6±0.3	A2
32	PhLF75	E	G	4.3±0.3	6.3±0.3	5.6±0.3	A2
33	PhMI81	E	G	4.3±0.3	5.0±0.6	6.3±0.3	A1
34	PhSB94	E	G	4.6±0.3	4.3±0.3	6.0±0.0	A1

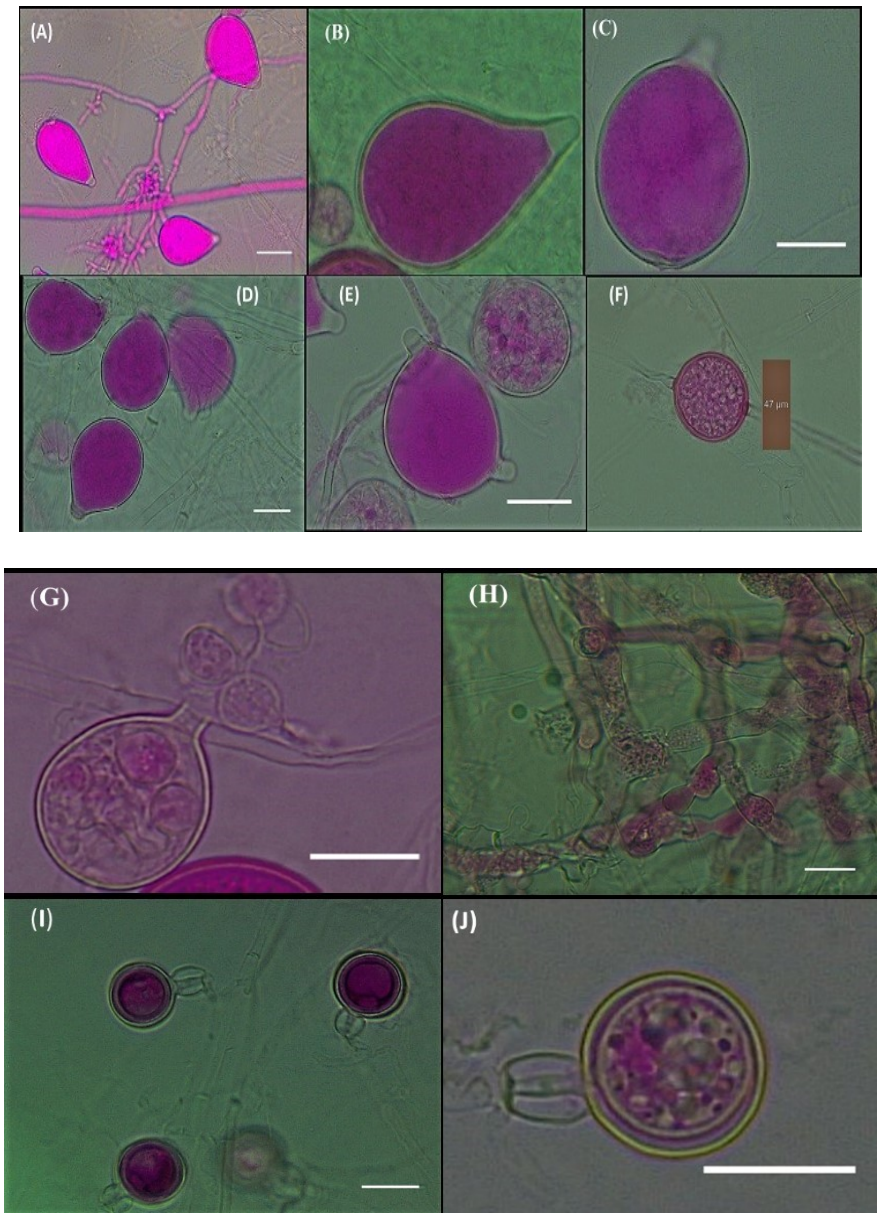

Figure 14. Asexual and sexual reproductive structure and mycelial characteristics of *Phytophthora nicotianae* representative of isolates obtained from South Texas (400X). A: ovoid sporangia, B: turbinate sporangia with prominent papilla, C&D: spherical sporangia, E: bi-papillate sporangia, F: intercalary chlamydospore. G: Sporangia are releasing zoospore, H: hyphal swellings, I&J- oogonia with amphigynous antheridia ad oospore. Bar = 25 µm

Sporangial size varied considerably between the isolates, mean sporangium length and breadth ranged from 23.0±1.2 to 47.5±2.5 µm x 28.0±1.2 to 65.7±3.3 µm. Isolates PhO44 and PhD54 recorded the smallest and largest length x breadth, respectively. Mean sporangial length x breadth for all isolates was observed as 47.7±0.76µm x 36.0±0.52 µm (Table 5, Table 6). Mean length to breadth ratio for isolates ranged from 1.2 to 1.4 with mean L/B ratio for all isolates recorded as 1.3:1. Only two isolates (PhD6 and PhE48) had 1.5:1 L/B ratio. Mean chlamydospore diameter for individual isolates ranged from 23.0±1.2 to 50.7±1.9 µm with a mean diameter for all isolates being 36.1µm. Smooth and spherical amphigynous oogonia were formed in all the isolates when mated with opposite mating type of *P. capsici* or *P. nicotianae* tester isolates.

Sexual structures on the plates were formed within 10 days of mating in the zone where the opposite mating types met. No self-fertility was observed in the testers or the isolates when mated with themselves. The oogonia diameter ranged from 22.6±1µm to 26.8±0.4µm, and the largest and the smallest diameter was observed in isolates PbRP24 and PhH1, respectively. The average oogonial diameter for all the isolates was recorded as 24.9±0.16 µm. Antheridium was observed as amphigynous and almost spherical in most isolates. Antheridial diameter ranged from 8.0±1.2µm to 13.4±0.6 µm with the largest and smallest diameters observed in isolates PhMC28 and PhH66, respectively. Oospores were aplerotic and ranged in diameter from 17.5±0.5 to 22.8±0.4 µm. Largest and smallest oospore diameters were recorded in isolate PhMC28 and PhH66, respectively (Table 4)

Table 5. Dimensions of the asexual and sexual reproductive structures of *Phytophthora nicotianae* isolates collected from citrus rhizosphere soil and roots of commercial citrus orchards of South Texas.

	Sporangia (µm)			Chlamydospore Diameter (µm)	Sexual structure (µm)		
Isolate ID	Length (L)	breadth (B)	LB ratio		Oogonia	Antheridium	Oospore
PhH1	42.2±1.5	30.4±1.0	1.4:1±0.0	41.2±1.7	26.8±0.4	11.2±0.8	21.8±0.4
PhLF2	52.2±1.0	37.2±0.6	1.4:1±0.02	44.2±2.3	24.0±0.7	12.4±0.5	20.6±0.5
PhD4	48.6±1.7	37.4±1.2	1.3:1±0.03	36.0±4.0	24.2±0.3	11.6±0.7	21.0±0.3
PhC5	47.8±3.9	38.2±3.1	1.3:1±0.05	34.4±2.4	24.4±0.6	12.0±0.7	20.4±0.8
PhD6	60.8±3.6	43.6±4.1	1.5:1±0.2	43.6±4.1	22.6±0.7	11.6±0.4	19.6±0.5
PhW13	54.8±6.2	39.0±3.7	1.4:1±0.05	29.0±3.5	25.2±1.4	13.3±0.6	20.6±0.6
PhSB14	45.4±2.1	33.8±1.5	1.3:1±0.04	24.2±0.3	23.5±1.2	12.5±1.5	20.2±1.5
PhC15	47.4±2.0	39.2±2.4	1.2:1±0.1	33.4±0.7	24.4±0.6	12.0±0.7	20.4±0.8
PhO20	36.4±2.5	28.4±1.6	1.3:1±0.03	26.4±1.0	26.4±1.1	12.7±0.7	20.0±1.0
PhA-21	35.0±1.5	27.0±2.0	1.3:1±0.06	27.0±1.2	24.6±1.2	9.60±0.3	18.8±0.4
PhSJ23	54.8±1.7	42.4±1.9	1.3:1±0.02	48.0±3.0	24.6±0.9	10.6±0.3	20.3±0.9
PhRP24	46.0±1.7	33.2±1.3	1.4:1±0.02	38.4±1.2	22.6±1.0	12.6±2.2	19.0±0.7
PhLF25	40.4±2.7	34.0±2.6	1.2:1±0.03	30.2±3.0	26.4±0.2	12.8±0.3	22.2±0.5
PhMC26	50.4±1.9	37.0±0.9	1.3:1±0.04	36.2±0.9	25.6±0.6	12.0±0.4	19.2±0.5
PhMC28	47.0±3.4	34.6±2.1	1.4:10.06	33.3±3.1	25.6±0.5	11.2±0.3	22.8±0.4
PhE32	47.6±2.5	33.8±1.9	1.4:1±.05	34.0±1.2	25.0±0.5	11.8±0.4	20.0±0.8
PhE41	47.0±3.6	36.0±1.9	1.3:1±.04	41.2±2.0	NM	NM	NM
PhO44	28.0±1.2	23.0±1.2	1.2:1±.02	23.0±1.2	23.0±1.4	12.0±0	18.5±1.5
PhE48	48.4±1.5	33.6±0.6	1.5:1±0.05	33.8±1.6	26.4±1.0	12.6±0.2	18.0±1.3
PhE52	58.6±2.7	41.4±0.6	1.4:1±0.05	35.0±2.9	26.0±1.4	10.5±0.9	21.2±0.6
PhD53	50.6±0.4	38.4±0.8	1.3:1±0.02	43.0±2.2	26.0±0.5	10.0±0.0	21.0±1.0
PhD54	65.7±3.3	47.5±2.5	1.4:1±0.05	50.7±1.9	23.6±0.3	10.6±0.3	18.3±1.4
PhMC56	54.6±2.8	40±2.9	1.4±0.04	40.0±1.5	25.8±0.4	12.0±1.5	17.6±0.6
PhMC59	53.6±1.6	40.6±2.0	1.4:1±0.05	43.4±3.4	26.2±0.7	11.8±0.6	21.8±0.2
PhLF60	55.0±1.7	43.0±1.9	1.2:1±0.5	38.0±1.3	25.4±0.9	13.2±0.5	20.2±1.5
PhH62	40.4±2.1	33.4±2.2	1.2:1±0.04	30.0±2.8	25.4±0.5	11.2±0.5	21.2±0.4

Table 5 Continued

	Sporangia (µm)			Chlamydospore Diameter (µm)	Sexual structure (µm)		
Isolate ID	Length (L)	breadth (B)	LB ratio		Oogonia	Antheridium	Oospore
PhCC63	31.6±1.9	25.4±1.7	1.2:1±0.04	27.2±1.1	27.0±0.54	13.4±0.6	22.0±0.3
PhC65	49.2±5.1	37.4±2.9	1.3:1±0.06	35.8±3.8	23.6±0.33	10.3±1.3	19.6±0.6
PhH66	37.6±1.8	32.0±2.2	1.2:1±0.02	28.8±1.2	24.2±0.8	8.0±1.2	17.5±0.5
PhMC72	56.2±2.3	43.0±2.0	1.3:1±0.05	49.0±2.5	24.6±0.8	11.0±1.0	19.6±1.8
PhRP74	49.0±3.3	35.6±2.7	1.4:1±0.03	38.2±1.4	23.2±0.8	10.4±0.5	20.8±0.7
PhLF75	35.8±3.1	39.8±1.1	1.3:1±0.08	38.2±1.2	23.0±0.6	11.00	NM
PhMI81	32.0±1.5	25.4±1.4	1.2:1±0.02	30.8±2.0	26.6±0.8	10.6±1.2	22.0±1.0
PhSB94	60.2±2.9	42.2±1.2	1.4:1±0.03	44.6±3.0	23.6±0.3	9.3±0.6	19.6±0.6

Isolate characteristics of 29 isolates from citrus orchards of LRGV were compared with 5 isolates obtained from cities outside the LRGV and located along the gulf coast of South Texas. Significant difference for chlamydospore diameter, sporangial length and width were recorded. Isolates obtained from sites along the Gulf Coast had smaller mean sporangia length, breadth, and chlamydospore diameter when compared to those from the LRGV sites. Sexual reproductive structures like oogonia, antheridia, oospore, growth rate and length to breadth ratio of sporangia were not significantly different (Table 5, Table 6).

3.35 Mefenoxam sensitivity

Growth inhibition results of mefenoxam-amended media at different concentrations are presented in Table 3.6. The t-ratio of the slope for all isolates was significant at *P>0.05*, which show a significant dose-response line. Six isolates were inhibited by more than 95%, 20 by 90 to 95%, and seven by 50 to 90% at 1.0 µg/ml concentration of mefenoxam. These isolates were rated as highly sensitive, sensitive, and intermediate sensitive at 1.0 µg/ml concentration of mefenoxam. Only one isolate (PhCC63) grew at 100 µg/ml producing growth similar to the non-amended control. The EC_{50} and EC_{90} values for this isolate measured 143.6 µg/ml and 2615 µg/ml, respectively. Average EC_{50} values for all the highly sensitive to intermediate sensitive isolates was 0.09µg/ml, with a range of <0.01 to 144 µg/ml. Isolates PhH1, PhA21, PhC15, PhRP24, PhE32, and PhC65 showed high sensitivity to mefenoxam and measured EC_{50} values below 0.01. The EC_{90} value for all isolates, except PhCC63, ranged from 0.1 to 19 µg/ml with a mean of 3.5 µg/ml (Table 7, Table 8)

Table 6. Comparative morphological characteristics of *Phytophthora nicotianae* from sites located in the Lower Rio Grande Valley (LRGV) with those obtained from sites along the Gulf Coast of South Texas. Five isolates from Corpus Christi, Orange Grove, and Rockport were compared to isolates from the LRGV.

Site/ # of isolates	No	Chlamydospore diameter (µm)	Sporangia length (L) (µm)	Sporangia breadth (B) (µm)	LB ratio	Oogonia diameter (µm)	Oospore Diameter (µm)	Antheridium Diameter (µm)	Growth rate mm/day
LRGV (29)	513	36.5±0.38[a]	42.28±0.42[a]	36.4±0.3[a]	1.32±0.01	24.9±0.16	20.3±0.18	11.4±0.14	5.6±0.08
Others (5)	147	28.1±.63[b]	38.4±0.81[b]	29.1±0.58[b]	1.30±0.01	24.5±0.56	20.3±0.42	12.2±0.46	5.4±0.4

Means followed by different letters are significant at $p<0.05$

Table 7. Dose-inhibition regression results for 50 and 90% concentration (EC_{50} & EC_{90}) of 34 *Phytophthora nicotianae* isolates. *P. nicotianae* isolates were obtained from the citrus rhizosphere. Fiducial (FL) upper and lower limits are reported below with EC_{50} and EC_{90} values. For isolates where EC_{50} is measured as 0.001 or less, the EC_{90} value was not determined and is represented as (--) in the table.

Isolate ID	Slope ±SE	Intercept	EC_{50}	95% FL (low-upper)	EC_{90}	95% FL (low-upper)
PhH1	0.434±0.15	6.188	0.002	0.001-0.04	1.64	0.0824-3.278
PhLF2	0.86±0.14	5.997	0.07	0.038-0.130	1.71	0.930-3.152
PhD4	0.82±0.13	6.238	0.03	0.018-0.058	0.90	0.498-1.624
PhC5	1.17±0.13	5.646	0.28	0.156-0.511	2.96	1.638-5.352
PhD6	0.84±0.09	5.858	0.10	0.065-0.144	2.53	1.701-3.771
PhW13	0.86±0.11	5.887	0.09	0.057-0.156	2.30	1.393-3.797
PhSB14	0.89±0.09	5.775	0.14	0.090-0.205	2.99	1.976-4.532
PhC15	0.29±0.03	6.536	<0.001	--	0.07	0.062-0.082
PhO20	0.62±0.16	6.037	0.02	0.010-0.045	1.83	0.874-3.812

Table 7 Continued

Isolate ID	Slope ±SE	Intercept	EC_{50}	95% FL (low-upper)	EC_{90}	95% FL (low-upper)
PhA21	0.50±0.21	6.633	0.001	--	0.14	0.053-0.364
PhSJ23	0.91±0.08	5.862	0.11	0.080-0.162	2.34	1.643-3.342
PhRP24	0.14±	6.510	0.00	0.000	0.01	0.004-0.017
PhLF25	0.63±0.08	6.147	0.02	0.011-0.023	1.21	0.839-1.755
PhMC26	0.77±0.08	6.005	0.05	0.035-0.71	1.79	1.254-2.554
PhMC28	0.92±0.11	5.729	0.16	0.097-0.273	3.23	1.928-5.416
PhE32	0.06	6.715	<0.001	--	--	
PhE41	0.71±0.06	5.845	0.07	0.052-0.086	3.12	2.426-4.018
PhO44	0.61±0.12	5.699	0.07	0.041-0.126	6.61	3.775-11.589
PhE48	0.64±0.09	6.117	0.02	0.012-0.026	1.35	0.915-1.993
PhE52	0.99±0.11	5.796	0.16	0.096-0.258	2.55	1.558-4.169

Continued Table 7

Isolate ID	Slope ±SE	Intercept	EC_{50}	95% FL (low-upper)	EC_{90}	95% FL (low-upper)
PhD53	1.11±0.11	5.671	0.25	0.154-0.401	3.00	1.860-4.839
PhD54	0.54±0.09	5.733	0.17	0.118-0.266	3.06	2.040-4.583
PhMC56	0.77±0.08	5.929	0.06	0.043-0.090	2.24	1.558-3.228
PhMC59	0.78±0.06	5.988	0.05	0.041-0.073	1.87	1.395-2.499
PhLF60	0.53±0.08	5.600	0.07	0.052-0.104	13.52	9.609-19.012
PhH62	0.91±0.16	5.326	0.44	0.209-0.923	9.07	4.311-19.062
PhCC63	0.95±0.12	2.956	142.36	81.1-249.8	2615	1490.1-4588.3
PhC65	0.22±0.03	6.442	<0.001	--	0.08	0.073-0.094
PhH66	0.63±0.09	5.840	0.05	0.030-0.071	3.73	2.447-5.700
PhMC72	0.92±0.20	5.761	0.15	0.060-0.373	2.99	1.198-7.472
PhRP74	1.57±0.16	6.131	0.19	0.092-0.392	1.11	0.537-2.280

Continued Table 7

Isolate ID	Slope ±SE	Intercept	EC_{50}	95% FL (low-upper)	EC_{90}	95% FL (low-upper)
PhLF75	0.74±0.14	5.746	0.10	0.053-0.185	4.09	2.184-7.650
PhMI81	0.69±0.08	6.256	0.02	0.011-0.022	0.83	0.583-1.185
PhSB94	0.96±0.06	6.044	0.08	0.062-0.110	1.45	1.089-1.932

Table 8. Summary of mefenoxam sensitivity assessment of *Phytophthora nicotianae* isolates obtained from citrus orchards of South Texas.

Percent growth in CV8 amended with mefenoxam at 1µg/ml compared to control	Sensitivity rating	Number of isolates	EC_{50} value
No growth	Highly sensitive	6	<0.001µg/ml
Less than 10% growth	Sensitive	20	0.01 to 0.1 µg/ml
10 to <50% growth	Intermediate sensitive	7	0.2 to <1µg/ml
50 to 90% growth	Resistant	1	More than 1µg/ml

3.36 Pathogenicity of *P.nicotianae* isolates on citrus and non-citrus hosts

The pathogenicity of 17, 20, 23, 17 and 24 out of 34 *P. nicotianae* isolates was determined on five non-citrus hosts such as *Lupinus* (cv. Russel Hybrid), bean, tomato, tobacco, and squash, respectively. The presence of lesion, mortality and successful re-isolation of *P. nicotianae* from infected and symptomatic tissue were checked to confirm pathogenicity. The highest pathogenicity of isolates was observed in *Lupinus* as 18 out of 19 isolates were tested pathogenic and caused 85% mortality. Pathogenicity of *P. nicotianae* isolates on tobacco was tested low as they caused only 13% mortality. Five out of 17 isolates tested were obtained in sub-culture from leaf and stem tissue in tobacco. Only one isolate PhLF25 showed moderate pathogenicity as it caused leaf lesions. Mortality of tobacco seedlings was observed in 3 isolates, but *P. nicotianae w*as re-isolated from stem tissue for only one isolate (PhMC56) in sub-culture. Therefore, it cannot be concluded with certainty the cause of death was *P. nicotianae* infection.

In the case of bean, *Phytophthora* was isolated from stems and roots with no visible lesions showing on plant parts. Isolate PhLF25 proved pathogenic on all five hosts whereas PhE32 did not cause disease on any of the five hosts tested (see A- 6). Eighteen out of 19 isolates tested pathogenic and caused 88% mortality of the seedlings. The mortality of *Lupinus* seedlings was observed within 4 days of inoculation with *P. nicotianae* zoospores of several isolates.

Lupinus expressed high susceptibility, and water-soaked lesions were observed on all parts of the plant. The pathogen was isolated from all parts of the seedling when plated on selective medium PARPH-CMA (Figure 15, C&D).

The number of *Phytophthora* isolates pathogenic on squash and tomato seedlings, were nearly equal, however higher mortality was observed in tomato as 21 out of 23 (91%) isolates tested caused 33 to 100 % mortality. Isolate PhE32, PhLF2 and PhLF75 were not pathogenic on tomato. On the other hand, in squash 83% (20/24 isolates) caused between 33 to 100% mortality with average 58% mortality when all tested isolates were taken into consideration. Isolate PhE32, PhMC56 and PhLF60 were not pathogenic on squash. Successful isolation of *Phytophthora* was made from both stem and root tissues in squash, but plating of leaf tissue did not isolate any colony. Fifty percent of the inoculated squash seedlings died within one week of inoculation whereas only 10% of remaining live seedlings were dead at the time of harvesting. Roots, stems, and cotyledons of squash seedlings showed distinct water-soaked lesions compared to the control (Figure 15, H). *P. nicotianae* isolates were weakly pathogenic on tobacco and bean. Unlike Lupinus, tomato, and squash, no mortality was observed within a week of inoculation in tobacco or beans. Water soaked lesions were not observed, though *Phytophthora* was successfully isolated from these hosts (A- 6, Table 9).

