Huanglongbing Increases Diplodia Stem End Rot in
Citrus sinensis

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Huanglongbing (HLB), one of the most devastating diseases of citrus is caused by the α-Proteobacteria Candidatus Liberibacter. Diplodia natalensis Pole-Evans is a fungal pathogen which has been known to cause a postharvest stem-end rot of citrus, the pathogen infects citrus fruit under the calyx, and the fruit decay typically occurs following harvest and is exacerbated by exposure to ethylene. In this study, we report that high incidence of Diplodia infection in HLB-symptomatic fruit. The incidence of SER in 300 ‘Hamlin’ and 300 ‘Valencia’ orange fruit, with or without ethylene treatment was determined. Two weeks following exposure to ethylene (10 ppm, four days), the incidence of SER in HLB-symptomatic fruit was as high as 66.7% (‘Hamlin’) and 58.7% (‘Valencia’); whereas for asymptomatic fruit, less than 10% of the fruit were affected by SER. Confirmation of Diplodia in calyx abscission zone was by qPCR validation of the isolates and morphology of conidia.

Huanglongbing (HLB), also known as citrus greening, is one of the most devastating diseases of citrus that has spread throughout the major citrus producing regions in Asia, Africa and the Americas, and causes great losses for the citrus industry worldwide (Gottwald, 2010). HLB is associated with Candidatus Liberibacter spp., a Gram-negative, phloem-limited bacterium (Jagoueix et al., 1994). The Asian form of the disease is currently present in the United States, and the causal pathogen, bacterium Candidatus Liberibacter asiaticus (CLas), was first confirmed in Southeast Florida in 2005, and now is prevalent in all Florida citrus-growing areas in the state. It has been reported that since 2006, HLB has cost Florida’s economy an estimated $3.63 billion in lost revenues and 6,611 jobs within five years (2006–07 to 2010–11) by reducing orange juice production (Hodges and Spreen, 2012). HLB causes dramatic symptoms in citrus. Leaf symptoms include yellowing and an asymmetrical chlorosis referred to as “blotchy mottle” (Bové, 2006). The yellowing may first appear on a single shoot or branch, and usually spreads throughout the tree over the year. Twig dieback, tree decline, and tree mortality occur several months to years after infection. Fruit symptoms include reduction in size, asymmetrical shape and a curved central core. Color development is poor and may only ‘break’ on the stem end, leaving the majority of the fruit surface green. A brown stain may be present in the fruit calyx abscission zone located at the pedicel–fruit interface (Bové, 2006). It has also been observed that HLB-diseased trees are more adversely affected by extremes of temperature and moisture than healthy trees. Consequently, symptoms of stress (e.g., excessive leaf loss and premature fruit drop) occur in HLB-diseased trees (Wang and Trivedi, 2013).

Studies have shown that HLB not only affects plant health but also manifests profound effects on the structure and composition of the bacterial community associated with citrus leaves (Sagram et al., 2009), roots (Trivedi et al., 2010) and rhizospheres (Trivedi et al., 2012). In our investigation, conducted to identify fungal species in orange juice from HLB fruit, Diplodia natalensis Fr. (synonyms: Botryodiplodia theobromae Pat., Botryosphaeria rhodina, Lasiodiplodia theobromae; hereafter referred to as “Diplodia”) was consistently present in juice from HLB symptomatic (HLB) fruit, but not in juice from healthy fruit (CLas negative). Diplodia natalensis is the causal agent of citrus stem end rot (SER), and is typically seen on fruit following harvest (Brown, 1986). The occurrence of SER is greatly enhanced by ethylene treatment (Baremore and Brown, 1985), commonly used in Florida to improve rind color in a process called “de-greening”. Symptoms of SER on citrus fruit are softening of the rind around the button followed by a brown discoloration of affected areas. Typically, decay is formed at the stem end followed by the stylar end before involving the entire fruit. This is because Diplodia spreads rapidly through the spongy central axis of the fruit, reaching the stylar end much sooner by this route than through rind.