The pathogenicity of the isolates on sour orange varied considerably. Not all *P.nicotianae* isolates tested caused the same severity of infections in the roots of sour orange

seedlings. Sour orange inoculated with different *P. nicotianae* isolates looked healthy with no visible sign of wilting or yellowing of leaves. There were visible lesions on the main root, but the fine fibrous roots were devoid of lesions. Sixty-eight percent (19 out of 28) isolates tested caused some level of infection. The percent of root tips testing positive for *P. nicotianae* infection varied amongst the isolates from 0 to 23% (Figure 17 B).

Out of the 28 isolates tested on sour orange, 10 isolates were chosen to test on grapefruit twigs for pathogenicity. Isolates were categorized into four groups, based on the severity of infection on sour orange from 0 severity to the highest severity of the infection (Figure 17 B). All isolates caused lesions on grapefruit twigs after 10-days of incubation in the dark and moist conditions. Some isolates also produced gummy ooze. Significantly ($P<0.05$) largest and smallest lesion sizes were produced by PhH1 and PhE42 respectively **(**Figure 16 & Figure 17 B)

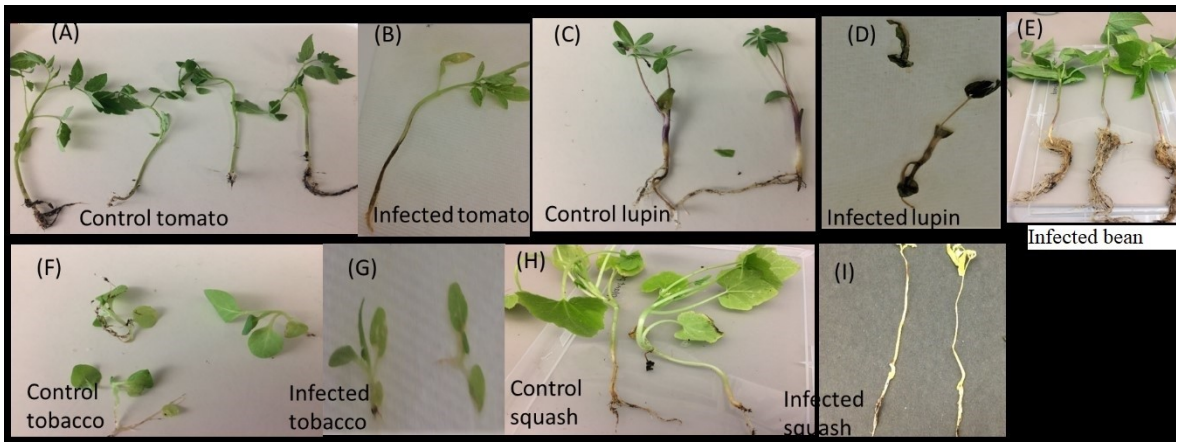

Figure 15. Pathogenicity test results of *Phytophthora nicotianae* isolate obtained from citrus rhizosphere to different non-citrus hosts. Seedlings are showing symptoms of disease caused by *P. nicotianae* isolates tested on tomato (A&B), Lupin (C&D), beans, tobacco (F&G) and squash seedlings (H&I). Each host was inoculated with *P. nicotianae* zoospore suspension and control plant was treated with sterilized distilled water. Infected roots, stems, and leaves were plated on 10% CV8A.

Table 9. Summary of pathogenicity of *Phytophthora nicotianae* isolates from citrus rhizosphere soil and roots tested on the different non-citrus host.

Host	Plant parts tested positive for isolation of *P. nicotianae*	Number of Isolates tested	Mortality	Percentage of isolates pathogenic
Tomato	Roots, stem, and leaves	23	80%	83%
Lupin	Roots, stem, leaves, cotyledon	19	88%	95%
Bean	Stem, roots	25	1.6%	32%
Tobacco	Leaves, stem, roots	13	12.5%	38%
Squash	stem, roots, cotyledon	24	58%	87%

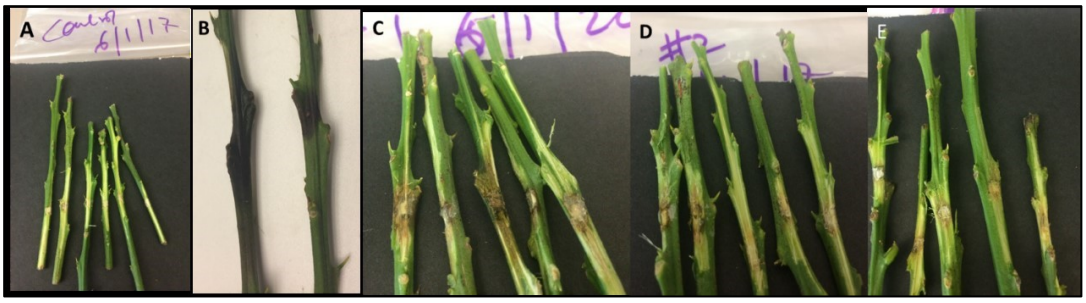

Figure 16. Grapefruit twigs, showing lesions caused by *Phytophthora nicotianae* infection. A-control, B- water-soaked lesion visible from outside after 10 days of inoculation with a 5mm agar culture of *P. nicotianae.* C through E represent highest, intermediate and lowest lesion size formed by isolate PhH1, PhLF2, and PhE42 respectively.

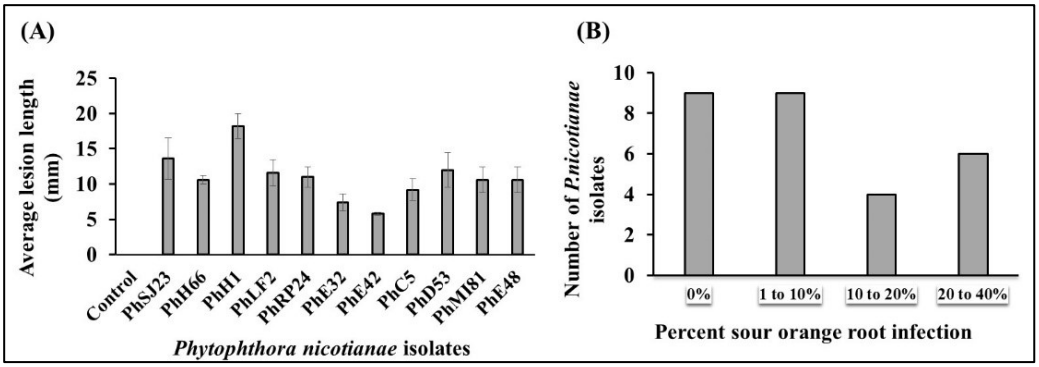

Figure 17. Pathogenicity tests of *Phytophthora nicotianae* on sour orange and grapefruit. (A) Lesion sizes measured in grapefruit twigs inoculated with *P. nicotianae* isolates**.** (B) Bar height represents the number of the isolates in each category of percent root infection in sour orange

3.4 Discussion

ITS sequence analysis of 80 out of 93 isolates showed 99 to 100% identity to *P. parasitica,* accession number KR827692, and 9 isolates showed 97 to 98% identity to *P. nicotianae* KJ755109, and KJ754387.1 Since both *P. parasitica* and *P. nicotianae* are synonymous (Gallegly and Hong 2008), it is concluded that the only species isolated from root and soil samples from citrus rhizosphere was *P.nicotianae*. No other species of the *Phytophthora* was isolated from the sampled soil or plant tissue. It can be proposed from this work that *P. nicotianae* is the most prevalent species of *Phytophthora* found in the citrus orchard soils in the LRGV. *P. nicotianae* has been confirmed as most common species of *Phytophthora* found in the citrus orchards in Egypt (Ahmed et al. 2012), India (Das et al. 2016), Thailand (Hung et al. 2015), São Paulo, Brazil (Seiiti Urashima et al. 2016), Florida (Donahoo et al. 2013) and also in Texas (Timmer 1973, Kunta et al. 2012 and RoyChowdhury et al. 2016) *P. citrophthora* was reported present in the rhizosphere soil from 16 commercial orchard sites in the LRGV region (RoyChowdhury et al. 2016). Out of 16 sites that tested positive for *P. citrophthora* in the study, three sampling sites McAllen #3, Weslaco#28 and Harlingen #46 (A-1) were surveyed by me but none of them were positive for *P. citrophthora*.

Wide variation in sporangial shape and size was *P. nicotianae* isolates. Ovoid, turbinate, spherical and bi-papillate shapes were observed in different identified *P.nicotianae*

isolates. These shapes of sporangia are reported for *P. nicotianae* species in earlier observations (Erwin and Ribeiro 1996). Wide variations in length and width of sporangia were observed, with an average length x width being 47.7 x 36 μm and length to width ratio of 1.3:1. These measurements were close to those reported in the literature for length and breadth in *P. nicotianae* (Gallegly and Hong 2008; Erwin and Ribeiro 1996). Length to breadth ratio also matches earlier measurements recorded for isolates from this region (Timmer 1973). Sporangial length and breadth exhibited the most diversity, indicating that morphological characteristics such as sporangia length and width or chlamydospore diameter cannot be used as stand-alone identification for these species. Five different types of colony motif were observed on CV8A media. These characteristics are helpful for initial diagnosis.

The growth of *P.nicotianae* species at a temperature above 35^0 C is also a useful characteristic for distinguishing it from *P. citrophthora*. All isolates in this study grew at 37^0 C, thereby ruling out *P. citropthora* as a possible species amongst the isolates tested. *P. citropthora* has lower temperature optimum 24 to 28^0C, and maximum temperature it can tolerate is 32 to 33^0C (Erwin and Ribeiro 1996). It is difficult to identify *Phytophthora* species based on morphological differences alone as there is quite an overlap of morphological characteristics between closely related species. Due to this limitation, molecular identification based on ITS non-coding region was also considered.

Phylogenetic analysis using the ITS region did not show the genetic difference in the ITS region amongst the Phytophthora isolates. Microsatellites or simple sequence repeats (SSR) markers to study intraspecific variations in *P. nicotianae* have been successfully tested (Biasi et al. 2015). Future studies can use molecular tools like SSR markers to study variation within the *P. nicotianae* population in the region.

All isolates of *P. nicotianae* produced oospores when paired with opposite mating types of *P. capsici* or *P. nicotianae*. No self-fertile isolates were detected. It is known that in heterothallic species of *Phytophthora*, sexual reproduction is regulated by hormones produced by opposite mating types. Heterothallic species of *Phytophthora* can form oospores even with morphologically and physiologically different species (Ko 1988). Formation of natural hybrids of *Phytophthora* species *P. cactorum* and *P. nicotianae* has been confirmed (Bonants et al. 2000). Mating type findings in this study showed that both mating types are found in some orchards and that all the isolates formed oospores in paired culture.

The occurrence of sexual reproduction can introduce more virulence into the pathogen population (Erwin and Ribeiro 1996). No tested isolates were found to be sterile, and no oospore production was observed in single culture either. Twenty-nine percent of the tested isolates were A1, and 71% were A2 mating types. These results are unlike earlier findings from Texas which reported the detection of 3 times more A1 compared to A2

mating types (Timmer 1973). The difference in observation could be due to a lower number of isolates (17) and sites tested (7) in the earlier work. It could be due to also a shift in the mating type populations over the 40 years period since the Timmer (1973) study was conducted. This study also confirmed the occurrence of both mating types in the same orchard at two different sites from LRGV and one site located outside the LRGV region. It may be more common but could not be confirmed from all sites studied as not many isolates from each site were tested. Nevertheless, there is a possibility of occurrence of sexual reproduction amongst the isolates due to the presence of both mating types in the same orchard. This could lead to an increase of fitness in the *P. nicotianae* isolates regarding resistance to the fungicide.

The mefenoxam study on 34 isolates of *P. nicotianae* from citrus orchards from the LRGV and three sites from Orange Grove, Corpus Christi, and Rockport is the first report on mefenoxam efficacy test for *Phytophthora* isolates from citrus orchards in Texas. Based on this study, four groups of isolates, highly sensitive, sensitive, intermediate sensitive and insensitive at 1µg/ml of mefenoxam were identified. EC_{50} values for all isolates were below 1µg/ml except for one isolate. Isolates of opposite mating type obtained form same site showed sensitivity to mefenoxam at 1µg/ ml concentration. Isolates PhC5, PhD53, and PhH62 from LRGV sites exhibited intermediate sensitivity. One *P. nicotianae* isolate from citrus rhizosphere in a residential site at Corpus Christi site had an EC_{50} up to 142.36. This

isolate also demonstrated different colony motif compared to all other isolates. The difference at the molecular level could not be resolved by ITS sequence alone. It is likely that this isolate was introduced through the infected plant or soil material to the site in Corpus Christi. The absence of resistant isolates from LRGV orchard soils implies that mefenoxam can still be an effective control for *Phytophthora* diseases.

Nevertheless, 7 out of 34 isolates were of intermediate sensitivity at 1µg/ml and measured 50 to 90% inhibition on mefenoxam-amended medium, which can be a matter of concern for future use due to development of resistance. Presence of both mating type in the same orchards create possibilities of passing on the resistant genes to the progeny. Although isolates of opposite mating type obtained from same sites in this study were found to be highly sensitive to mefenoxam. Some isolates were only intermediate in susceptibility to mefenoxam which could be concerning as they can give rise to resistant isolates. Resistance to mefenoxam is controlled by a single gene, crossing over and segregation during meiosis can give rise to homozygous isolates (Shattok 1988). Some *Phytophthora* isolates can also develop resistance naturally without any prior exposure to mefenoxam. Nevertheless, it is important to monitor detection of resistant isolates to such an important fungicide.

P. nicotianae isolates have been known to possess a broad host range, and many horticultural crops from ornamental flowers to vegetable crops have been reported to be affected by them (Erwin and Ribeiro 1996). This study attempted to verify the pathogenicity

of *P. nicotianae* isolates obtained from citrus rhizosphere soil and roots on non-citrus hosts. All the isolates tested were highly pathogenic to *Lupinus* Russel hybrid, tomato and Summer squash. *P. nicotianae* isolates were weakly pathogenic on tobacco and bean. Citrus isolates not pathogenic on tobacco could be due to the production of elicitors like parasiticein that induce necrosis in the tobacco host and prevent black-shank disease in tobacco (Ricci et al. 1992). Isolates of *P. nicotianae* from citrus have shown high pathogenicity to tomato but not vice–versa in similar experiments (Matheron and Matejka 1990).

CHAPTER IV

EVALUATION OF GRAPEFRUIT TREES AFFECTED BY FOOT ROT AND *CANDIDATUS* LIBERIBACTER ASIATICUS

4.1 Introduction

Huanglongbing (HLB) is the most destructive disease of citrus with no effective control available to stop the damage to citrus trees. In the USA, HLB is caused by '*Candidatus* Liberibacter asiaticus (*C*Las), a phloem-inhabiting, fastidious α-proteobacteria that is efficiently transmitted by the Asian citrus psyllid (ACP). Unfortunately, all commercial citrus species and cultivars are susceptible to HLB. Trees with HLB symptoms are characterized by leaves with blotchy mottling with green islands; yellow stems with small and lopsided fruit with inverted coloration (Bové 2006). HLB has adversely affected the citrus industry worldwide by reducing the productive lifespan of trees, and the yield and quality of the fruit. In Florida, the introduction of HLB in 2005 has had a profound impact on the overall gross domestic product (GDP), reducing employment and closing industries related to citrus (Hodges and Spreen 2012). Additionally, the average percentage of yield loss that Florida growers attribute to HLB is 41% (Singerman and Useche, 2015). These losses are not only due to tree decline and tree death but also to the severe reduction in fruit production and quality, affecting both the citrus fresh and juice industries.

Since the first identification of *C*Las in a commercial orchard in Texas in 2012 (da Graça et al. 2015), HLB management became a priority in the state. A three-pronged management approach was implemented to reduce pathogen sources by removing infected trees, control psyllid populations with area-wide coordinated sprays, and production of disease-free seedlings under screen house conditions. However, even with those measures, HLB continues to spread in the citrus producing region of Texas and eradication efforts by growers in mature orchards has stopped.

Studies in Florida have reported that pre-symptomatic *C*Las positive trees have fibrous root decline, suggesting that upon pathogen acquisition, the bacterium moves to the roots and spreads to leaves when new foliar flushes become sink tissue (Graham et al. 2013; Johnson et al. 2014). Additionally, these reports have linked HLB-related fibrous root damage to the aggravation of *Phytophthora* root rot disease (Wu et al. 2018; Johnson et al. 2014; Graham et al. 2013). The *C*Las pathogen can induce nutrient deficiency by interfering with translocation and nutrient uptake from the soil, but nutrient imbalances have also been reported in avocado trees due to *Phytophthora* infection (Labanauskas et al. 1975).

The plant-pathogen interaction causes photosynthetic impairment and significant effect on carbohydrate metabolism. That could lead to turning a source organ like leaves into a sink. Extracellular invertase activity can increase to cause a change in sugar in the cell and apoplast (Berger et al. 2007). Increase in hexose especially glucose is associated to

plant defense response as it participates in hexokinase signaling pathway (Chou et al. 2009, Boava et al. 2017).

Foot rot disease is a chronic and slow progressing disease commonly seen in mature citrus orchards in Texas. The foot rot pathogen *Phytophthora* spp. damages the inner bark, and cambium and thereby affects the translocation of nutrients due to partial girdling of the trunk in mature trees (Timmer et al. 2000). The tree may not die, but foot rot negatively affects tree health and causes decline over time. Below ground, *Phytophthora* root rot damages fibrous roots needed for nutrient and water uptake. Sour orange is the most common rootstock and is considered moderately tolerant *to Phytophthora*. Therefore foot rot infection depends more on the susceptibility of the scion species and environmental factors. *C*Las infection has been shown to increase *Phytophthora* damage of the fibrous roots, but its interaction with foot rot and gummosis caused by the same pathogen is not studied. The objective of this study was to evaluate root health, nutrient and carbohydrate status of *C*Las and foot rot affected grapefruit trees to improve our understanding of the effect of both diseases, or individually on the host grapefruit trees.

4.2 Materials and Methods

4.21 Orchard characteristics and experimental design

This study was conducted on four grapefruit orchards located in a 308.6 ha plot at Donna, Texas (26^0 14'N 98^0 02'W) in the year 2014 and 2016 between September to December (Figure 18, A- 8). All orchards consist of grapefruit trees on sour orange rootstock of the same age and under similar management program. Young and replanted trees were avoided and not sampled. Mean annual air temperature of Donna was 64.4 to 82.4^0F and mean annual precipitation was 67. 6 cm during the study period. These orchards were chosen because of high (>40%) foot rot incidence and trees showing leaves with the characteristic blotchy mottled symptom of HLB.

Grapefruit trees were scouted for symptomatic leaves characteristic of HLB symptoms. Symptomatic leaves from 382 trees were tested for the presence of *C*Las by qPCR at the Texas A&M University Kingsville, Citrus Center Diagnostic Lab, Weslaco. The same trees were also rated for foot rot and gummosis on a scale of 0 to 5 for foot rot symptom (See page number 20 for rating scale). Four categories of trees were identified from the 382 scouted trees; they are *C*Las positive and negative trees with or without foot rot and gummosis disease (*C*Las+ FR+ FR+, *C*Las+ FR-, *C*Las-FR+, *C*Las-FR-). Tree observations comprised of 2x2 factorial design, with 2 factor comprising presence and absence of *C*Las and foot rot disease. The combination of these 2 factors determined four group of trees: *C*Las positive tree with foot rot, *C*Las positive tree without foot rot, *C*Las negative

trees with foot rot and *C*Las negative trees without foot rot disease. Each orchard served as one block with 3 replicates of each category of trees. Twelve trees in orchard D3, D7 and D2 and 10 from D9 with a total of 46 trees were evaluated **(**Figure 18**,** Table 10**).**

All trees chosen in this study were tested for *C*Las again in 2016 to check for trees that may have acquired *C*Las bacterium from the time of first testing. Nine *C*Las positive and 7 *C*Las negative trees studied from 2014 were not studied again in 2016. This was due to the removal of *C*Las positive trees and negative trees, only 30 trees from original 46 trees studied in 2014 were studied again in 2016 (A- 8).

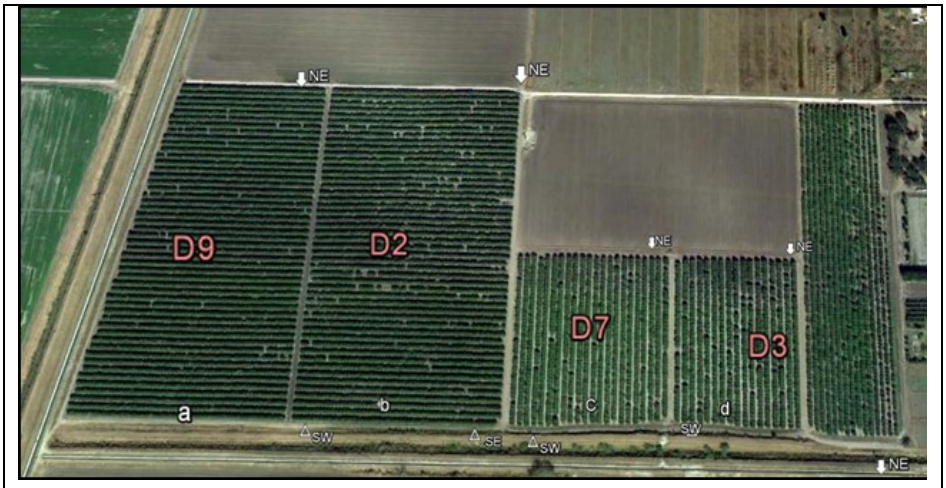

Figure 18. Grapefruit orchards located at Donna, Texas was selected to study *C*Las positive trees and negative trees affected with or without foot rot and gummosis disease. The study was conducted in 2014 and 2016. All grapefruit trees are grafted on sour orange rootstock.

Table 10. Summary of the *C*Las positive (*C*Las+) and negative (*C*Las-) trees with foot rot (FR+) and without foot rot (FR-) disease studied in 2014 and 2016.

Year of survey	Total number of trees surveyed	*C*Las + FR+	*C*Las + FR-	*C*Las – FR+	*C*Las – FR-
2014	46	10	12	12	12
2016	30	7	6	8	9

4.22 Leaf, fibrous root and soil sample collection for nutrient and carbohydrate analysis

Ten symptomatic leaves from each quadrant of the tree were collected to make one composite sample of 40 leaves per tree for nutrient and carbohydrate analysis. Soil samples were collected with the help of an Auger under the canopy at 45 cm distance from the trunk in four different directions around the trunk. Soil core of diameter 7.5 cm at 17- 18 cm depth with 796 cm^3/core soil was taken under the canopy. Four soil cores were composited into one sample per tree. All root, leaf and soil samples were transported to the lab on ice. Sampling was done in both 2014 and 2016.

4.23 DNA extraction and quantitative PCR detection of *C*las

Two to three leaves showing characteristic HLB symptoms were collected from each quadrant of the tree to make one composite sample. Composite leaf samples from each tree were tested for the presence of *C*Las by qPCR. Leaves were rinsed thoroughly

with tap water and air dried for 30 minutes at room temperature. DNA was extracted using QIAGEN DNAeasy mini kit from 200 mg of chopped midrib and petiole of leaf tissue. The tissue was placed in a lysing tube and pulverized using Mini-Bedbeater-96 (Biospec Products Inc, Bartlesville, OK). The total DNA was eluted in 100 µl nuclease-free water. For the detection of CLas, qPCR assay was performed using HLB primer-probe (Li et al. 2006) set on 2 µl DNA extract in 25 µl reaction mixture using a Smart Cycler II (Cepheid, Sunnyvale, CA). Citrus mitochondrial cytochrome oxidase (COX) primer-probe set COXfpr (Li et al. 2006) was used as internal control. *C*Las positive control DNA and non-template water control were also included. The presence of *C*Las was confirmed based on the threshold cycle (Ct) value cut off at 34 cycle.

4.24 Quantification of *Phytophthora* propagules in the soil

From each composite soil sample, 100 cm^3 of soil without the roots were soaked in a foam cup with a drainage hole at the bottom, placed in another foam cup, and allowed to drain for 48 hours. Ten cm^3 of soil slurry was taken from this sample and diluted with 90 mL of double distilled sterilized water. One mL of diluted soil sample was plated each on five plates containing 17 g/L corn meal agar amended with 10 µg/ml Rifamycin-SV (sodium salt), 71.4 µg/ml Tachigaren (70% Hymexazol), 10 µg/ml Delvocid (50% Pimaricin), 66.7µg /ml of 75% PCNB, 250 µg / ml of Ampicillin) (Jeffers 2006). *Phytophthora* colonies were counted after visual inspection following 5 days of incubation of the plates in the

dark and the number of propagules per milliliter of soil slurry was calculated cfu/cm^3. Each soil sample was evaluated twice and the average of the count was taken.

4.25 Soil sample collection and root measurements

Soil was collected at a depth of 16 to 18 cm under the canopy from around the trunk from north, south, east and west side. Composite soil samples were made from four cores of soil taken. All roots were removed from the composited soil from each tree by passing through a sieve with 5 mm pore size and very fine roots were removed manually. All isolated roots were washed thoroughly with tap water and air dried. Root lengths were measured. Roots were spread on a plexiglass tray of WinRhizo flatbed scanner (EPSON STD 4800).

The image was acquired and root length was analyzed with the software WinRhizo 2013 image analysis system (Reagent Instruments Inc., Canada) (Costa et al. 2000). Three measurements of each root sample were taken after rearranging the roots in the tray. Average root length value was used to calculate root length density (RLD) by dividing the root length value out of total soil volume assayed and reported in units of cm/cm^3. Roots were separated into two groups of <2mm and >2mm diameter. The roots were dried in an oven at 65^0 C for 48 hrs. Dry weight was measured for each group separately, less than 2 mm diameter roots was considered as fibrous or fine roots and fibrous root density (FRD) was calculated by dividing the fibrous root dry weight (mg) by the amount of soil assayed (3500 cm^3). Total root density (RD) was calculated by dividing the dry mass of total root

weight in milligrams by total volume of soil assayed (Eissenstat 1991). *Phytophthora* propagule per root weight was calculated by dividing the *Phytophthora* propagule per cm^3 of soil by root density

4.26 Leaf area index measurement and tree canopy rating

The canopy was rated, and leaf area index was measured in the 2016 evaluation. Trees were rated for canopy thinning on a rating scale of 1 to 4, where 1 represents healthy canopy with no sign of thinning and 4 represent severe thinning and dieback of the canopy (rating scale modified from Klooster et al. 2013) (Figure 19). Leaf area index was measured with the help of a Ceptometer (model AccuPAR LP-80, Decagon Devices, Pullman, USA). The readings were taken between 11:00 to 13:00 hours on a clear day for all the trees. Two readings per tree were taken, and the average was calculated.

Figure 19. Canopy rating scale of grapefruit trees. The rating represents different levels of canopy thinning due to *C*Las infection or foot rot and gummosis disease. Visual rating of 1, is healthy canopy whereas 4 represents the highest canopy thinning.

4.27 Leaves and fibrous roots nutrient and non-structural carbohydrate measurements

Leaves were soaked in 1% HCl solution for 2 minutes and then washed for 5 minutes under tap water to remove all residues of dirt and pesticides. Leaves were air dried at the room temperature and stored in the brown paper bag once they were thoroughly dry (Obreza and Morgan 2008). Macro and micronutrient analysis were done by the Soil, Water and Forage Testing Laboratory at Texas A&M AgriLife Extension, College Station, TX.

For starch, glucose and sucrose analysis, 15 leaves after cleaning as above were dried in the oven at 60°C for 48 hrs. The dried leaf tissue was ground in an electric grinder and stored in sterile 100 ml centrifuge tubes. Twenty milligrams of lyophilized tissue was taken in a screw-cap microcentrifuge tube, and 1 ml of 80% ethanol was added to it and a half immersed in a hot water bath at 80° C for 20 min or until the tissue was free of pigment. The tubes were then spun down for 1 min at 13,000 rpm, and the supernatant (1 ml) was transferred into a new tube. The residue remaining in the tube was washed and 1 ml 80% of ethanol was added again and extracted as outlined above. Supernatants from the first and second extractions were combined and used to test soluble sugars, glucose, and sucrose. The extract was tested for glucose and sucrose. Forty µl of the extract was added

to a microplate well along with the glucose and sucrose standards and dried down at a temperature of 55° C in a hot water bath. To this dried residue, 160 µl of distilled water was added to all tubes except blank wells.

The background absorbance was read on a microplate reader (Model VMAX Molecular Devices, Sunnyvale, California, US) at wavelength 490 nm. Reducing sugar glucose was assayed first, and then sucrose was released by the action of invertase enzyme. To release the glucose in the extract, 40 µl of peroxidase (10.0 U peroxidase/ ml)/glucose oxidase (36.5 U / ml) reagent (PGO) was added. The plate was incubated in the dark for 30 min. Absorbance at 490 nm was re-read to measure the amount of glucose in the solution. Glucose content, was calculated by subtracting background absorbance from the absorbance reading obtained after adding PGO. To evaluate sucrose, to the same plate 40 µl of invertase solution (825 U/ ml invertase prepared in 45Mm acetate buffer, pH4.6) was added and was left to incubate for 60 min at 55°C. Absorbance was read at 490 nm; sucrose was quantified by subtracting the background absorbance from the absorbance obtained after addition of invertase solution (Hendrix 1993).

Linear graph of known standards concentration and their absorbance at 490 nm wavelength was plotted, and the intercept and slope values from the equation were used to calculate the sucrose and glucose content of unknown samples according to the formula, $y= mx + c$. Here “y” is the concentration of the unknown sample, “m” is the intercept and “x” is the absorbance reading of the unknown sample, and “c” is the slope reading obtained

from the absorbance plotted against known concentrations of known standards. The mass of glucose or sucrose was reported in mg/g dry weight. Known standards of glucose and sucrose were prepared at 0, 2, 4, 6, 8, 10 and 15 µg/ml concentration in distilled water. Starch was quantified from the residue left after extracting the soluble sugar extract from lyophilized leaf or root tissue. To evaluate for starch quantity, a set of starch standards (0-2 mg) was prepared. 200 µl of distilled water was added to all tubes including the standards. Tubes were autoclaved at 2 atm, 125°C for 30 min to get the starch. Tubes were allowed to sit on lab benches so that they can return to room temperature and 800 µl of amyloglucosidase solution (10 U/ml) was added to all tubes. Tubes were incubated at 55° C for 6 to 12 hours with occasional mixing and shaking to keep the residue and enzyme in contact. From the tube, 100 µl aliquots were added to a new tube, and 1 ml of distilled water was added and mixed thoroughly. With this new solution, 20 µl aliquots were added to microplate wells including the glucose (0-15 µg) and starch standards. Further steps were similar to how glucose was assayed, and final starch quantity was reported in mg starch/DWT of leaf or root tissue (Haissig and Dickson 1979; Batey and North Ryde 1982).

4.28 Data analysis

Data were analyzed with PROC GLM (SAS version 9.4 for Windows, SAS Institute, Cary, NC) to test significance. Data was log transformed, and percent data was ARCSIN converted as box plot showed not normally distributed data sets. When "f" value for the Model

was found significant, mean separations test was performed with Student-Newman-Keuls multiple range test at alpha value, $p< 0.05$. PROC REG and PROC COR were used for correlation analysis and to determine the significance of correlation respectively. PROC t-test was performed to determine the uniformity of variance of data and compare the 2014 and 2016 measurements. If the significant interaction between *C*Las and foot rot was not observed, the main effects of the individual factors were evaluated.

4.3 Results

4.31 *Phytophthora* propagule counts in the soil

No interaction was observed between foot rot and *C*Las for the propagule counts in the rhizosphere soil in both years. Therefore, the primary effect of *C*Las and foot rot was evaluated. A dataset of all *C*Las positive trees was compared with *C*Las negative trees, the same comparison was made for foot rot and gummosis affected versus not affected trees. In both years *C*Las-positive trees irrespective of foot rot disease measured significantly higher propagule counts in the rhizosphere soil. (Figure 20**).** Propagule per unit mass (g) of fibrous roots (<2mm) was also significantly higher in *C*Las-positive trees compared to a negative tree **(**Figure 20 C& D**).** No significant effect of the presence of foot rot on the trunk on propagule counts in the soil was observed. Nevertheless, *C*Las-positive trees with foot rot measured the highest propagule counts.

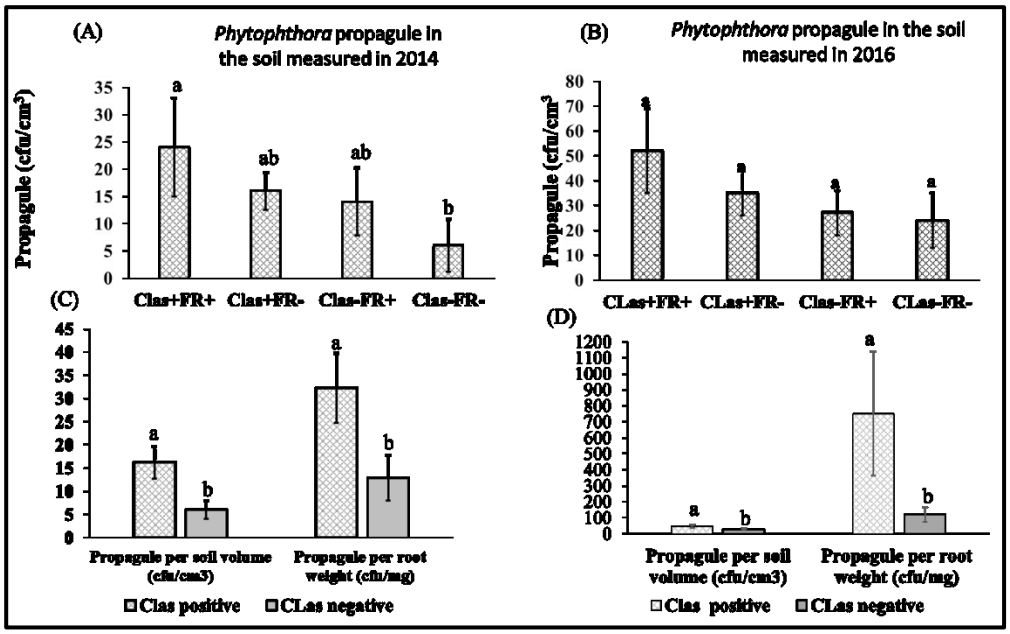

Figure 20 *Phytophthora* propagule (cfu/cm3) counts of *C*Las positive and negative grapefruit trees affected with or without foot rot and gummosis. *C*Las + and *C*Las – represents trees tested positive and negative for *C*Las and FR+ and FR- stand for a tree with or without foot rot and gummosis disease (n= as shown in table 1) A and B shows *Phytophthora* propagule counts of *C*Las positive and negative trees with and without foot rot disease measured in 2014 and 2016 respectively. B & D *Phytophthora* propagule in soil and per unit mass of fibrous root of *C*Las positive and negative grapefruit trees measured in 2014 and 2016 respectively. Error bars with different letters are significant at $P<0.05$ according to the Student-Newman-Keuls test.

4.32 Root densities

No interaction between *C*Las and foot rot disease was observed for root densities, and hence the main effects of *C*Las and foot rot disease were evaluated. Total root density (RD), root length density (RLD) and fibrous root density (FRD) of *C*Las positive and negative tree with and without foot rot were compared with each other (Figure 21).

The 2014 measurements significantly higher FRD was measured in *C*Las positive trees compared to *C*Las negative trees (Figure 21, Figure 22). No main effect of foot rot was observed for FRD, RLD, and RD in the 2014 measurements although foot rot positive trees had lower FRD and RLD **(**Figure 22). In the 2016 measurements, no main effect of *C*Las was observed for FRD, RLD, and RD. Although the main effects of foot rot were significant for FRD, RLD, and RD. Foot rot positive trees measured significantly lower FRD, RLD, and RD compared to the foot rot negative trees. *C*Las negative trees with foot rot measured significantly lower FRD and RLD compared to *C*Las positive with and without foot rot and *C*Las negative trees without foot rot disease in 2016 (Figure 22). Significant main effect of *C*Las was observed for increasing FRD in 2014, no main effect was observed for FRD, RLD, and RD in 2016 (Figure 22)

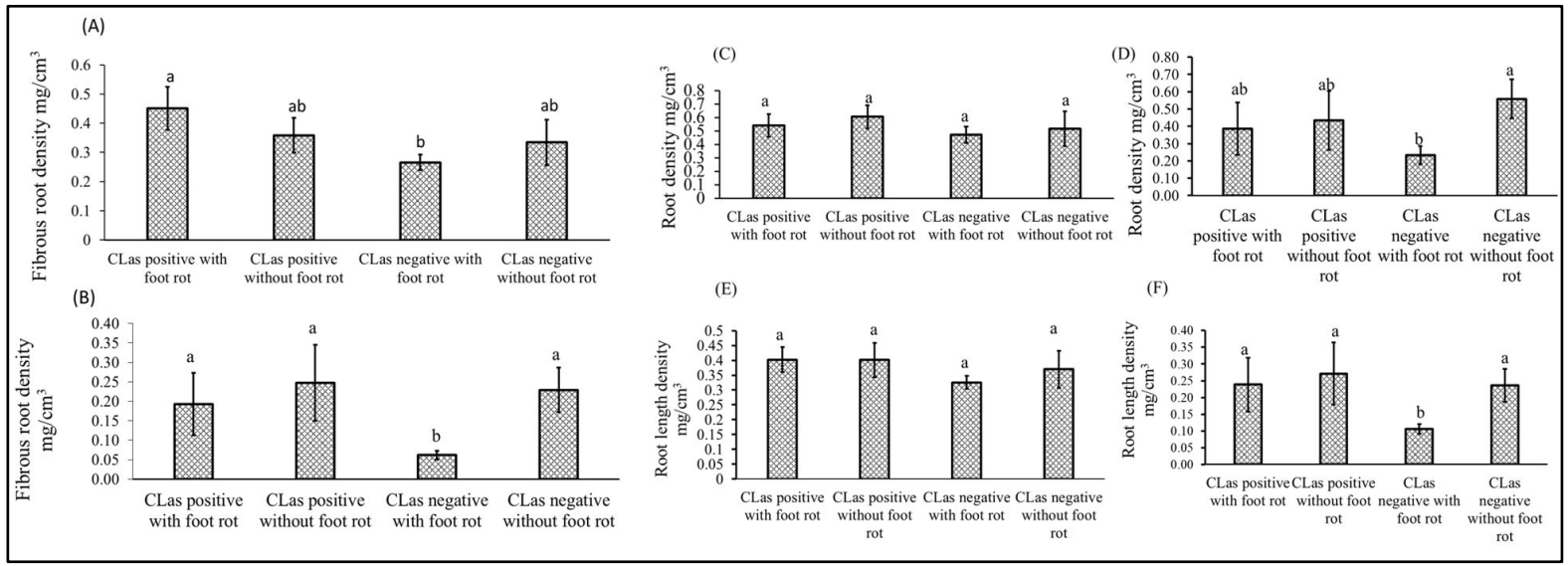

Figure 21. Fibrous root density, root density and root length density of *C*Las positive and negative grapefruit trees with or without foot rot disease. Measurements in 2014 (A, C, & D) and 2016 (B, E, & F). A&B - FRD, C&D- RD, E&F- RLD, Number of samples is as shown in Table 1. Error bar with different letters is significant at $p<0.05$ according to Student-Newman-Keuls test.

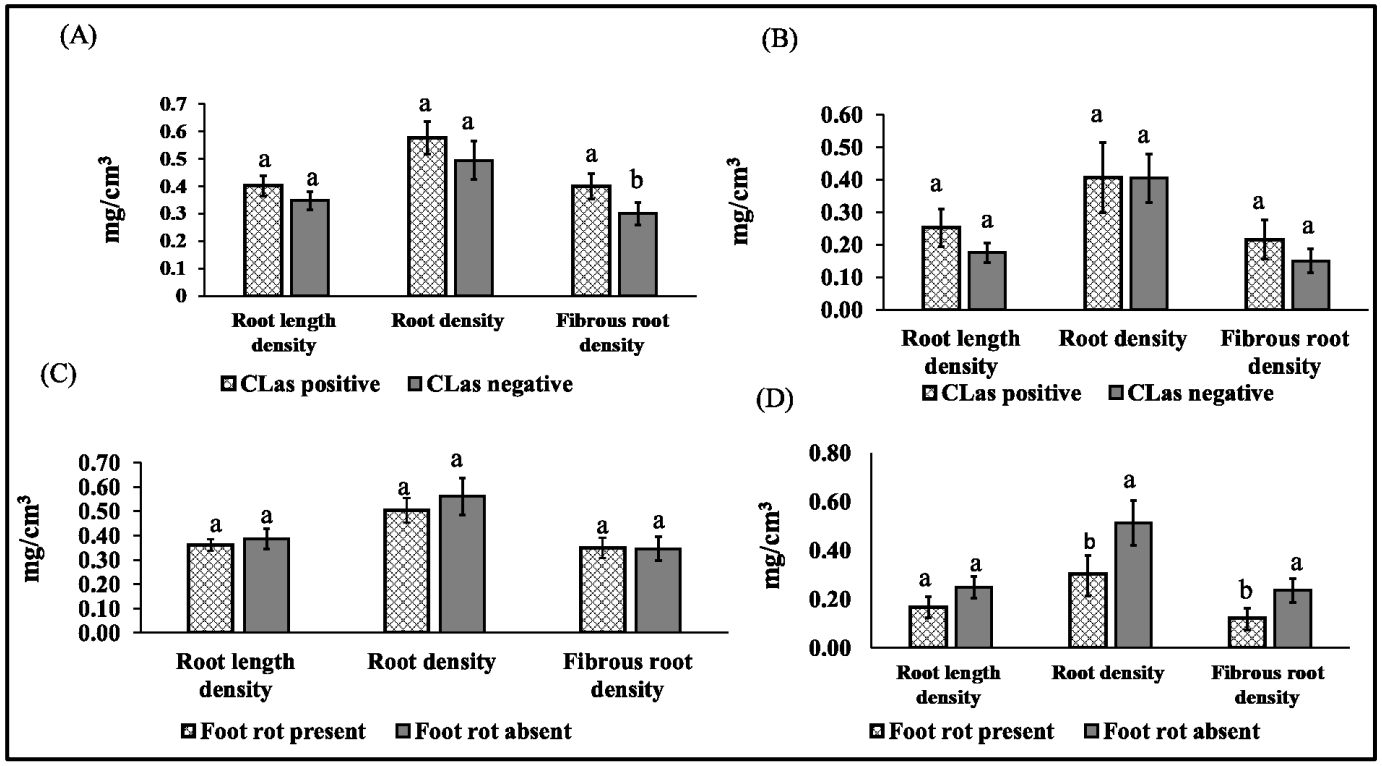

Figure 22. Main effect of CLas or foot rot on root density, root length density, and fibrous root density. The graph represents data from 2014 (A&C) and 2016 (B&D).

4.33 Leaf area index and canopy thinning

Leaf area index (LAI) and canopy thinning evaluation were only done in 2016. *C*Las positive trees with foot rot disease showed the highest rating on the visual rating scale for canopy thinning (P=0.001) whereas it was lowest in the *C*Las negative trees. Both foot rot and *C*Las have a significant effect on canopy thinning (Figure 23). Interaction effect (P =0.04) between *C*Las and foot rot disease on thinning of the tree canopy was also observed. LAI was not significantly different although lowest values were measured in the *C*Las positive trees with foot rot disease. The values of visual rating and LAI measurements were correlated. The correlation coefficient between Leaf area index and visual rating of canopy thinning was negative and significant (P=0.04, R^2 = 0.14) but the r^2 value was low (Figure 24). Trees with a higher visual rating for canopy decline measured lower leaf area index (LAI).