Species of Diplodia are pathogens with worldwide distribution in tropical and subtropical regions causing different types of diseases in many woody plants including fruit and tree crops (Phillips et al., 2012). Expression of the diseases usually follows the onset of some type of stress due to factors other than the Diplodia infection itself (Schoeneweiss, 1981).

The high incidence of Diplodia in juice from HLB fruit indicates that HLB-affected orange fruit may be more vulnerable than healthy fruit to Diplodia infection. To verify the coincidence of HLB and Diplodia infection in orange fruit, decay rate was compared between asymptomatic (AS) and HLB-symptomatic (HLBs) sweet orange fruit (‘Hamlin’ and ‘Valencia’).

**Materials and Methods**

**Plant Material**

Asymptomatic (AS) and HLB-symptomatic (HLBs) fruit samples were collected from ‘Hamlin’ and ‘Valencia’ sweet orange trees in Vero Beach, FL. Selection of AS and HLB fruit in the field was by visual observation. AS fruit were collected from trees showing no symptoms for HLB; while HLB fruit were smaller in size, poorly colored, misshapen and attached to HLB-symptomatic branches or canopy sectors of a symptomatic tree (yellowing or blotchy mottled leaves and twig dieback). When
the fruit were brought to lab, the presence or absence of CLas was verified by qPCR analysis as described below. qPCR analysis revealed that some of AS fruit were CLas negative, while others were mildly positive but without visual symptoms; and all the HLB fruit were CLas positive. Three harvests of ‘Hamlin’ and ‘Valencia’ fruit were conducted between 2013 Dec. and 2014 Jan.; and between 2014 Feb. and 2014 May, respectively. Fruit were harvested in the field with 15–20 cm attached stems and immediately transported to the laboratory where the samples were processed for fungal isolation within 2 h.

DNA Extraction

The fruit side of the fruit abscission zone was removed using a #4 cork borer and used for DNA extraction. DNA was extracted from 100-mg plant tissue using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). The plant tissues were initially chopped and ground into powder in liquid nitrogen with mortar & pestle, and then the manufacturer’s isolation protocol was followed for the DNA isolation.

Primers and Real-time PCR

Real-time PCR amplifications were performed in a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). For CLas detection, primers HLBASF and HLBBr and probe HLBp was used targeting 16S rRNA genes of CLas (Li et al., 2006): HLBAS (F): TCGAGCGGTATGCAATACG; HLBBr (R): GCGTTATCCCGTAGAAAAGGTAG; HLBp (Probe): 6-FAM-AGACGGTGTAGTACACGGC-MGBNFQ. For Diplodia detection, specific primers targeting Diplodia β-tubulin gene (GenBank #DQ458858.1) were designed with software Primer Express 3.0.1. TB-F: ATGGCTCCCGTGTTGTTAAGTG; TB-R: TGCTACAGGTGAGTAACGCG. PCR mixtures, with a total volume of 15 μl contained 7.5 μl of TaqMan PCR master mix or SYBR Green PCR Master Mix (Applied Biosystems), 250 nM each primer, 150 nM probe (for CLas detection), and 100 ng of template DNA. The qPCR parameters are as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 seconds, and 60 °C for 1 min, with fluorescence signal capture at each stage of 60 °C. For the SYBR® Green Real-Time PCR reaction, the default Melt Curve (disassociation) stage is continued after the 40 cycles of PCR reaction. Cycle threshold (Ct) values are analyzed using ABI 7500 Software version 2.0.6 (Applied Biosystems, Inc., Carlsbad, CA) with a manually set threshold at 0.02 and automated baseline settings.

Fruit Decay Assay

The AS and HLBs ‘Hamlin’ and ‘Valencia’ fruit were respectively divided into two groups randomly for treatment with ethylene or air. Each group consisted of 25 fruit, and three batches of fruit were tested for both orange varieties. Ethylene treatment was applied at 10 ppm, at 27.8 °C (82 °F) and 90% to 95% relative humidity for 4 d. Fruit not treated with ethylene were held at the same temperature and humidity for 4 d as ethylene treated group. Following ethylene treatment, fruit were transferred to ambient conditions for 2 weeks. Incidence of decay was recorded on days 3, 7, and 14 posttreatment.