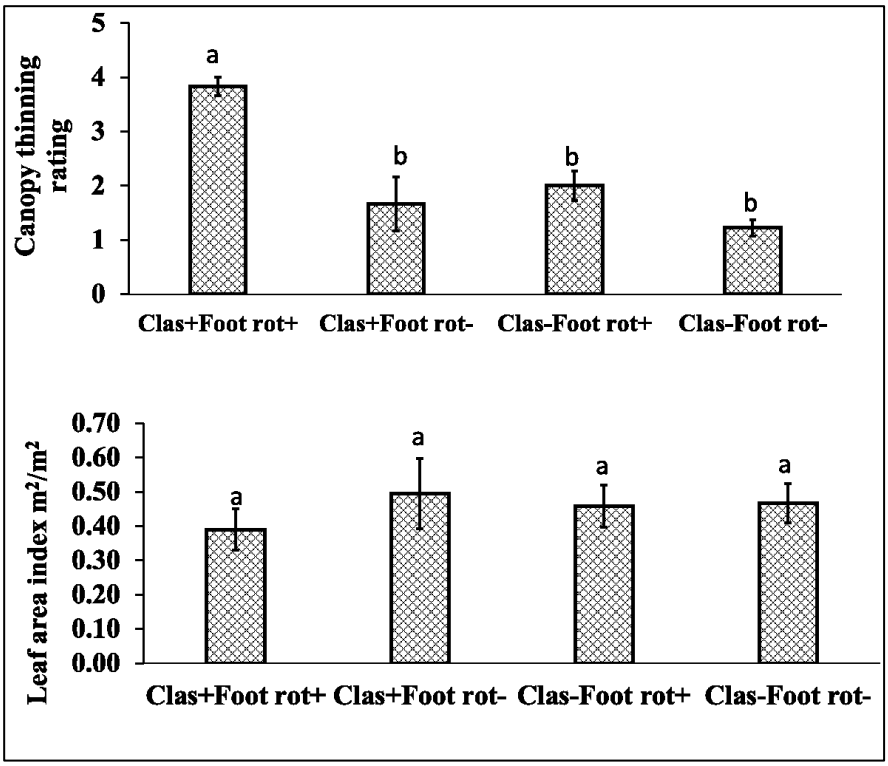

Figure 23. Canopy thinning rating scale of *C*Las positive and negative grapefruit trees with and without foot rot on a scale of 1 to 4 is represented. A rating scale of 1 represents a healthy-looking canopy, and 4 is highly thinned out canopy. Leaf area index was measured to compare with the visual rating scale.

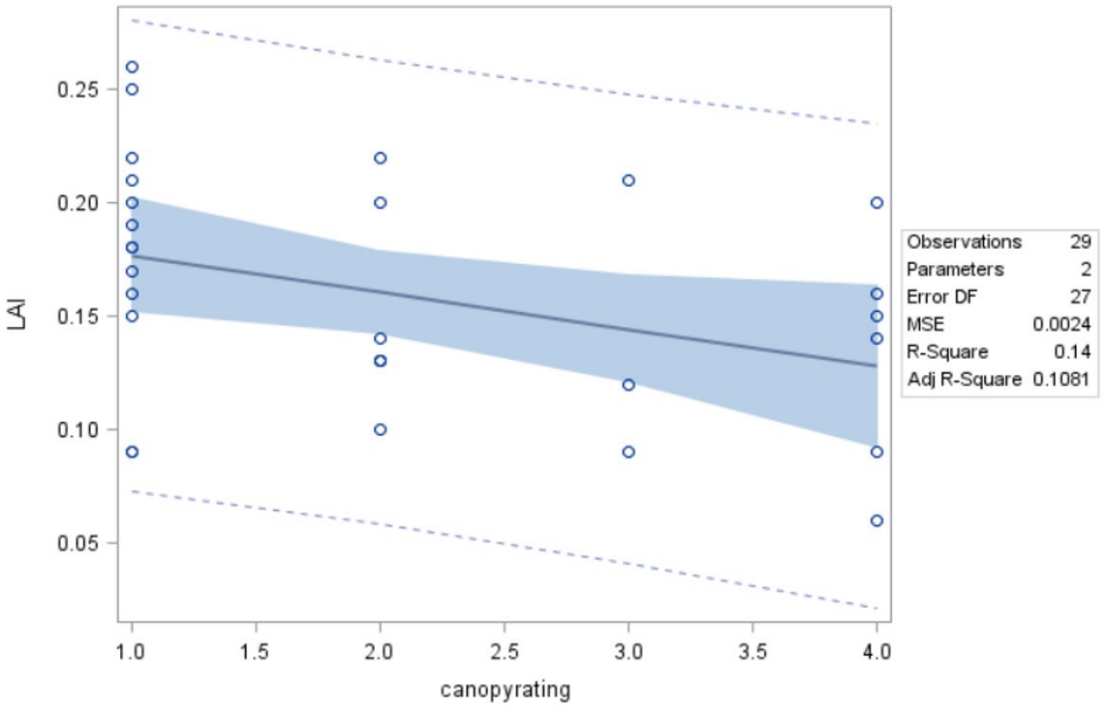

Figure 24. Correlation of visual canopy rating with Leaf area index of *C*Las positive and negative grapefruit trees with or without foot rot disease (t=-2.41, df=1, *P*>0.04).

4.34 Leaf nutrition

Asymptomatic leaves

Significant effect of the orchard was observed on the P, Ca, Zn, Cu, Mn, and B levels in the asymptomatic leaves of *C*Las positive and negative trees affected with or without foot rot disease. A significant interaction between *C*Las and foot rot was observed for the level of Mn in the asymptomatic leaves as lowest Mn was measured in *C*Las positive trees with foot rot disease (A- 9). No significant interaction was observed for other

macro and micronutrients. The main effect of *C*Las was observed for the level of Ca as it measured significantly lower in the asymptomatic leaves of *C*Las positive trees compared to *C*Las negative trees. *C*Las positive trees with foot rot disease measured significantly lowest Ca compared to all other groups of trees (A- 9). Nevertheless, it was still within the threshold levels (3.0-5.0 %) (Koo et al. 1984). Boron measured significantly lower in foot rot positive trees (A-10).

Symptomatic leaves

Nitrogen (N): Significant effect of the orchard was noted only in 2014 measurements. During 2014 irrespective of the *C*Las or foot rot, N was deficient in the symptomatic leaves of all trees (>2.5% threshold level). In 2016, N was significantly higher ($P<0.05$) in the symptomatic leaves of *C*Las positive trees though it still falls within the threshold levels (2.5 – 2.6%) (Koo et al. 1984) compared to *C*Las negative trees (Table 11). No interaction or foot rot effect was observed for the level of nitrogen in the symptomatic leaves in 2014 and 2016.

Phosphorus (P)*:* Significant effect of the orchard was not observed in both 2014 or 2016 and no significant interaction of P level in the symptomatic leaves for both years (Table 11**,** Figure 25). Although P measured low in *C*Las positive trees compared to negative trees in 2016.

Potassium (K): Significant effect of the orchard was observed only in 2016 for K measurements in symptomatic leaves. No significant interaction or main effects of *C*Las and foot

rot was observed for levels of K in symptomatic leaves in both 2014 and 2016. In both years potassium was less than threshold levels in the *C*Las positive trees (Less than 1.1 %) (Koo et al. 1984) compared to *C*Las negative trees. Some effect of foot rot disease was also observed as K percent of symptomatic leaves of grapefruit trees without foot rot, or *C*Las measured significantly higher potassium (Table 11).

Calcium (Ca): Significant effect of the orchard was observed for Ca levels in the symptomatic leaves only during 2016. Although no significant interaction, *C*Las, and foot rot effect were observed for Ca levels in the symptomatic leaves for both 2014 and 2016 measurements.

Magnesium (Mg): Significant effect of the orchard was observed only in 2016 on Mg percent in the symptomatic leaves. No significant interaction or main effect of *C*Las and foot rot was observed for Mg levels in the symptomatic leaves in 2014. The main effect of *C*Las was observed in 2016 as *C*Las positive trees have a significantly lower percent of Mg. Measurement of Mg was significantly lower (13%) in the symptomatic leaves of *C*Las positive trees during 2016 **(**Figure 25 B, Table 12), although it was still within the threshold (0.30 – 0.50 %) (Koo et al. 1984).

Sodium (Na): Significant effect of the orchard was observed on Na percent in the symptomatic leaves only in 2016. In 2014 significant interaction of *C*Las and foot rot diseases was observed on Na levels in the leaves. Also in 2014 simple effect of foot rot dis-

ease was observed for Na levels in the symptomatic leaves. Foot rot positive trees measured 70% and 45% lower Na compared to trees without foot rot in 2014 and 2016 respectively **(**Figure 26 A & B**,** Table 12). No interaction effect was observed in 2016 although *C*Las positive trees measured significantly higher Na (Table 12).

Sulfur (S): Significant effect of the orchard was observed in both year on the percent of S in the symptomatic leaves. In 2014 significant interaction was observed between *C*Las and foot rot disease on S percent. Trees with foot rot disease measured significantly lower S in the symptomatic leaves compared to trees without foot rot disease. During 2014 measurement lowest sulfur was detected in the trees with both *C*Las and foot rot diseases compared to trees without *C*Las or foot rot (Table 11). Compared to healthy control all trees with either *C*Las or foot rot disease or both measured significantly lower Sulfur in the symptomatic leaves compared to trees without foot rot disease (Table 12). The percent was significantly lower compared to the healthy control although it was still within threshold levels in 2014 (0.20% to 0.40%) (Table 12). In 2016, the only the main effect of *C*Las was observed as *C*Las positive trees measured significantly lower S compared to *C*Las negative trees (Table 12). Also in 2016 *C*Las negative trees without foot rot have more than threshold levels of sulfur (> 0.40 %) (Koo et al. 1984) (Table 12).

Zinc (Zn): Significant effect of the orchard was observed on the Zn percent in the symptomatic leaves in both 2014 and 2016. No significant interaction of *C*Las and foot rot or

main effects were observed for Zn levels in the symptomatic leaf tissues in both 2014 and 2016 (Table 13, Table 14).

Iron (Fe): No significant effect of the orchard, the interaction between *C*Las and foot rot or main effect foot rot was observed on the Fe percent in the symptomatic leaves in 2014. Although the significant effect of *C*Las was observed only in 2014 as *C*Las positive trees measured higher Fe compared to negative trees. The levels of Fe were not observed to be different in *C*Las positive and negative trees with or without foot rot disease in 2016 (Table 13, Table 14).

Copper (Cu): Significant effect of the orchard was observed on Cu levels in symptomatic leaves only during 2016. In 2014 significant interaction ($P<0.05$) between *C*Las and foot rot disease was observed on Cu level and simple effect of *C*Las was also recorded. During 2014, it was also less than the threshold (6–16 ppm) in trees with one or both diseases. In both 2014 and 2016 trees with *C*Las, or foot rot or both had lower copper in the symptomatic leaves compared to grapefruit trees without *C*Las or foot rot (Table 13, Table 14). In 2016 only main effect of *C*Las was observed such that CLas positive trees have significantly lower Cu compared to negative trees. Compared to the symptomatic leaves of *C*Las negative trees *C*Las positive trees measured 27% lower Cu in 2016 (Figure 25).

Manganese (Mn): Significant effect of the orchard was observed on Mn levels in the symptomatic leaves only during 2016. In both 2014 and 2016 significant interaction, *C*Las, or foot rot effect was not observed on the Mn levels in the symptomatic leaves of the trees.

Boron (B): Significant effect of the orchard was observed in both 2014 and 2016. In 2014 significant interaction between *C*Las and foot rot disease was observed for levels of B in the symptomatic leaves (Table 13). B was significantly higher in *C*Las positive trees without foot rot disease. No simple effect of *C*Las or foot rot was observed on B levels in the leaf tissue. The lowest level of boron was measured in the *C*Las negative trees with foot rot disease in 2014 (Table 13). In 2016 no main effect of *C*Las or foot rot on B level was measured. In both years though, B was above threshold levels (<100 ppm) (Koo et al. 1984) in both asymptomatic and symptomatic leaves.

Soil analysis was also done to check if the macro and micronutrient were deficient or in excess in the soil of the orchards studied. Except for sodium and nitrate, all macro and micronutrients were found to be present in moderate to slightly high percent (A-7). Pairwise t-test results of each group of trees analyzed in both 2014 and 2016 concerning the nutrients in the symptomatic leaf tissues were also done. Compared to 2014 measurements higher nitrogen, calcium, magnesium, sulfur, boron, and iron were measured in symptomatic leaves in the year 2016 irrespective of the treatments. Only Zinc was deficient in 2016 in all the blocks and all the treatment.

Table 11. Macronutrient N, P and K (%) analysis of symptomatic leaves of grapefruit trees affected with CLas and or foot rot and gummosis compared to healthy trees evaluated in 2014 and 2016.

	N		P		K	
	2014	2016	2014	2016	2014	2016
*C*Las						
Positive	2.1[a]	2.5[a]	0.14[a]	0.14[a]	0.9[a]	1.1 [a]
Negative	2.1[a]	2.4[b]	0.15[a]	0.15[a]	1.0[a]	1.4 [a]
Foot rot						
Present	2.1[a]	2.5[a]	0.14[a]	0.15 [a]	0.9[a]	1.2 [a]
Absent	2.1[a]	2.3[a]	0.14[a]	0.15 [a]	1.0[a]	1.3 [a]
*C*Las X foot rot						
*C*Las positive x foot rot present	2.1[a]	2.5[a]	0.13[a]	0.14 [a]	0.96[a]	1.0 [a]
*C*Las positive x foot rot absent	2.1[a]	2.4[a]	0.14[a]	0.14 [a]	0.97[a]	1.1 [a]
*C*Las negative x foot rot present	2.1[a]	2.4[a]	0.14[a]	0.15 [a]	0.92[a]	1.2 [a]
*C*Las negative x foot rot absent	2.1[a]	2.4[a]	0.14[a]	0.15 [a]	1.20[a]	1.6 [a]

Means in the column represented by different letters are significant at $P<0.05$.

Table 12. Macronutrient Ca, Mg, Na and S (%) analysis of symptomatic leaves of grapefruit trees affected with ***C*Las** and or foot rot and gummosis compared to healthy trees evaluated in 2014 and 2016**.**

	Ca		Mg[z]		Na		S	
***C*Las**	2014	2016	2014	2016	2014	2016	2014	2016
Positive	3.3[a]	4.7 [a]	0.24[a]	0.33[b]	0.14[a]	0.17 [a]	0.29[a]	0.39[b]
Negative	3.3[a]	4.7 [a]	0.23[a]	0.38[a]	0.14[a]	0.11 [b]	0.32[a]	0.42[a]
Foot rot								
Present	3.1[a]	4.7 [a]	0.23[a]	0.36 [a]	0.17[a]	0.16 [a]	0.28[b]	0.39 [a]
Absent	3.4[a]	4.7 [a]	0.24[a]	0.35 [a]	0.10[b]	0.11 [b]	0.32[a]	0.42 [a]
***C*Las X foot rot**								
*C*Las positive x foot rot	3.2[a]	4.8[a]	0.23[a]	0.34[ab]	0.15[ab]	0.19 [a]	0.30[b]	0.38 [a]
CLas positive x no foot rot	3.4[a]	4.6[a]	0.25[a]	0.31[b]	0.12[ab]	0.14 [a]	0.29[b]	0.40 [a]
*C*Las negative x foot rot	3.0[a]	4.7[a]	0.25[a]	0.38[ab]	0.21[a]	0.13 [a]	0.27[b]	0.39 [a]
*C*Las negative x no foot rot	3.5[a]	4.7[a]	0.22[b]	0.41[a]	0.10[b]	0.08[b]	0.37[a]	0.45 [a]

Means in the column represented by different letters are significant at $P<0.05$.
[z] Represent significant interaction between HLB and foot rot disease

Table 13. Micronutrient (ppm) analysis of symptomatic leaves of grapefruit trees affected with *C*Las and or foot rot and gummosis compared to healthy trees evaluated in 2014.

Percent Micronutrient	Zn	Fe	Cu[z]	Mn	B[z]
***C*Las**					
HLB positive	30.5[a]	45.1[a]	5.1[a]	35.5[a]	162.7[a]
HLB negative	29.5[a]	38.0[b]	5.3[a]	32.6[a]	159.3[a]
Foot rot					
Present	28.0[a]	41.0[a]	4.7[b]	32.4[a]	149.8[a]
Absent	31.9[a]	41.7[a]	5.6[a]	36.1[a]	171.4[a]
***C*Las X foot rot**					
*C*Las positive x foot rot present	28.7[a]	44.0[a]	5.05[b]	33.5[a]	159.0[ab]
*C*Las positive x foot rot absent	32.0[a]	46.0[a]	5.26[b]	37.1[a]	165.6[ab]
*C*Las negative x foot rot present	27.1[a]	37.0[a]	4.45[b]	30.9[a]	137.5[b]
*C*Las negative x foot rot absent	37.0[a]	34.4[a]	6.30[a]	34.4[a]	181.5[a]

Means in the column represented by different letters are significant at $P<0.05$.

[z] Represent significant ($P>0.05$.) interaction between *C*Las and foot rot disease

Table 14. Micronutrient (ppm) analysis of symptomatic leaves of grapefruit trees affected with CLas and or foot rot and gummosis compared to healthy trees evaluated in 2016.

Percent Micronutrient	Zn	Fe	Cu	Mn	B
***C*Las**					
CLas positive	14.9 [a]	67.3 [a]	09.0 [b]	31.0 [a]	255.2 [a]
CLas negative	12.5 [a]	60.0 [a]	12.4 [a]	30.0 [a]	228.0 [a]
Foot rot					
Present	14.4 [a]	63.8 [a]	10.7 [a]	30.8 [a]	237.1 [a]
Absent	12.8 [a]	62.9 [a]	10.8 [a]	30.0 [a]	245.7 [a]
***C*Las X foot rot**					
*C*Las positive x foot rot present	16.0 [a]	66.2 [a]	10.0[bc]	31.0 [a]	242.0[ab]
*C*Las positive x foot rot absent	14.0 [a]	69.0 [a]	8.10[c]	31.0 [a]	270.2[a]
*C*Las negative x foot rot present	13.2 [a]	62.0 [a]	12.0[ab]	31.0 [a]	232.4[b]
*C*Las negative x foot rot absent	12.0 [a]	57.3 [a]	13.4[a]	29.0 [a]	221.2[b]

Means in the column represented by different letters are significant at $P<0.05$.

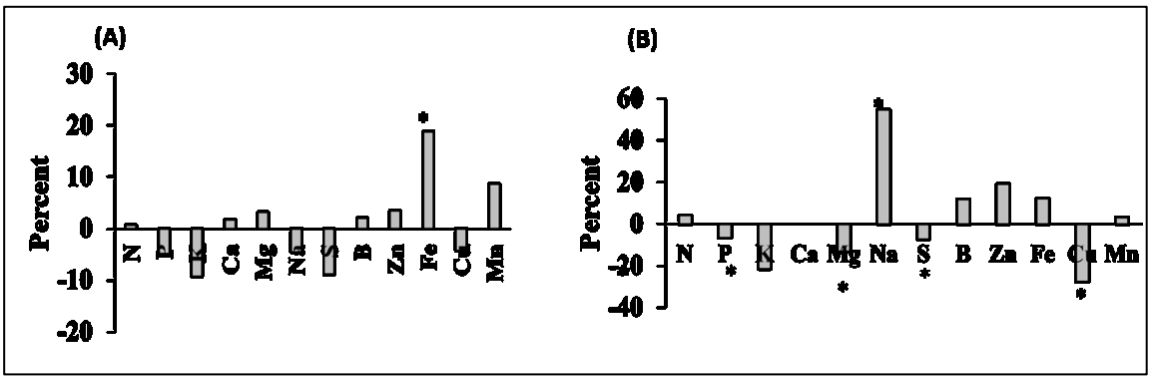

Figure 25. Percent difference in the macro and micronutrient measured in the symptomatic leaves of *C*Las positive trees in comparison to *C*Las negative trees. (A) 2014 (B) 2016. Bars with an asterisk are at the significant difference (P<0.05).

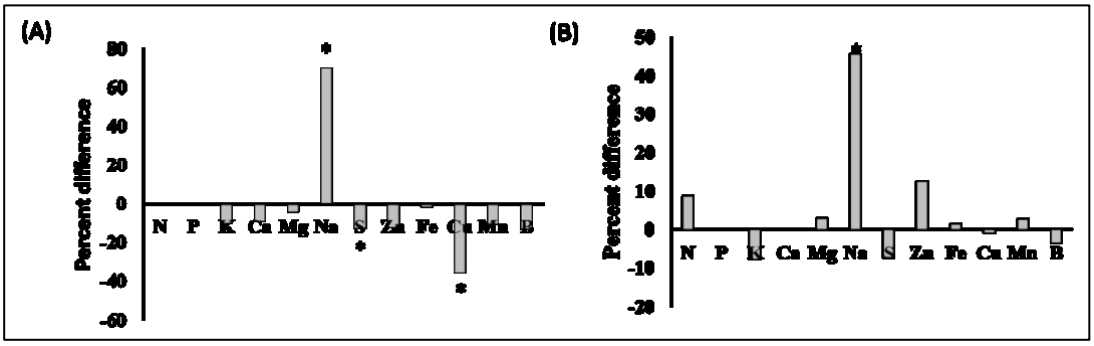

Figure 26. Percent difference in the macro and micronutrient of symptomatic leaves of grapefruit trees with or without foot rot disease. Percent difference in the macro and micronutrient measured in the symptomatic leaves of grapefruit trees with foot rot disease in comparison to trees without foot rot. (A) 2014 and (B) 2016. Bars with an asterisk are at the significant difference (P<0.05).

4.35 Fibrous root nutrient analysis

The interaction between *C*Las and foot rot was evaluated on the macro and micro-nutrient content in the fibrous root of grapefruit trees. The simple effect of *C*Las or foot rot was evaluated if the interaction was the significant otherwise main effect is tested if the interaction is not significant (Figure 27).

2014 measurement

Effect of blocks was significant for P, K, Mg, Fe, Cu, Mn and B. No significant effect of interaction between *C*Las and foot rot was observed for any of the measured macro and micronutrients in the fibrous root tissue. The main effect of *C*Las was observed for percent P and Mg in the fibrous roots. Both P (21%, p=0.001) and Mg measured significantly lower (15%, p = 0.002) in the *C*Las positive compared to negative trees (Figure 29 A). Significant main effect of foot rot was observed only for Mn. Foot rot positive trees measured higher Mn and *C*Las positive trees with foot rot measured significantly higher compared to positive trees without foot rot disease (Figure 28 A, Figure 30 A). Copper was significantly lower in trees with both *C*Las and foot rot or either compared to *C*Las negative trees without foot rot (Figure 28 A**).** Although no significant main effect of *C*Las or foot rot was observed on Cu percent nevertheless *C*Las positive trees had 18% lower Cu in the fibrous roots compared to *C*Las negative trees (Figure 29 A)

2016 measurement

Orchard sites have a significant effect on Mg, Zn, Fe, Cu levels in the fibrous root tissue. No significant interaction between *C*Las and foot rot was observed on macro or micronutrient content in the fibrous roots. The main effect of *C*Las was observed on the Mg, and Cu content in the fibrous root tissue. Mg and Cu measured significantly lower (14%, p= 0.03) and (33%, p=0.01) in the fibrous roots of the *C*Las positive trees compared to *C*Las negative trees in 2016 respectively (Figure 27 D, Figure 29, B). A significant effect of both *C*Las and foot rot was observed on the Mn content of fibrous roots. *C*Las positive trees measured significantly higher Mn compared to *C*Las negative trees. Similarly, foot rot positive trees measured significantly higher Mn. Highest Mn was measured in the *C*Las positive trees with foot rot (Figure 27 D, Figure 30). Zinc measurements were significantly lower (*P*= 0.007) in 2016, and CLas positive trees showed 16% lower zinc in the fibrous roots compared to *C*Las negative trees (Figure 27, Figure 29) .

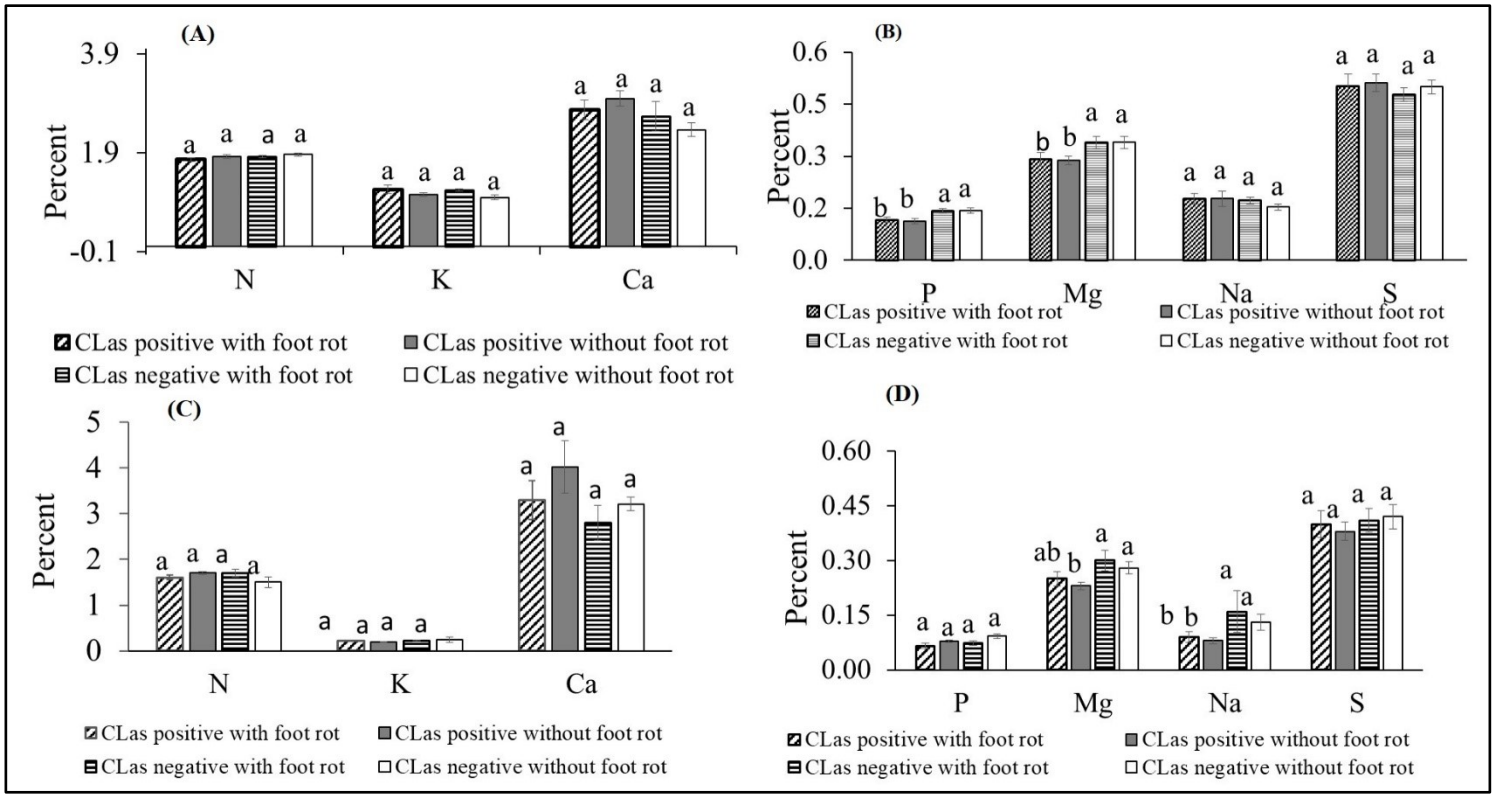

Figure 27 Measurement of macronutrients in the fibrous roots of *C*Las positive and negative grapefruit trees affected with and without foot rot disease. A & B 2014, C & D 2016.

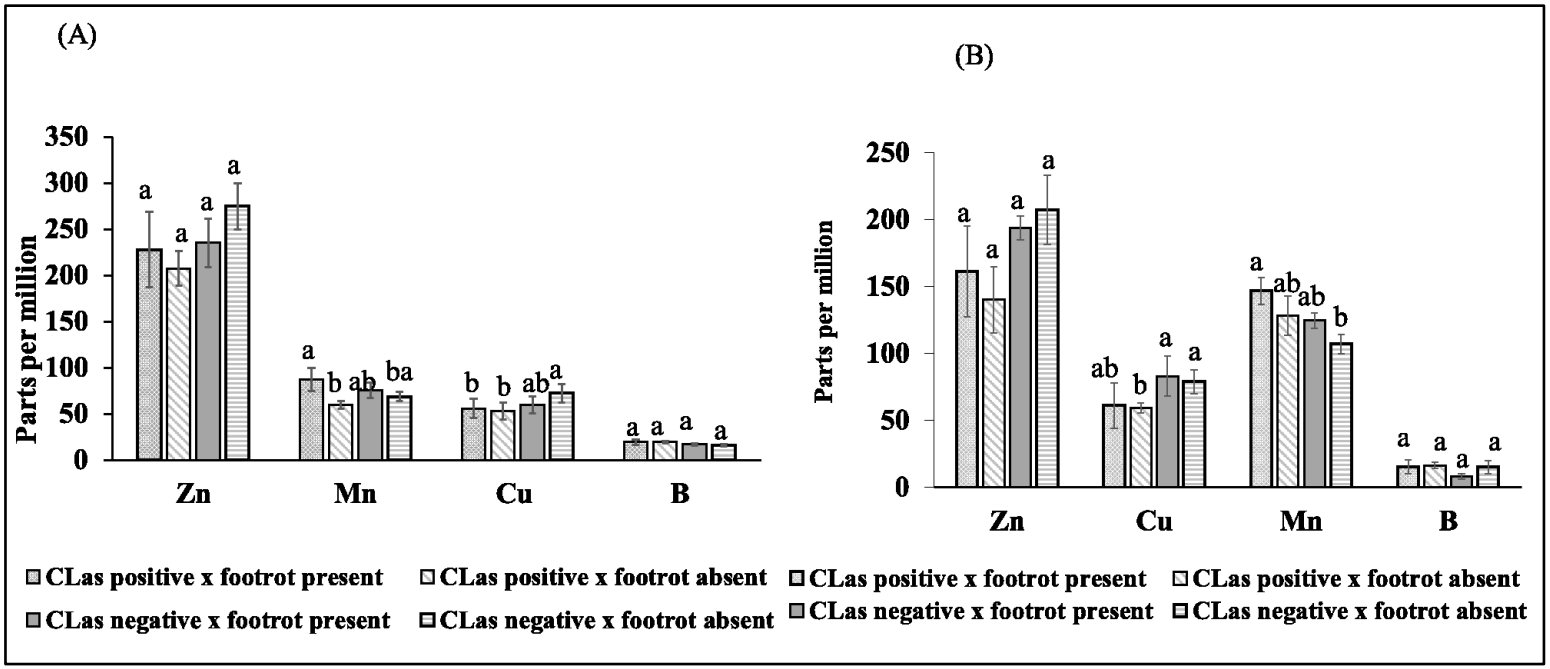

Figure 28. Measurement of micronutrients zinc, manganese, copper, and boron in the fibrous roots of *C*Las positive and negative grapefruit trees with and without foot rot disease. A :2014 & B 2016.

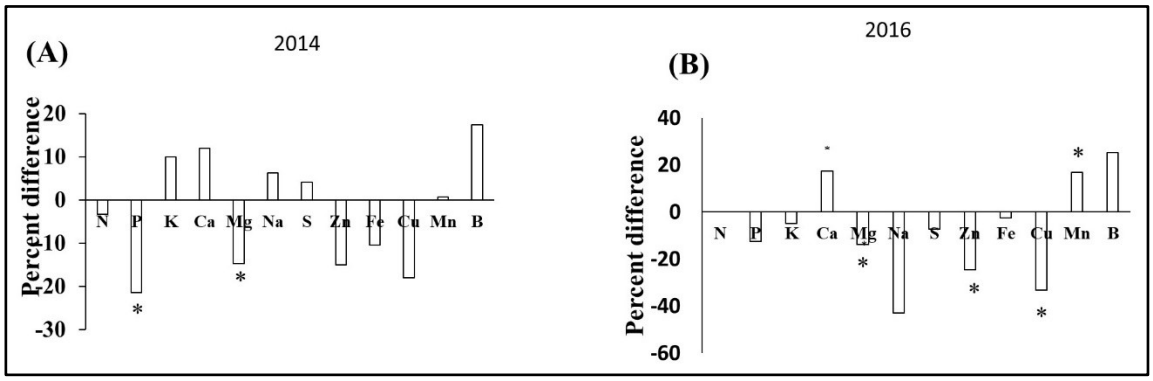

Figure 29. Difference in the macro and micronutrient in the fibrous roots of *C*Las positive trees in comparison to *C*Las negative grapefruit trees. 2014 (A) and 2016 (B). Bars with an asterisk are at the significant difference ($p<0.05$).

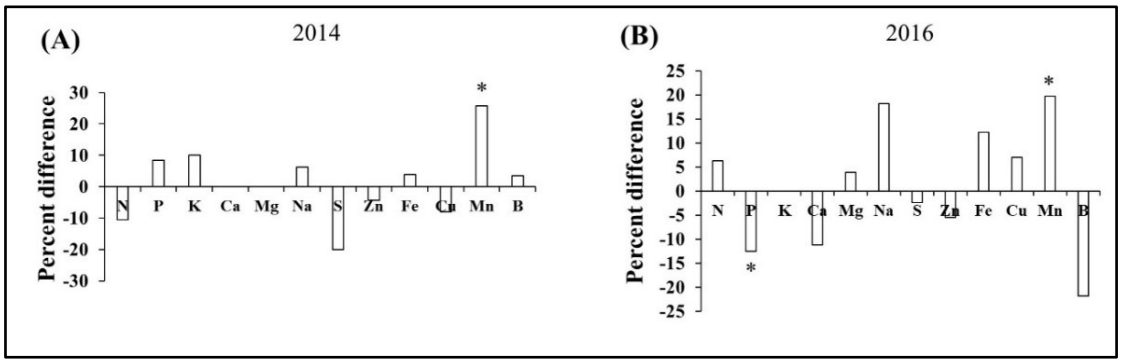

Figure 30. Difference in the macro and micronutrient in the fibrous roots of foot rot positive grapefruit trees in comparison to grapefruit trees without foot rot. 2014 (A) and 2016 (B). Bars with an asterisk are at the significant difference ($P<0.05$).

4.36 Leaf carbohydrate measurements

Starch, sucrose and glucose content were not significantly affected by *C*Las, foot rot or interaction between the two in the asymptomatic leaves. No significant interaction between *C*Las and foot rot disease was observed for the level of starch, glucose, and sucrose in the symptomatic leaves in both 2014 and 2016, therefore the only main effect is explained here. Block effect was not observed in the carbohydrate levels in 2014. *C*Las positive trees measured significantly high ($P<0.001$) starch in the symptomatic leaves compared to *C*Las negative trees irrespective of foot rot. *C*Las positive trees with foot rot measured significantly higher starch compared to all groups of trees. Although it was not significantly different from *C*Las without foot rot trees (Figure 31**,** Figure 32).

The main effect of foot rot or interaction between foot rot and *C*Las was observed for the starch level in the symptomatic leaves. Glucose and sucrose content were not affected by the *C*Las (Figure 31) or foot rot or the interaction of the two diseases in 2014. Symptomatic leaves of the same set of trees measured significantly higher starch, glucose, and sucrose compared to the *C*Las negative trees in 2016 (Figure 31 D to F, Figure 32). Block effect was weakly significant only for starch content in the symptomatic leaves ($P<0.05$). The main effect of foot rot was not observed for starch, glucose and sucrose levels in the symptomatic leaves. *C*Las positive trees with or without foot rot measured high starch, sucrose and glucose compared to *C*Las negative with foot rot or control trees without *C*Las or foot rot disease (Figure 31, D to F).

4.37 Root carbohydrate measurements

Significant block effect on glucose and sucrose content of fibrous root was observed in both 2014 and 2016. In 2014 measurement no significant effect of *C*Las and foot rot or interaction of the two was observed on starch and sucrose. Nevertheless, foot rot had a significant effect on glucose content in the fibrous root. As foot rot positive trees fibrous roots have significantly higher glucose content compared to foot rot negative trees. In 2016 except for sucrose significant effect of *C*Las or foot rot or their interaction was not observed on the levels of starch and glucose in the fibrous roots. Sucrose measured significantly lower in the roots of *C*Las positive trees in 2016 compared to negative trees. Trees, affected with foot rot, on the other hand, had significantly higher sucrose in the fibrous roots in both years. *C*Las and foot rot showed main effects on root sucrose content independent of each other. Presence of *C*Las may induce reduction where foot rot leads to increase sucrose. Therefore, *C*Las positive trees without foot rot measured the lowest sucrose in the fibrous roots. *C*Las or foot rot disease did not show a significant effect on starch, but a trend of lower starch due to *C*Las was observed in the 2016 measurements (Figure 33).

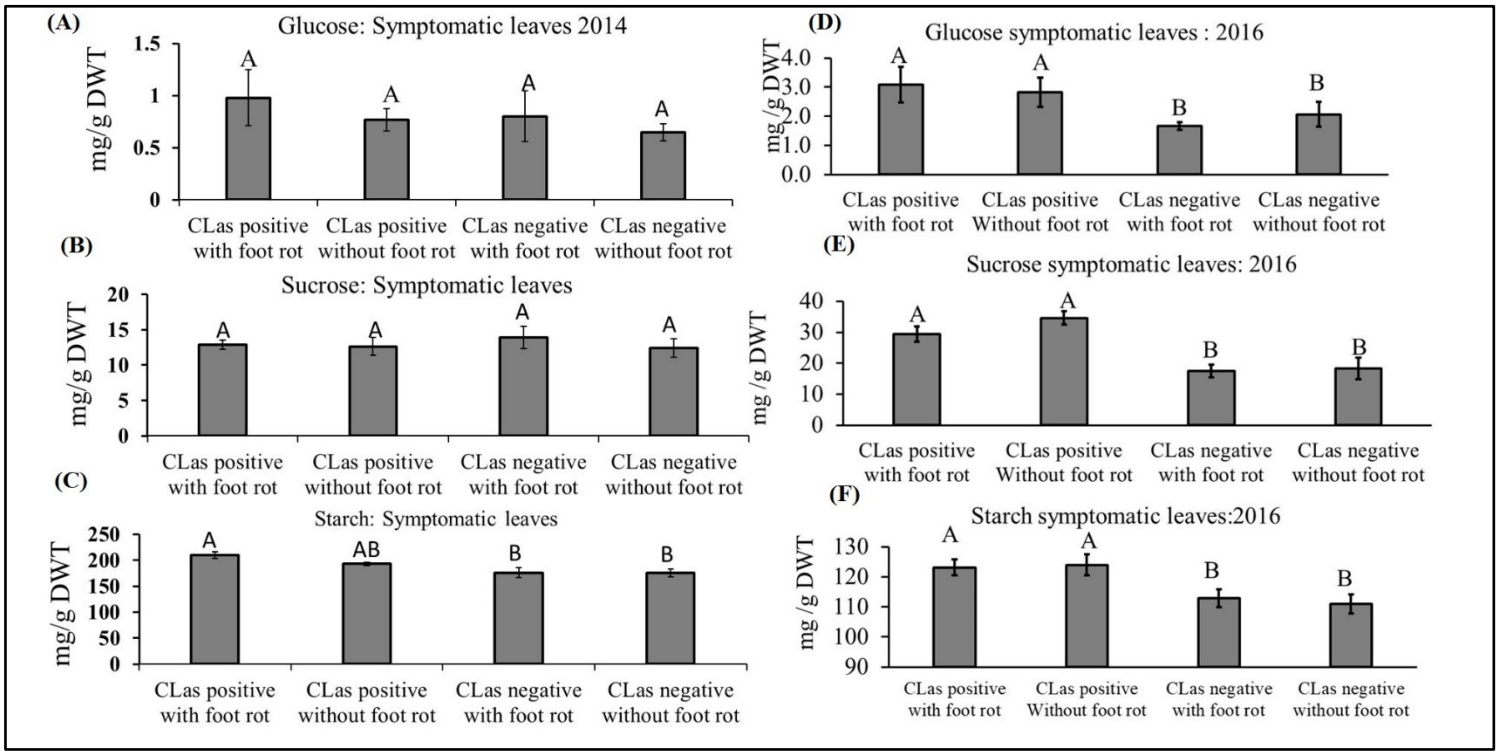

Figure 31. Starch, glucose and sucrose contents in symptomatic leaves of grapefruit trees of *C*Las positive and negative trees with and without foot rot or gummosis disease. A to C, are measurements taken of the symptomatic leaves samples collected in 2014 and D to F are measurements on foliar samples collected for the same set of trees between August to September in 2016. Error bars with different letters are significant at $P<0.05$.

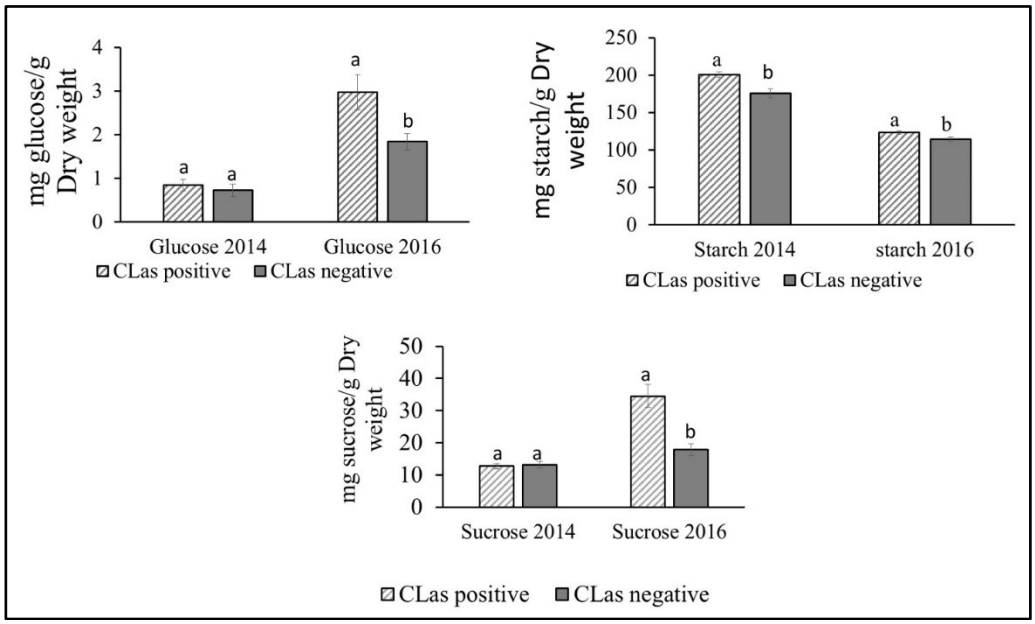

Figure 32. Starch, sucrose, and glucose measured in symptomatic leaves of *C*Las positive and negative grapefruit trees measured in 2014 and 2016. Bars with different letters are significant at $P<0.05$.

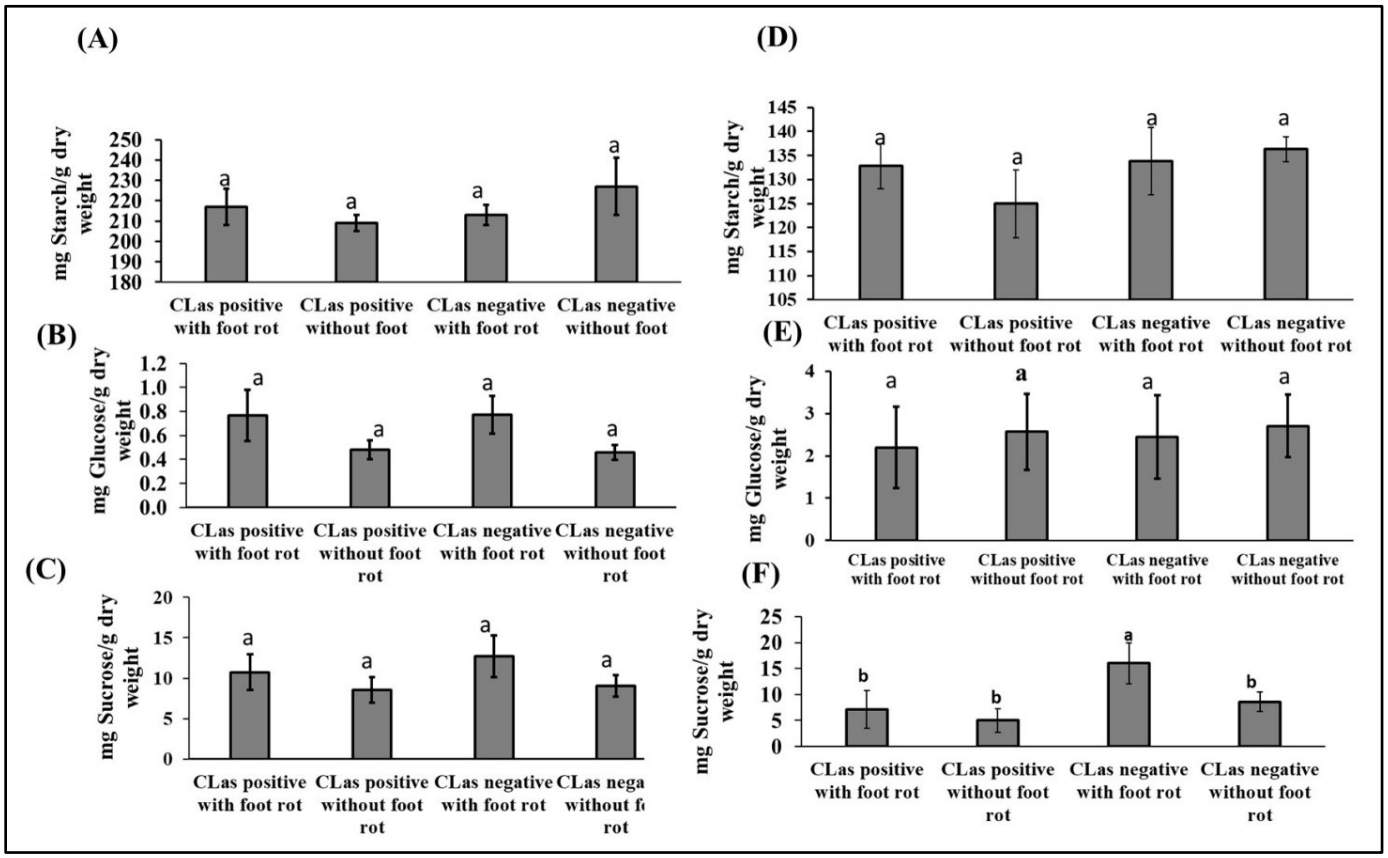

Figure 33. Starch, glucose and sucrose contents in the fibrous roots of CLas positive and negative trees with and without foot rot or gummosis disease. Error bar with same letters represent means not significantly different at $P< 0.05$. Bar graphs in the set A to C and D to F represent measurements taken in 2014 and 2016.

Table 15. Summary of the interaction of CLas and foot rot disease in grapefruit or their main effects of on starch, sucrose, and glucose in the symptomatic leaves and fibrous roots. The symbol "↑" means significant (P<0.05) increase, "↓" means significant (P<0.05) decrease and "NS" means not significant (P < 0.05) respectively.

	Symptomatic leaves					
	Glucose		Sucrose		Starch	
	2014	2016	2014	2016	2014	2016
CLas	NS	↑	NS	↑	↑	↑
Foot rot	NS	NS	NS	NS	NS	NS
CLasx Foot rot	NS	NS	NS	NS	NS	NS
	Fibrous roots					
	Glucose		Sucrose		Starch	
	2014	2016	2014	2016	2014	2016
CLas	NS	NS	NS	↓	NS	NS
Foot rot	↑	NS	↑	↑	NS	NS
CLas x Foot rot	NS	NS	NS	NS	NS	NS

Table 16. Summary of the interaction of *C*Las and foot rot disease in grapefruit or their main effects of on the macro and micronutrient content of the symptomatic leaves and fibrous roots. The symbol "↑" means significant (P<0.05) increase, "↓" means significant (P<0.05) decrease and "NS" means not significant (P < 0.05) respectively.

	Symptomatic leaves 2014											
	N	P	K	Mg	Ca	Na	S	Cu	B	Zn	Mn	Fe
CLas	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	↑
Foot rot	NS	NS	NS	NS	NS	↑	↓	↓	NS	NS	NS	NS
CLas x Foot rot	NS	NS	NS	NS	NS	Sig.	Sig.	NS	Sig.	NS	NS	NS
	Symptomatic leaves 2016											
	N	P	K	Mg	Ca	Na	S	Cu	B	Zn	Mn	Fe
CLas	↑	↓	NS	↓	NS	↑	↓	↓	NS	NS	NS	NS
Foot rot	NS	NS	NS	NS	NS	↑	NS	NS	NS	NS	NS	NS
CLas x Foot rot	NS	NS	NS	Sig	NS	NS	NS	NS	NS	NS	NS	NS
	Fibrous roots 2014											
	N	P	K	Mg	Ca	Na	S	Cu	B	Zn	Mn	Fe
CLas	NS	↓	NS	↓	NS	NS	NS	NS	NS	NS	NS	NS
Foot rot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	↓	NS
CLas x Foot rot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Table 16 Continued

	Fibrous roots 2016											
	N	P	K	Mg	Ca	Na	S	Cu	B	Zn	Mn	Fe
CLas	NS	NS	NS	↓	↑	↓	NS	↓	↑	↓	↑	NS
Foot rot	NS	↓	NS	NS	NS	NS	NS	↑	NS	↓	↑	↑
CLas x Foot rot	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Table 17. Summary of the main effect of CLas and or foot rot and gummosis and their interaction in grapefruit trees on root density, root length density, fibrous root density, canopy thinning and leaf area index. The symbol "↑" means significant (P<0.05) increase, "↓" means significant (P<0.05) decrease and "NS" means not significantly different.

	Root density		Root length density		Fibrous root density		Canopy thinning visual rating	Leaf area index (LAI)
	2014	2016	2014	2016	2014	2016	2016	2016
CLas	NS	NS	NS	NS	↑	NS	↑	NS
Foot rot	NS	↓	NS	↓	NS	↓	↑	NS
CLas x Foot rot	NS	NS	NS	NS	NS	NS	Sig.	NS

4.4 Discussion

Observation in this study was done to evaluate the effect of *C*Las infection and foot rot disease in the grapefruit trees. It was predicted that both *C*Las and foot rot disease would interfere with effective nutrient translocation so there should be imbalances of nutrients that could cause lower root density and higher canopy thinning. I looked into the symptomatic leaves since *C*Las titer in asymptomatic leaves could be too low to register any changes due to phloem damage. Damaging levels (15 to 20 cfu/cm$^{3)}$ *Phytophthora* counts were made consistently for both years in the rhizosphere of *C*Las positive tree compared to negative trees. Main effects of *C*Las on *Phytophthora* propagule counts in the rhizosphere were observed.

Reports of an increase in *P. nicotianae* propagule from symptomatic *C*Las positive trees rhizosphere have been confirmed from *C*Las-infected citrus trees in Florida (Graham et al. 2013). *C*Las negative trees with foot rot disease did not measure significantly higher *Phytophthora* propagule compared to negative trees without foot rot disease. The results were similar to what was observed in the survey study where foot rot incidence and soil propagule levels were not found to be correlated (Chapter 2). It is known that foot rot does not add inoculum to the soil and could explain why foot rot affected trees did not show higher propagules in the soil (Timmer et al. 2000). Although the same pathogen also causes root rot, which has been shown to be affected by soil propagule levels of *Phytophthora* and higher root damage is observed when propagule levels are high in the soil. Infection on rootstock and scion are independent of each other as the two are made of different cultivar type and have different susceptibility to *Phytophthora*.

The high number of *Phytophthora* propagules in the soil can cause severe damage to fibrous roots, and the HLB-causing bacterium *C*Las can also cause pre-symptomatic fibrous root damage in the affected trees (Graham et al. 2013). Despite high counts of *Phytophthora* propagules in the rhizosphere soil of *C*Las positive trees, root densities did not significantly vary between *C*Las positive and negative trees in either year. Nevertheless, grapefruit trees affected only by foot rot disease measured significantly lower root density, fibrous root density, and root length density compared to trees without foot rot in 2016. Foot rot or gummosis lesions do not usually produce inoculum for subsequent infections and do not have epidemiological significance (Timmer et al. 2000). In this study, it was found that foot rot affected trees have lower root densities. It necessitates further testing on larger number of trees samples **(Table** 17). One possible reason for lower densities could be impaired translocation of photosynthates to root tissue in such trees.

It is known that foot rot and gummosis causes partial girdling of the trunk (Timmer et al. 2000) which may inhibit transport of photosynthates to the roots and interfere with the translocation of water and nutrients to the above-ground tissues, thereby affecting both root and shoot systems. In the above ground manifestation of the disease, where it is severe, leaf chlorosis and canopy thinning are observed (Graham et al. 2014). Results of the root density measurements in this study did not register an interaction between *C*Las and foot rot. Nevertheless, based on the root density means, *C*Las positive trees show some increase in the fibrous root density where as in 2016 observation foot rot affected trees showed significant lower FRD. This can be stated since, in both years, *C*Las negative trees with foot rot disease reported the lowest FRD means and *C*Las positive without foot rot disease reported the highest means for FRD. These results compare to a greenhouse trial on

artificial inoculation of *C*Las on citrus Swingle citrumelo showed an increase in fibrous root biomass whereas *P. nicotianae* caused reduction of the canopy and fibrous root biomass within 5 weeks of inoculation (Wu et al. 2017). Higher number of *Phytophthora* propagules in the rhizosphere of *C*Las positive trees is not clearly understood. Based on the results of this study, it can be proposed that temporary increase in fibrous roots in these trees may favor higher propagule densities. *C*Las is known to pre-disposes trees to *Phytophthora* related fibrous root damage (Graham et al. 2013; Johnson et al. 2014). Effect of foot rot was observed for overall reduction in the root biomass and not just fine fibrous roots (< 2mm). Significant effect of foot rot effect on root density was only observed in 2016.

During 2016 visual observation rating of the canopy of *C*Las positive and negative trees with or without foot rot was done. Leaf area index (LAI) was measured to support the visual rating of canopy density, as it is a good morphometric indication of the amount of leaf cover of the trees. Canopy ratings of *C*Las positive trees with or without foot rot disease showed a significant difference. *C*Las positive trees with foot rot showed the most canopy decline, in comparison to trees affected with only *C*Las or foot rot or no disease. LAI was not significantly different between the various tree categories, but *C*Las positive trees with foot rot disease had a lower LAI. There is a limitation of my study as I have observed these trees only for the 2-year period and the sample size was smaller in 2016 with 6 to 8 trees in each category of tree observed. The other important limitation was the studied trees were not tested for other possible pathogens.

As it was a field study, the presence of other factors could not be ruled out. Nevertheless some critical information about canopy thinning was observed in trees when

both foot rot and *C*Las is present. From the results of this study it can be suggested that canopy decline rating with leaf area index measurements can serve as a useful tool to monitor *C*Las affected trees when evaluating the disease progression under field conditions. Previous studies have shown that *C*Las infected trees have an altered nutritional profile (Koen and Langenegger 1970; Nwugo et al. 2013; Masaoka et al. 2011). Altered nutrient profile indicates physiological metabolic imbalances caused by pathogen and are sometime related with the disease symptoms (Srivastava 2013). In *C*Las positive trees the altered nutrient could be due to the phloem blockage due to callose formation which perhaps severely affect the translocation of nutrients and carbohydrates to the sink tissue (Boava et al. 2017; Achor et al. 2010). Negative feedback can lower the rate of photosynthesis due to the accumulation of starch, glucose, and sucrose.

Translocation of nutrients will also be affected due to severe foot rot in the trunk of the tree. I observed a reduction of more than one macro and micronutrients in the symptomatic leaves caused due to *C*Las including P, Mg, S, and Cu in 2016 except N and Na which measured higher (Table 16). Both *C*Las and foot rot affected Na and S. Both diseases caused an increase in sodium and a decrease in sulfur and copper in the symptomatic leaves. The main effects of *C*Las were most significant for Mg as both were significantly lower in *C*Las positive trees. Reduction of several nutrients in the *C*Las positive trees are well documented (Nwugo et al. 2013), but nutritional changes about foot rot have not been studied yet. This is important, as it was observed that grapefruit trees affected severely by foot rot disease and *C*Las showed a different nutrient profile in comparison to trees that were only affected by *C*Las or foot rot. Despite the changes brought by *C*Las or foot rot disease or both in the nutrient status of grapefruit trees, most nutrients in the symptomatic

leaves were within the normal range as mentioned in the literature (Obreza and Morgan 2008). For potassium, though it was not the case, the normal standards found in the leaves of the healthy citrus tree should be in the range of 1.2–1.7% (Obreza and Morgan 2008).

Although not significant, both *C*Las positive and foot rot affected trees had lower potassium compared to healthy control. Potassium is linked with host susceptibility to the pathogen as potassium play a key role in metabolism. Results from this study differ from earlier reports where an increase in potassium was observed in the symptomatic leaves (Spann et al. 2011). Reduction of sulfur and magnesium is reported by the earlier researcher in *C*Las positive trees (Spann et al. 2011). Magnesium is a component of chlorophyll and plays important role in photosynthesis and beside another important role it is required for P uptake and transport. Sulfur is a structural component of amino acid, protein, and chlorophyll (Srivastava 2013).

In my study, a significant interaction between *C*Las and foot rot was found on the percent of S in the symptomatic leaf tissue. Presence of both *C*Las and foot rot reduced Sulfur. Additionally, both significant interaction and primary effect of *C*Las was observed in the reduction of Mg in *C*Las positive compared to negative trees. Chlorophyll chelates Mg which affects chlorophyll synthesis and is probably why symptomatic leaves have yellow patchy regions with no chlorophyll. However, since HLB management is shifting more toward root diagnosis and root health management, understanding the *C*Las and *Phytophthora* interaction has received more attention (Johnson et al. 2014; J. Wu et al. 2017). In the absence of information on threshold levels of these nutrients in the fibrous root, the inference has to be made comparing the treatments to each other and control trees without

*C*Las or foot rot. *C*Las showed a significant effect such that positive trees have significantly lower Zn, Cu, Mg, and P whereas Na, Ca, and Mn measured significantly higher in comparison to negative trees (Table 16). Foot rot effect was only observed for significantly higher Mn and lower P in the fibrous roots. No significant interaction was observed for nutrient levels in the fibrous root between *C*Las and foot rot disease in both years.

It should be noted that the nutrient test of soil in the four orchards studied measured adequate nutrients (A-7). Therefore, deficiency or excess of the nutrients in the root or leaves is possibly not attributed to the presence of their lower or higher levels in the soil. Another critical aspect of *C*Las-related changes in citrus trees is an altered carbohydrate status. Changes in the non-structural carbohydrates are attributed to up-regulation of carbohydrate synthesis genes and down-regulation of photosynthesis genes (Nwugo et al. 2013). In 2014, only starch measured high in the symptomatic leaves of *C*Las positive trees, but in 2016 all of the three types of carbohydrates measured were significantly high. Foot rot effects on starch, sucrose and glucose content of symptomatic leaves were not significantly different, nor were any interaction observed in *C*Las positive trees with foot rot disease.

A significant effect of *C*Las on carbohydrates levels confirms the effect *C*Las have on genes that regulate starch synthesis or glucose and sucrose assimilation pathways (Fan et al. 2010; Nwugo et al. 2013). Overall results from this study support earlier find on the increase in starch levels in the symptomatic leaves (Table 15). A significant increase of glucose and sucrose was also measured in the symptomatic leaves of *C*Las positive trees. A possible cause of the increase is associated with increased activity of cell wall invertase (Berger et al. 2007; Fan et al. 2010).

Starch and glucose in the fibrous roots did not measure significantly different between *C*Las positive and negative trees with or without foot rot disease. The only effect was observed in the sucrose level in the *C*Las positive trees where roots measured significantly higher sucrose compared to negative trees.

This study attempted to evaluate the root health and nutrient status of naturally infected *C*Las positive trees also affected by foot rot disease. *C*Las effect on the trees was pronounced for *Phytophthora* propagule counts in the rhizosphere, nutrition and carbohydrate status. The most significant effect of foot rot was observed for lower root densities. Results of this study could be significant for managing *C*Las positive trees as trees with both *C*Las and foot rot disease can have increased root loss and faster decline. Most importantly *Phytophthora* management in orchards should be done, as the results show higher propagule count in *C*Las positive trees rhizosphere soil. Although several limitations are found in field studies, much valuable information was obtained from this work that improves our understanding of *C*Las effect in conjunction with foot rot disease. The results obtained in this study come from field observations of the already infected perennial trees. It is possible that these trees are in the early stage of *C*Las infection.

Nevertheless, we found some critical changes regarding nutrition in symptomatic leaves in conjunction with foot rot; leaf nutrition dynamics are altered. An altered nutrient status is more like a post-mortem analysis rather than a cause as it is an effect of metabolic imbalance caused by pathogen. I found S to be consistently changed due to individual effects or interaction of *C*Las and foot rot in both years. S along with C, H, O, and N are major constituents of organic materials involved in enzymatic processes and oxidation and reduction reactions (Srivastava 2013). Future extension of this work can be extended to

studying the effect of *C*Las and foot rot on fruit yield. I propose based on the results of my study that the presence of both CLas and foot rot interact to reduce canopy and affect nutrient like S, Cu, and Mg in the leaf tissue. Independent of foot rot, *C*Las affects carbohydrate content and reduce several macro and micronutrient in the leaves and may have different metabolic effects. Foot rot, on the other hand, may have adverse effect on root densities and reduce S, and Mg and increase Na. The overall presence of both factors in the grapefruit tree has greater adverse effect compared to when presenting individually.

CHAPTER V

DISCUSSION AND FINAL CONCLUSIONS

The studies undertaken in this dissertation were driven by the need to manage *Phytophthora* diseases effectively for the commercial citrus orchards in the Lower Rio Grande Valley of South Texas. As reported in Chapter 2, I found that foot rot disease is quite common as 96% of the orchards has foot rot incidence, which is affecting trunks of the trees. Not mentioning the fact that of all the 400 trees studied in 2017, 54% reported lesions on branches and 34% of trees had a lesion on the trunk (Chapter 2, Figure 7). So even when some trees have no lesion on the trunk of the tree, branch lesion may still occur. Grapefruit proved to be more susceptible when compared to sweet orange and tangerine. Although sample size for sweet orange and tangerine was small to make a meaningful conclusion. Since almost 70% of citrus cultivars grown in LRGV consist of grapefruit, this statistic cannot be ignored and effective orchard management to reduce gummosis is very important for this region.

Orchards with micro-sprinklers had a high incidence of foot rot even in young orchards. The number of the orchards was very few with this type of irrigation, therefore no statistical comparison could be made. This can be an important point to consider, since it may not be feasible in the future to irrigate the orchards with flood irrigation due to water scarcity and partial drought-like conditions in the LRGV. Alternative water-saving irrigation will need to be explored that saves water but does not increase *Phytophthora* infection of the trunk. Right now, more than 95% of the Valley citrus is grown on sour orange rootstock (Sauls 2008). Compared to other rootstocks, sour orange performs better in highly calcareous and saline soils of the LRGV (Louzada et al. 2008). It was noted in this

study that 21% of trunk lesions originated from below the bud union (A-2), suggesting infection starting in the rootstock. This might indicate the tolerance of sour orange to *Phytophthora* may not be very satisfactory under some management and soil conditions. The results of this study are also supported by the almost ubiquitous detection of *Phytophthora* propagules in the soil of all the orchards studied. In fact, 76% reported higher than 10 propagule/ cm^3 of soil. It is clear that *Phytophthora* is very common in the soil, though its number fluctuates and that is reflected in the counts obtained in 2015 and 2017. The counts were significantly higher in 2015, which was due to high rainfall in the spring of 2015. This also confirms that more than any other factor, soil moisture is the most critical factor in increasing *Phytophthora* in the soil, and the number of *Phytophthora* propagules can increase very suddenly under suitable conditions.

Huanglongbing or HLB has become a major concern for the commercial citrus industry in Texas as it has already spread to all counties in the LRGV. Right now, the *C*Las affected trees are not showing major visual symptoms of decline though characteristic HLB symptoms, like mottled leaves and yellow shoots, are visible in some trees. The fact that so many trees are also affected by the slow-acting but the chronic presence of foot rot is a matter of concern. The objective of this dissertation was to improve understanding of *C*Las positive tree's physiological response to the disease and also to look at the response of trees affected with the dual infection of *C*Las and severe foot rot (Chapter IV).

Field observation of four grapefruit orchards in Donna, Texas presented *C*Las positive grapefruit trees with foot rot disease and results of this study can be applied to help in the decision for managing *C*Las in the region. The most important result was to find that *Phytophthora* propagules were significantly higher in *C*Las positive trees irrespective of

foot rot. It means aggressive *Phytophthora* management, and application of fungicide even as a prophylactic manner on confirmed *C*Las positive trees need to be considered. Another significant find was that foot rot had a significant effect on the fibrous root density. Athough the affected part of the plant is above ground, roots are somehow affected. It can be stated based on the results of this study, that characteristic canopy thinning observed in association with severe gummosis could be due to impaired root growth.

HLB pathogen *Candidatus* Liberibacter *asiaticus* (*C*Las) promotes root infection by *Phytophthora* by attracting zoospores (Wu et al. 2017). This can shift the disease management of HLB toward maintaining root health, and *Phytophthora* control should be of primary importance as both root rot and gummosis will affect root health. Though main effects of *C*Las and foot rot were noted for propagule count and root density respectively, no interaction between the two diseases was observed that affected the fine roots.

Interaction of the two diseases was observed in altering macronutrients and micronutrients in the symptomatic leaves and fibrous roots. *C*Las positive trees measured significantly lower potassium, phosphorus, magnesium and copper and higher nitrogen and sodium in the symptomatic leaves when compared to *C*Las negative trees (Table 16). Potassium was at a deficient level in *C*Las positive trees. A significant interaction of the two diseases was seen for boron and Sulfur levels in the symptomatic leaves of *C*Las positive trees. Both diseases lowered Sulfur whereas *C*Las infection caused an increase and foot rot disease led to decreased boron in the symptomatic leaves.

Root nutrient status was significantly affected by *C*Las infection or foot rot. Magnesium and sodium were lower, and calcium and boron measured higher in *C*Las positive trees. Micronutrients, zinc, copper, and manganese were measured lower due to *C*Las or

foot rot independent of each other. The one common broad statement that can be made is more than one micronutrient are lowered in leaves and fibrous roots due to *C*Las or foot rot acting independently of each other. This imbalance is perhaps the physiological effect that each has on the tree, with *C*Las affecting the photosynthetic machinery by causing accumulation of the non-structural carbohydrates. On the other hand, foot rot may be interfering with the effective translocation by damaging the cambial and cortical tissues in the trunk and causing nutritional imbalance. Nevertheless, the culmination of the two diseases results in alteration of the nutrient status of the tree concerning trees not affected with either *C*Las infection or foot rot disease or both.

The visual rating of canopy thinning was significantly higher in the *C*Las positive trees with foot rot disease which was also supported by leaf area index values. Ratings for canopy thinning were negatively correlated with leaf area index. Canopy thinning could be due to root loss of foot rot positive trees paired with nutrient and photosynthesis alteration due to *C*Las and foot rot disease. The critical point is that canopy of trees affected only with *C*Las or foot rot was not significantly different from the healthy control, but in conjunction with infection by both diseases, the canopy thinning is significant. An interaction between the two diseases for this factor was significant ($P<0.05$).

The principal question to improve our understanding of the *Phytophthora* diseases in the commercial citrus orchards was: which prevalent species of *Phytophthor*a is the causative agent? Although *P.citrophthora's* presence in the soil was confirmed with molecular technique, the pathogen has not been isolated and studied (RoyChowdhury 2016). I tried to address this knowledge gap in trying to isolate and culture other *Phytophthora* spe-

cies from soil and plant tissue and study biological characteristics. My investigation addressed this question by looking at a fairly large number of isolates (93) covering 30 orchards from all the citrus-growing counties in South Texas. Eighty-nine out of the 93 isolates were confirmed to be *P. nicotianae* through cultural and molecular confirmation. Four isolates were found to be *Pythium* species. My study was not able to isolate and identify *P. citrophthora* in the orchards. Significant find in this study was to confirm the presence of both the mating types in the same orchards. The occurrence of both mating type in the same orchard can lead to the formation of highly virulent strains of *P. nicotianae* as sexual reproduction will be more frequent in a heterothallic species like *P. nicotianae* (Erwin and Ribeiro 1996).

Mefenoxam resistant isolates were not found at least from the soil of commercial orchards of LRGV, but one such isolate was detected from a citrus rhizosphere from Corpus Christi. Cultural characteristics of this isolate were also different as the rate of growth, and asexual structures length and breadth of sporangia and diameter of chlamydospore were significantly different from all other isolates. This suggests it might have been introduced through plant or soil material movement from out of the state.

Characterization of 34 isolates of *P. nicotianae* indicates biological diversity, but genetic diversity was not confirmed through Phylogenetic analysis using the ITS region of the genome. Nevertheless, virulence tests on non-citrus hosts gave some impressive results. Most were highly virulent on tomato, squash, and Lupin. Whereas bean and tobacco were quite resistant to the majority of isolates and hardly any mortality occurred in these two hosts. The reports from my study suggest that *P.nicotianae* from citrus orchards do not

have a high degree of host specialization as I tested them to be pathogenic on more than one non-citrus hosts.

Greenhouse pathogenicity tests of *P. nicotianae* isolates produced only mild lesions on roots and no mortality in the inoculated sour orange seedlings (Chapter III). Virulence results on grapefruit were different. All of the 9 isolates tested caused lesions on grapefruit stem, but some isolates were more pathogenic compared to others based on lesion size formed. We cannot compare these two studies, as sour orange intact seedling roots were subjected to zoospore inoculum and in case of grapefruit, an injury was made on the stem to put an inoculum plug. An open wound is easy access to pathogen entry and will cause infection even in a resistant host. Although it can be concluded that not all isolates showed the same level of pathogenicity toward grapefruit stems tested. I find this could be important as depending on the isolate virulence type, different level of damage to the grapefruit host is expected with the same propagule load in the rhizosphere. So not only soil moisture, rootstock and scion type but also isolates virulence will be a factor for causing defense.

Based on the results of this study, some recommendations can be made for the effective management of *Phytophthora* diseases and more importantly HLB. Regular evaluation of *Phytophthora* propagules in the soil, especially in orchards with poor drainage of water and high incidence of foot rot disease is recommended. This is extremely important for orchards having *C*Las positive trees. Mefenoxam may be used in orchards with high counts of *Phytophthora* and where *C*Las positive trees are detected. Foot rot disease in the orchards can be reduced if factors that create excess moisture near the tree trunk can be curbed. Since Scion is mostly sensitive, any contact of scions with the soil increase the

chance of infection on the trunk. Therefore *Phytophthora* management needs to integrate with cultural practices that decrease the risk factors of *Phytophthora* infection. Future study needs to evaluate the effect of foot rot and gummosis in conjunction with HLB on fruit yield and quality. I propose it should adversely reduce yield and affect fruit quality even in asymptomatic *C*Las positive trees affected with foot rot and gummosis. My study showed that when studying *Phytophthora* interaction with *C*Las causing HLB disease, above ground manifestation of the disease foot rot should be considered, as *Phytophthora* may infect scion independent of fibrous root and have an adverse effect on tree health in conjunction with *C*Las.

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APPENDIX

A-1 Location and orchard characteristics of surveyed commercial citrus orchards from Lower Rio Grande Valley, Texas.

No.	Orchard ID	County	Longitude	Latitude	Scion	Age (year)	Irrigation
1.	Mission-40	Hidalgo	98.2629993	26.2942297	Grapefruit	>20	Flood
2.	Mission-38	Hidalgo	98.2960622	26.3008834	Grapefruit	>20	Flood
3.	Mission-39	Hidalgo	98.2629993	26.2942297	Grapefruit	>20	Flood
4.	Mission-50	Hidalgo	98.3311197	26.2924795	Grapefruit	>20	flood
5.	McAllen-5	Hidalgo	98.25274114	26.24712985	Grapefruit	>20	flood
6.	McAllen-3	Hidalgo	98.25511176	26.24087442	Grapefruit	>20	flood
7.	McAllen-2	Hidalgo	98.18922541	26.23521871	Orange	>20	flood
8.	McAllen-48	Hidalgo	98.2162917	26.2735552	Grapefruit	>20	flood
9.	Edinburg-12a	Hidalgo	98.0861616	26.3143483	Grapefruit	>20	flood
10.	Edinburg-12b	Hidalgo	98.0861616	26.3143483	Tangerine	15-20	flood
11.	Edinburg-25	Hidalgo	98.1438308	26.3133235	Grapefruit	5-10	flood
12.	Edinburg-26	Hidalgo	98.0782686	26.315172	Tangerine	>20	flood
13.	Hargil-21	Hidalgo	98.02259886	26.44434748	Orange	15	flood
14.	Hargil-20	Hidalgo	97.98880373	26.44108463	Orange	15	drip
15.	Alamo-1	Hidalgo	98.11431351	26.2242903	Grapefruit	>20	flood
16.	San Juan-30	Hidalgo	98.14964198	26.17327358	Grapefruit	>20	flood
17.	Donna-29	Hidalgo	97.96594	26.13023	Orange	>20	flood
18.	Donna-14	Hidalgo	98.04775458	26.2348208	Grapefruit	>20	flood
19.	Weslaco- D1	Hidalgo	97.95069516	26.13640663	Grapefruit	>20	flood
20.	Weslaco-28	Hidalgo			Grapefruit	15-20	flood
21.	La Feria -44	Cameron	97.82468129	26.18569346	Grapefruit	>20	flood
22.	La Feria -42	Cameron	97.8141632	26.20277786	Grapefruit	>20	flood
23.	Combes city-52	Cameron	97.74152039	26.24527574	Grapefruit	>20	drip
24.	Combes city-53	Cameron	97.71035939	26.25028197	Grapefruit	>20	flood
25.	Los Fresnos-51	Cameron	97.522651	26.0877203	Orange	>20	flood
26.	Harlingen-45	Cameron	97.78882	26.24798	Grapefruit	>20	flood
27.	Harlinge-46	Cameron			Grapefruit	>20	flood
28.	Raymondville-17	Willacy	97.91507184	26.44663027	Grapefruit	6	sprinkler
29.	Raymondville-18	Willacy	97.91839615	26.44396458	Grapefruit	6	drip
30.	San Benito -55	Cameron	97.5736083	26.0794448	Grapefruit	15-20	flood

A-2 Occurrence of branch rot, foot rot and sources of each recorded in the 400 citrus trees surveyed in 20 orchards during spring 2017**.** [a]Rootstock is part of the tree from ground to bud union, [b]Part of the tree starting above bud union to main branch and c is part of the tree referring to all branches and secondary branches.

No.	Orchard ID	Per-cent branch rot	Percent Foot rot and gummo-sis inci-dence	Percent foot rot from root-stock[a]	Percent foot rot and gum-mosis from trunk[b]	Per-cent branch rot from trunk[b]	Percent branch rot from branch[c]
1.	Mission#40	70	35	5	30	0	70
2.	Mission#38	35	45	15	15	15	15
3.	Mission#39	30	25	0	25	0	30
4.	McAllen#5	40	25	10	15	5	35
5.	McAllen#3	90	15	5	10	0	90
6.	McAllen#48	40	35	15	15	0	40
7.	Edinburg12a	50	55	20	35	10	40
8.	Edinburg12b	55	45	15	30	0	55
9.	Edinburg25	20	15	0	15	5	15
10.	Edinburg26	75	45	0	45	5	70
11.	Alamo#1	75	45	0	45	5	70
12.	San Juan#30	80	15	5	10	15	65
13.	Donna#29	85	30	0	30	5	80
14.	Donna#14	60	15	5	10	5	55
15.	Weslaco D1	65	40	0	40	5	60
16.	La Feria #44	80	35	20	15	20	60
17.	La Feria #42	10	10	0	10	0	10
18.	Combes city#53	55	70	30	40	25	25
19.	Harlingen#45	30	10	0	10	0	30
20.	Harlingen#46	42	85	70	0	70	40

A- 3 Average temperature and precipitation data for year 2015 and 2017 of three cities representative of upper*, middle** and lower valley*** of Lower Rio Grande Valley of Texas.

City	**Year**	**Average Temperature** (°F) **& Precipitation** (inch)	**Jan**	**Feb**	**March**	**April**	**May**	**June**
McAllen*	2015	Temperature Precipitation	52.05 2.99	58.35 1.08	63.05 2.97	71.3 5.08	79.3 4.61	90 5.5
McAllen	2017	Temperature Precipitation	61 1.06	64.5 1.1	71 1.0	76.5 1.34	82 2.2	86 2.6
Weslaco**	2015	Temperature Precipitation	58.3 0.38	53.6 1.33	60.5 3.2	73.5 0.96	72 2.3	81 1.8
Weslaco	2017	Temperature Precipitation	60 1.59	63 0.75	69 1.37	75 0	80.5 1.78	84.5 2.52
Brownsville***	2015	Temperature Precipitation	55.7 3.56	61.5 0.76	66.6 4.74	77.1 1.73	81.1 9.72	83.9 0.76
Brownsville	2017	Temperature Precipitation	66.3 0.18	73.2 1.36	74.2 1.84	78.6 0.63	82.15 1.85	86.2 0.0

A- 4 Site of commercial citrus orchards in the Lower Rio Grande Valley (LRGV), location and number of *Phytophthora* isolates selected from each site. Sites with asterisks, are cities located outside the LRGV region located along the Gulf of Mexico of South Texas.

No.	Sites name	Longitude	Lattitude	# of Isolate selected
1	Mission-40	98.2629993	26.2942297	2
2	McAllen-5	98.25274114	26.24712985	8
3	McAllen-3	98.25511176	26.24087442	2
4	McAllen-2	98.18922541	26.23521871	2
5	McAllen-48	98.2162917	26.2735552	2
6	Edinburg-12a	97.5736083	26.0794448	2
7	Edinburg-12b	98.0861616	26.3143483	1
8	Edinburg-25	98.1438308	26.3133235	1
9	Edinburg-26	98.0782686	26.315172	3
10	Hargil-20	97.98880373	26.44108463	3
11	Alamo-1	98.11431351	26.2242903	2
12	San Juan-30	98.14964198	26.17327358	4
13	Donna-29	97.96594	26.13023	5
14	Donna-14	98.04775458	26.2348208	3
15	Donna 131/2			1
16	Weslaco- D1	97.95069516	26.13640663	2
17	Weslaco-28	97.96594	26.13023	3
18	La Feria-44	97.82468129	26.18569346	2
19	La Feria-42	97.8141632	26.20277786	5
20	Combes-52	97.74152039	26.24527574	6

A- 4 continued

No.	Sites name	Longitude	Lattitude	# of Isolate selected
21	Combes-53	97.71035939	26.25028197	4
22	Los Fresnos-51	97.522651	26.0877203	3
23	Harlingen-45	97.78882	26.24798	3
24	Harlingen-46	97.77255	26.14057	2
25	San Benito -55	97.5736083	26.0794448	6
26	Corpus Christi*	97.23284	29.599937	2
27	Rockport*	97.05692	28.03376	4
28	Orange grove*	93.76297	30.126901	2
29	Galveston*			2
30	Palacios *	96.21044	28.71993	1
31	Alvin#8*	95.28304	29.442495	1

A- 5 Collection data and summary information about the *Phytophthora* sp. isolates examined in this study. Isolates with ** were chosen only for biological characterization and with * were chosen for both characterization and pathogenicity test.

Isolate ID	source	Host	City	Acc. No.	Gene bank Acc. No.	Homology	Identity (%)
PhH1*	Soil	Citrus	Harlingen	KR827692.1	MH290367	*Phytophthora parasitica*	99%
PhLF2*	Soil	Citrus	La Feria	KR827692.1	MH290378	*Phytophthora parasitica*	99%
PhRp3	Soil	Citrus	Rock Port	KR827692.1	MH290385	*Phytophthora parasitica*	99%
PhD4*	Soil	Citrus	Donna	KR827692.1	MH290394	*Phytophthora parasitica*	100%
PhC5*	Soil	Citrus	Combes	KX650279.1	MH341612	*Phytophthora nicotianae*	97%
PhD6*	Soil	Citrus	Donna	KR827692.1	MH290445	*Phytophthora parasitica*	99%
PhSB7	Soil	Citrus	San Benito	KR827692.1	MH290419	*Phytophthora parasitica*	99%
PhE8	Roots	Citrus	Edinburg	KJ755109.1	MH341613	*Phytophthora nicotianae*	97%
PhA9	Soil	Citrus	Alamo	KR827692.2	MH290438	*Phytophthora parasitica*	99%
PhLF10	Roots	Citrus	Los Fresnos	KR827692.1	MH290368	*Phytophthora parasitica*	99%
PhW11	Soil	Citrus	Weslaco	KR827692.1	MH290369	*Phytophthora parasitica*	99%
PhMC12	Soil	Citrus	McAllen	KR827692.1	MH290370	*Phytophthora parasitica*	99%
PhW13*	Roots	Citrus	Weslaco	KR827692.1	MH290371	*Phytophthora parasitica*	100%
PhSB14*	Soil	Citrus	San Benito	KR827692.1	MH290372	*Phytophthora parasitica*	99%

A- 5 continued

Isolate ID	source	Host	City	Acc. No.	Gene bank Acc. No.	Homology	Identity (%)
PhC15**	Soil	Citrus	Combes	KR827692.1	MH290373	*Phytophthora parasitica*	99%
PhW16	Roots	Citrus	Weslaco	KR827692.1	MH290374	*Phytophthora parasitica*	100%
PhD17	Roots	Citrus	Donna	KR827692.1	MH290375	*Phytophthora parasitica*	100%
PhC18	Soil	Citrus	Combes	KR827692.1	MH290376	*Phytophthora parasitica*	100%
PhMC19	Soil	Citrus	McAllen	KR827692.1	MH290377	*Phytophthora parasitica*	100%
PhO20**	Soil	Citrus	Orange Grove	KR827692.1	MH290379	*Phytophthora parasitica*	100%
PhA-21*	Soil	Citrus	Alamo	KJ506201.1	MH341614	*Phytophthora nicotianae*	97%
PhMC22	Soil	Citrus	McAllen	KR827692.1	MH290380	*Phytophthora parasitica*	99%
PhSJ23*	Soil	Citrus	San Juan	KR827692.1	MH290381	*Phytophthora parasitica*	99%
PhRP24*	Soil	Citrus	Rock Port	KR827692.1	MH290382	*Phytophthora parasitica*	99%
PhLF25*	Soil	Citrus	La Feria	EU660838.1	MH341615	*Phytophthora nicotianae*	99%
PhMC26*	Soil	Citrus	McAllen	KR827692.1	MH290383	*Phytophthora parasitica*	100%
PhSB27	Roots	Citrus	San Benito	KR827692.1	MH341616	*Phytophthora parasitica*	100%
PhMC28*	Soil	Citrus	McAllen	KR827692.1	MH290384	*Phytophthora parasitica*	99%
PhE30	Roots	Citrus	Edinburg	KR827692.1	MH290386	*Phytophthora parasitica*	100%

A- 5 continued

Isolate ID	source	Host	City	Acc. No.	Gene bank Acc. No.	Homology	Identity (%)
PhE32*	Roots	Citrus	Edinburg	KJ755109.1	MH341617	*Phytophthora nicotianae*	97%
PhCC33	Soil	Citrus	Corpus Christi	KR827692.1	MH290387	*Phytophthora parasitica*	99%
PhC34	Roots	Citrus	Combes	KR827692.1	MH290388	*Phytophthora parasitica*	100%
PhLF35	Soil	Citrus	La Feria	KR827692.1	MH290389	*Phytophthora parasitica*	99%
PhMC36	Soil	Citrus	McAllen	KR827692.1	MH290390	*Phytophthora parasitica*	99%
PhD37	Soil	Citrus	Donna	KR827692.1	MH290391	*Phytophthora parasitica*	99%
PhD38	Soil	Citrus	Donna	KR827692.1	MH290392	*Phytophthora parasitica*	100%
PhH39	Roots	Citrus	Harlingen	KR827692.1	MH290393	*Phytophthora parasitica*	99%
PhSB40	Soil	Citrus	San Benito	KR827692.1	MH290395	*Phytophthora parasitica*	99%
PhE41*	Soil	Citrus	Edinburg	KJ494913.1	MH341618	*Phytophthora nicotianae*	98%
PhHa43	Soil	Citrus	Hargil	KR827692.1	MH290396	*Phytophthora parasitica*	99%
PhO44*	Soil	Citrus	Orange Grove	KR827692.1	MH290397	*Phytophthora parasitica*	99%
PhC45	Roots	Citrus	Combes	KR827692.1	MH290399	*Phytophthora parasitica*	99%
PhSB46	Roots	Citrus	San Benito	KR827692.1	MH290400	*Phytophthora parasitica*	99%
PhMC47	Soil	Citrus	McAllen	KR827692.1	MH290401	*Phytophthora parasitica*	100%
PhE48*	Roots	Citrus	Edinburg	KR827692.1	MH290402	*Phytophthora parasitica*	99%

A- 5 continued

Isolate ID	source	Host	City	Acc. No.	Gene bank Acc. No.	Homology	Identity (%)
PhMC49	Soil	Citrus	McAllen	KR827692.1	MH290403	*Phytophthora parasitica*	99%
PhPA50	Soil	Citrus	Palacios	KR827692.1	MH290444	*Phytophthora parasitica*	99%
PhC51	Roots	Citrus	Combes	KR827692.1	MH290405	*Phytophthora parasitica*	99%
PhE52*	Roots	Citrus	Edinuburg	KR827692.1	MH290406	*Phytophthora parasitica*	99%
PhD53**	Soil	Citrus	Donna	KR827692.1	MH290407	*Phytophthora parasitica*	99%
PhD54*	Soil	Citrus	Donna	KR827692.1	MH290408	*Phytophthora parasitica*	99%
PhLF55	Soil	Citrus	La Feria	KJ754387.1	MH341619	*Phytophthora nicotianae*	98%
PhMC56*	Soil	Citrus	McAllen	KR827692.1	MH290409	*Phytophthora parasitica*	99%
PhSF57	Soil	Citrus	Weslaco	KR827692.1	MH290410	*Phytophthora parasitica*	99%
PhMC58	Soil	Citrus	McAllen	KR827692.1	MH290411	*Phytophthora parasitica*	99%
PhMC59**	Soil	Citrus	McAllen	KR827692.1	MH290412	*Phytophthora parasitica*	99%
PhLF60*	Soil	Citrus	Los Fresnos	KR827692.1	MH290413	*Phytophthora parasitica*	99%
PhLF61	Soil	Citrus	La Feria	KR827692.1	MH290414	*Phytophthora parasitica*	99%
PhH62**	Soil	Citrus	Harlingen	KR827692.1	MH290415	*Phytophthora parasitica*	99%
PhCC63*	Soil	Citrus	Corpus Christi	KR827692.1	MH290416	*Phytophthora parasitica*	99%

A-5 continued

Isolate ID	source	Host	City	Acc. No.	Gene bank Acc. No.	Homology	Identity (%)
PhC64	Roots	Citrus	Combes	KR827692.1	MH290417	*Phytophthora parasitica*	99%
PhC65*	Roots	Citrus	Combes	GU259311.1	MH341620	*Phytophthora nicotianae*	97%
PhH66*	Roots	Citrus	Harlingen	GU259311.1		*Phytophthora nicotianae*	97%
PhSJ68	Soil	Citrus	San Juan	KR827692.1	MH341621	*Phytophthora parasitica*	99%
PhSJ69	Soil	Citrus	San Juan	KR827692.1	MH341622	*Phytophthora parasitica*	99%
PhLF70	Soil	Citrus	La Feria	KR827692.1	MH290420	*Phytophthora parasitica*	99%
PhC71	Roots	Citrus	Combes	KR827692.1	MH290421	*Phytophthora parasitica*	100%
PhMC72*	Soil	Citrus	Mc Allen	KR827692.1	MH290422	*Phytophthora parasitica*	99%
PhH73	Soil	Citrus	Harlingen	KR827692.1	MH290423	*Phytophthora parasitica*	99%
PhRP74*	Soil	Citrus	Rockport	KR827692.1	MH290424	*Phytophthora parasitica*	99%
PhLF75*	Soil	Citrus	La Feria	KR827692.1	MH290425	*Phytophthora parasitica*	100%
PhRP76	Soil	Citrus	Rockport	KR827692.1	MH290426	*Phytophthora parasitica*	99%
PhD77	Soil	Citrus	Donna	KR827692.1	MH290427	*Phytophthora parasitica*	100%
PhLF79	Soil	Citrus	La Feria	KR827692.1	MH290428	*Phytophthora parasitica*	99%
PhRP80	Soil	Citrus	Rockport	KR827692.1	MH290429	*Phytophthora parasitica*	100%
PhMI81*	Soil	Citrus	Mission	KR827692.1	MH290430	*Phytophthora parasitica*	99%

A-5 continued

Isolate ID	source	Host	City	Acc. No.	Gene bank Acc. No.	Homology	Identity (%)
PhE82	Soil	Citrus	Edinburg	KR827692.1	MH290431	*Phytophthora parasitica*	99%
PhC83	Roots	Citrus	Combes	KR827692.1	MH290432	*Phytophthora parasitica*	99%
PhH84	Soil	Citrus	Harlingen	KR827692.1	MH290433	*Phytophthora parasitica*	100%
PhH85	Soil	Citrus	Harlingen	KR827692.1	MH290434	*Phytophthora parasitica*	99%
PhG86	Soil	Citrus	Galveston	KR827692.1	MH290435	*Phytophthora parasitica*	100%
PhMI87	Soil	Citrus	Mission	KJ494913.1	MH290436	*Phytophthora nicotianae*	97%
PhMC88	Soil	Citrus	Combes	KR827692.1	MH290437	*Phytophthora parasitica*	100%
PhLF89	Soil	Citrus	La Feria	KF147901.1	MH290446	*Phytophthora nicotianae*	97%
PhG90	Soil	Citrus	Galveston	KR827692.1	MH290439	*Phytophthora parasitica*	99%
PhW91	Soil	Citrus	Weslaco	KR827692.1	MH290440	*Phytophthora parasitica*	100%
PhHa92	Soil	Citrus	Hargil	KR827692.1	MH290441	*Phytophthora parasitica*	99%
Ph893	Soil	Citrus	Weslaco	KR827692.1	MH290442	*Phytophthora parasitica*	100%
PhSB94*	Soil	Citrus	San Benito	KJ494913.1	MH290443	*Phytophthora nicotianae*	99%
PyE1	Soil	Citrus	Edinburg	KU208728.1	MH341608	*Pythium nodosum*	99%
PyE3	Soil	Citrus	Edinburg	HQ643916.1	MH341609	*Pythium ultimum*	100%
PyE4	Soil	Citrus	Edinburg	HQ643916.1	MH341610	*Pythium ultimum*	99%
PyMI5	Soil	Citrus	Mission	GU133594.1	MH341611	*Phytophythium vexans*	92%

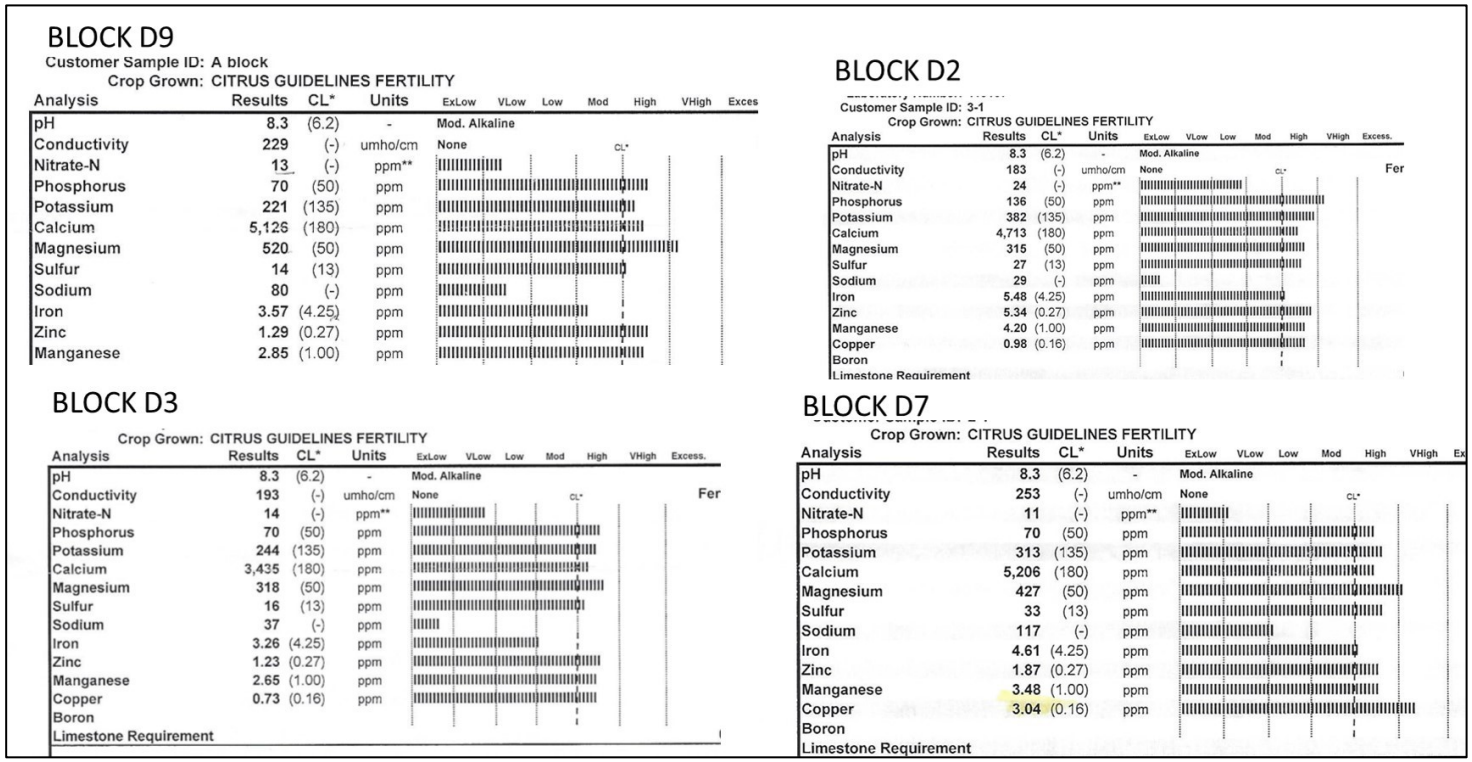
A- 6 Summary of pathogenicity of *Phytophthora nicotianae* isolates obtained from citrus host tested on non-citrus plants. Based on percent mortality and percent of plant segment give rise to *P. nicotianae* colonies, isolates were rated as, +++ highly pathogenic (>90% lesion, mortality and positive subculture), ++ moderately pathogenic (50 to 90% lesion, mortality and infected plant segment), + weakly pathogenic 1 to 50% lesion, mortality and same percent of infected plant segment), -- not tested and negative means not pathogenic. Part of the plant infected is recorded as part where lesion is observed and from where *Phytophthora nicotianae* was isolated after plating on selective medium PARPH-CMA.

No.	Isolate ID	Orchard site	Lupin	Tomato	Tobacco	Bean	Squash
1.	PhH1	Harlingen	----	----	----	negative	root, stem++
2.	PhLF2	La Feria	stem,root,leaf +++	Negative	negative	negative	stem,root++
3.	PhD4	Donna	stem,root,leaf +++	stem+++	----	root+	Stem,root +
4.	PhC5	Combes	----	----	----	negative	root, stem+++
5.	PhD6	Donna	root,+++	stem,root+++	leaf+	negative	Stem,root +
6.	PhW13	Weslaco	stem,root,leaf +++	stem,root++	leaf+	negative	root, stem++
7.	PhSB14	San Benito	stem,root,leaf +++	stem,root,leaf+++	----	negative	root, stem+++
8.	PhA21	Alamo	stem,root,leaf +++	stem,root+++	negative	stem,root+	root, stem+++
9.	PhSJ23	San Juan	stem,root,leaf +++	stem,root+++	leaf+	negative	root, stem+++
10.	PhRP24	Rockport	root, stem++	root,stem,leaf+	negative	negative	root, stem++
11	PhLF25	La Feria	stem,root,leaf +++	root,stem+++	stem,root+++	stem,root+	root, stem++
12.	PhMC26	McAllen	----	----	----	negative	root, stem+
13.	PhMC28	McAllen	stem,root,leaf +++	stem,root,leaf+++	negative	negative	root, stem+
14.	PhE32	Edinburg	Negative	Negative	negative	negative	Negative
15.	PhE41	Edinburg	----	----	----	negative	Stem, root +
16.	PhO44	Orange Grove	stem,root,leaf +++	stem,root,leaf +++	negative	stem,root+	Stem,root +
17.	PhE52	Edinburg	----	stem,root,leaf +++	----	----	----
18.	PhD54	Donna	----	----	----	stem,root+	root, stem++
19.	PhMC56	McAllen	stem,root,leaf +++	stem,root+++	leaf,stem+	negative	Negative
20.	PhLF60	La Feria	----	stem,root++	----	negative	Negative
21.	PhCC63	Corpus Christi	stem,root +	stem+	negative	negative	----

A-6 continued

No.	Isolate ID	Orchard site	Lupin	Tomato	Tobacco	Bean	Squash
22.	PhC65	Combes	stem,root,leaf +++	stem,root,leaf +++	----	root+	root, stem+++
23.	PhH66	Harlingen	stem,root,leaf +++	stem,root,leaf +++	----	----	root, stem++
24.	PhMC72	McAllen	stem,root,leaf +++	stem,root,leaf +++	----	stem+	root, stem++
25.	PhRP74	Rockport	----	----	----	----	root, stem++
26.	PhLF75	La Feria	stem,root,leaf +++	Negative	----	stem+	root, stem+
27.	PhMI81	Mission	stem,root,leaf +++	----	----	----	root, stem++
28.	PhSB94	San Benito	----	stem,root+++	----	----	----
29.	PhE48	Edinburg	----	----	----	negative	stem, roots++

A-7 Soil analysis report of the grapefruit orchards D9, D2, D7 and D3, located at Donna, Texas evaluated for CLas positive trees with and without foot rot disease.



BLOCK D9

Customer Sample ID: A block
Crop Grown: CITRUS GUIDELINES FERTILITY

Analysis	Results	CL*	Units	ExLow VLow Low Mod High VHigh Exces
pH	8.3	(6.2)	-	Mod. Alkaline
Conductivity	229	(-)	umho/cm	None
Nitrate-N	13	(-)	ppm**	
Phosphorus	70	(50)	ppm	
Potassium	221	(135)	ppm	
Calcium	5,126	(180)	ppm	
Magnesium	520	(50)	ppm	
Sulfur	14	(13)	ppm	
Sodium	80	(-)	ppm	
Iron	3.57	(4.25)	ppm	
Zinc	1.29	(0.27)	ppm	
Manganese	2.85	(1.00)	ppm	

BLOCK D2

Customer Sample ID: 3-1
Crop Grown: CITRUS GUIDELINES FERTILITY

Analysis	Results	CL*	Units	ExLow VLow Low Mod High VHigh Excess.
pH	8.3	(6.2)	-	Mod. Alkaline
Conductivity	183	(-)	umho/cm	None
Nitrate-N	24	(-)	ppm**	
Phosphorus	136	(50)	ppm	
Potassium	382	(135)	ppm	
Calcium	4,713	(180)	ppm	
Magnesium	315	(50)	ppm	
Sulfur	27	(13)	ppm	
Sodium	29	(-)	ppm	
Iron	5.48	(4.25)	ppm	
Zinc	5.34	(0.27)	ppm	
Manganese	4.20	(1.00)	ppm	
Copper	0.98	(0.16)	ppm	
Boron				
Limestone Requirement				

BLOCK D3

Crop Grown: CITRUS GUIDELINES FERTILITY

Analysis	Results	CL*	Units	ExLow VLow Low Mod High VHigh Excess.
pH	8.3	(6.2)	-	Mod. Alkaline
Conductivity	193	(-)	umho/cm	None
Nitrate-N	14	(-)	ppm**	
Phosphorus	70	(50)	ppm	
Potassium	244	(135)	ppm	
Calcium	3,435	(180)	ppm	
Magnesium	318	(50)	ppm	
Sulfur	16	(13)	ppm	
Sodium	37	(-)	ppm	
Iron	3.26	(4.25)	ppm	
Zinc	1.23	(0.27)	ppm	
Manganese	2.65	(1.00)	ppm	
Copper	0.73	(0.16)	ppm	
Boron				
Limestone Requirement				

BLOCK D7

Crop Grown: CITRUS GUIDELINES FERTILITY

Analysis	Results	CL*	Units	ExLow VLow Low Mod High VHigh Ex
pH	8.3	(6.2)	-	Mod. Alkaline
Conductivity	253	(-)	umho/cm	None
Nitrate-N	11	(-)	ppm**	
Phosphorus	70	(50)	ppm	
Potassium	313	(135)	ppm	
Calcium	5,206	(180)	ppm	
Magnesium	427	(50)	ppm	
Sulfur	33	(13)	ppm	
Sodium	117	(-)	ppm	
Iron	4.61	(4.25)	ppm	
Zinc	1.87	(0.27)	ppm	
Manganese	3.48	(1.00)	ppm	
Copper	3.04	(0.16)	ppm	
Boron				
Limestone Requirement				

A- 8 Summary of trees location, qPCR result for the test of *Candidatus* Liberibacter asciaticus and foot rot rating of grapefruit trees evaluated in 2014 and 2016. Symbol "nd" means not detected.

No.	BLOCK	Tree ID	Tree status	Latitude	Longitude	2014 HLB test and ct values	2016 HLB test and ct values	Foot rot rating 2014	Foot rot rating 2016
1	D9(A)	NER1T5	CLas positive, foot rot present	26.145711	98.025183	positive	28.15	3	3
2	D9(A)	SWR13T1	CLas negative, foot rot present	26.144832	98.025885	Nd	34	2	2
3	D9(A)	NWR7T4	CLas negative, foot rot present	26.145565	98.025718	Nd	Removed	4	-
4	D9(A)	NE R9 T32	CLas negative, foot rot present	26.249199	98.049154	Nd	35	4	2
5	D9(A)	SW R1 T38	CLas positive, foot rot absent	26.24586	98.04841	22.3	Removed	0	-
6	D9(A)	SW R1 T48	CLas positive, foot rot absent	26.24586	98.04803	24.1	Removed	0	-
7	D9(A)	SE R2 T6	CLas positive, foot rot absent	26.24372	98.04146	positive	Removed	0	-
8	D9(A)	NE R2 T20	CLas negative, foot rot absent	26.145718	98.025156	nd	38	0	0
9	D9(A)	NE R2 T29	CLas negative, foot rot absent	26.25427	98.04801	nd	34	0	0
10	D9(A)	NE R1 T39	CLas negative, foot rot absent	26.24999	98.04915	nd	29	0	0
11	D2(B)	NE R1T1	CLas positive, foot rot present	26.24922	98.04545	positive	Removed	3	-
12	D2(B)	NE R1 T34	CLas positive, foot rot present	26.24923	98.04675	positive	Removed	3	-
13	D2(B)	SE R1 T3	CLas positive, foot rot present	26.24589	98.04741	positive	Removed	1	-
14	D2(B)	NW R7 T36	CLas negative, foot rot present	26.248801	98.046239	nd	Nd	4	3
15	D2(B)	NW R7 T13	CLas negative, foot rot present	26.24884	98.04706	nd	Nd	4	-
16	D2(B)	NW R6 T30	CLas negative, foot rot present	26.248859	98.046836	nd	38.34	4	-
17	D2(B)	SE R1 T26	CLas positive, foot rot absent	26.24587	98.04623	positive	Removed	0	-
18	D2(B)	SE R2 T1	CLas positive, foot rot absent	26.144548	98.024375	25.4	Removed	0	-

A-8 continued

No.	BLOCK	Tree ID	Tree status	Latitude	Longitude	2014 HLB test and ct values	2016 HLB test and ct values	Foot rot rating 2014	Foot rot rating 2016
19	D2(B)	NE R1 T11	CLas positive, foot rot absent	26.24925	98.04586	positive	Removed	0	-
20	D2(B)	NE R3 T36	CLas negative, foot rot absent	26.249113	98.046836	nd	Nd	0	0
21	D2(B)	NE R4 T34	CLas negative, foot rot absent	26.249024	98.046744	nd	Nd	0	0
22	D2(B)	NE R4 T39	CLas negative, foot rot absent	26.249052	98.046949	nd	38.3	0	0
23	D7(C)	NE R8 T1	CLas positive, foot rot present	26.24750	98.04428	25.7	31.0	2	4
24	D7(C)	NE R8 T2	CLas positive, foot rot present	26.24744	98.04428	28.4	33.1	3	3
25	D7(C)	NE R1 T35	CLas positive, foot rot present	26.144880	98.023813	24.8	33	4	5
26	D7(C)	NE R4 T18	CLas negative foot rot present	26.24636	98.04375	nd	Nd	4	2
27	D7(C)	NE R3 T9	CLas negative foot rot present	26.247181	98.043917	nd	Nd	2	0
28	D7(C)	NE R6 T15	CLas negative foot rot present	26.24587	098.04552	nd	Nd	3	3
29	D7(C)	NE R2 T12	CLas positive foot rot absent	26.24706	98.04383	24.8	27	0	0
30	D7(C)	NE R1 T11	CLas positive foot rot absent	26.24716	98.04373	28.7	33	0	0
31	D7(C)	NE R7 T4	CLas positive foot rot absent	26.24740	98.04421	25.3	34	0	0
32	D7(C)	NE R4 T17	CLas negative foot rot absent	26.246275	98.042791	nd	Nd	0	0
33	D7(C)	SE R5 T15	CLas negative foot rot absent	30.959746	75.253276	nd	Nd	0	0
34	D7(C)	NE R2 T28	CLas negative foot rot absent	26.246493	98.043924	nd	Nd	0	0
35	D9(D)	NE R2 T1	CLas positive, foot rot present	26.24754	98.04256	29.7	32	4	4
36	D9(D)	NE R4 T1	CLas positive, foot rot present	26.24749	98.04267	29.4	27.8	2	2
37	D9(D)	NE R5 T3	CLas positive, foot rot present	26.24747	98.04274	26.6	26.9	3	3
38	D9(D)	NE R7 T1	CLas negative foot rot present	26.2475	98.042846	Nd	Nd	0	1
39	D9(D)	NE R2 T40	CLas negative foot rot present	26.24616	98.04250	Nd	Dead	3	-
40	D9(D)	NW R1 T11	CLas negative foot rot present	37.13284	95.78558	Nd	Nd	4	4
41	D9(D)	NE R1 T1	CLas positive foot rot absent	26.15596	97.96299	25.6	32.9	0	2
42	D9(D)	NE R5 T1	CLas positive foot rot absent	26.24752	98.04273	27.4	33	0	0
43	D9(D)	NE R9 T4	CLas positive foot rot absent	26.24737	98.04300	26.3	34	0	0
44	D9(D)	NE R5 T22	CLas negative foot rot absent	26.24678	98.04271	Nd	Nd	0	0

A-8 continued

No.	BLOCK	Tree ID	Tree status	Latitude	Longitude	2014 HLB test and ct values	2016 HLB test and ct values	Foot rot rating 2014	Foot rot rating 2016
45	D9(D)	SE R1 T2	CLas negative foot rot absent	26.24591	98.0424	Nd	Nd	0	1
46	D9(D)	SW R4 T1	CLas negative foot rot absent	26.24599	98.04375	Nd	33	0	0

A- 9 Macronutrient (percent) of the asymptomatic leaves of CLas positive and negative grapefruit trees with and without foot rot disease measured in 2014.

	N	P	K	Ca	Mg	Na	S
CLas							
Positive	2.3[a]	0.13[a]	0.99[a]	4.4[b]	0.32[a]	0.13[a]	0.39[a]
Negative	2.3[a]	0.13[a]	0.92[a]	4.5[a]	0.32[a]	0.12[a]	0.38[a]
Foot rot							
Present	2.3[a]	0.13[a]	0.92[a]	4.3[a]	0.31[a]	0.13[a]	0.37[a]
Absent	2.3[a]	0.13[a]	0.97[a]	4.5[a]	0.31[a]	0.12[a]	0.40[a]
CLas X Foot rot							
CLas positive x foot rot	2.4[a]	0.13[a]	0.97[a]	4.1[b]	0.32[a]	0.15[a]	0.36[a]
CLas positive x no foot rot	2.4[a]	0.13[a]	1.0[a]	4.6[a]	0.32[a]	0.12[a]	0.38[a]
CLas negative x foot rot	2.3[a]	0.13[a]	0.87[a]	4.5[ab]	0.32[a]	0.12[a]	0.39[a]
CLas negative x no foot rot	2.3[a]	0.14[a]	0.95[a]	4.4[ab]	0.32[a]	0.12[a]	0.41[a]

Mean with different letter is significant at $p<0.05$.

A-10 Micronutrient (parts per million) content of the asymptomatic leaf of CLas positive and negative grapefruit trees with or without foot rot disease measured in 2014. Means in the column with different letter are significant at $P<0.05$

Percent Micronutrient	**Zn**	**Fe**	**Cu**	**Mn[z]**	**B**
CLas					
CLas positive	25.8[a]	106.7[a]	5.3[a]	46.6[a]	225.1[a]
CLas negative	28.1[a]	90.6[a]	5.2[a]	51.8[a]	213.7[a]
Foot rot					
Present	26.4[a]	99.7[a]	5.3[a]	48.7[a]	209.8[b]
Absent	26.5[a]	97.2[a]	5.3[a]	49.9[a]	226.3[a]
CLas X foot rot					
CLas positive x foot rot	25.0[a]	115.3[a]	5.3[a]	39.7[b]	216.0[a]
CLas positive x no foot rot	24.4[a]	100.9[a]	6.6[a]	52.3[a]	232.0[a]
CLas negative x foot rot	27.5[a]	87.3[a]	6.3[a]	55.0[a]	192.2[a]
CLas negative x no foot rot	29.0[a]	93.5[a]	5.4[a]	48.4[a]	224.0[a]

[Z]Significant interaction between CLas and foot rot was observed. Mean with different letter is significant at $p<0.05$.