Isolation of Fungi from Plant Calyx Abscission Zone

Immediately before processing, pedicels were clipped to ~2 cm and pulled off from the fruit. To eliminate superficial microbes, pedicels with the calyx abscission zone were washed with tap water, then immersed in 70% ethanol for 1 min, 3.5% sodium hypochlorite for 2 min, 70% ethanol for 30 s and finally rinsed three times (3 min each) in sterile deionized water and then dried by blotting on sterile paper towel. Pedicels were cut into 5 mm sections from the calyx abscission zone side using a sterile razor blade, and then a thin layer (0.5 mm) of calyx abscission zone surface was cut and removed. The sections were then placed onto potato-dextrose agar (PDA), with the calyx abscission zone side facing the medium. Cultures were kept on the laboratory bench at about 20 °C to 25 °C for up to 30 d. The effectiveness of surface-sterilization was checked by spreading 100 μl of the final rinse water on PDA followed by incubation for 2 weeks at 20 °C to 25°C to confirm the absence of any microbial growth. The identities of the isolates were determined by PCR. The PCR-positive isolates were sub-cultured on water agar supplemented with autoclaved citrus twigs on the agar surface to induce conidia formation.

Statistical Analysis

SAS Version 9.3 (SAS Institute, Cart, NC) was used for analysis of data. One way ANOVA (PROC ANOVA) at the 95% (P = 0.05) confidence interval was used to determine statistical significance of differences in decay rates between AS and HLBs, where P < 0.05 was considered to be statistically significant.

Results and Discussion

Incidence of stem end rot in HLB symptomatic and HLB asymptomatic fruit

Since Diplodia infection is known to cause SER, and development of SER is exacerbated by exposure to ethylene (Baremore and Brown, 1985), SER incidence in AS and HLB ‘Hamlin’ and ‘Valencia’ fruit with and without ethylene treatment was determined. ‘Hamlin’ fruit used in the SER assay is shown in Fig. 1. The HLBs fruit had higher incidence of SER than did AS fruit (P < 0.001) (Fig. 2A and B) treatment with ethylene increased the incidence of SER in both AS and HLB fruit. Two weeks following exposure to ethylene, the incidence of SER rot in HLBs fruit was as high 67% in ‘Hamlin’ and 59% (‘Valencia’); while for AS fruit, the incidence of SER was less than 10%.

The increased incidence of SER in HLBs fruit may result from the biotic stress due to CLas infection. It has been reported that dramatic physiological and anatomical changes occur in CLas-infected citrus trees. Among them, cell wall aberration (Aritua et al., 2013), compromised plant defense (Kim et al., 2009; Nwugo et al., 2013) and physiological disorders (Fan et al., 2010). CLas infection may facilitate the Diplodia present in the grove environment to invade fruit tissue, and result in a high incidence of stem end rot after harvest. Swelling in the middle lamella, collapse of cell walls and separation of the adjacent cells were observed in CLas-infected citrus stems through transmission electron microscopy (Aritua et al., 2013). Cell walls are not only the first line of plant defense protecting against pathogen penetration, it is also a source of signals used by plants to induce defense mechanisms (Ferreira et al., 2007). Studies have shown that the plant defense system is suppressed by HLB disease. A gene product (salicylate hydroxylase) of CLas is able to reduce plant defenses by degrading salicylic acid (SA) (Wang and Trivedi, 2013), the molecule that has been known to mediate defense responses against pathogens in a number of plant species (Shah, 2003). A large number of defense-related genes were down-regulated and a general reduction in the production of defense-related proteins in CLas-infected citrus have been revealed by microarray studies and a proteomic study (Kim et al., 2009; Nwugo et al., 2013).
Isolation of Diplodia from HLBs fruit calyx abscission zone and confirmation of the identity by PCR and morphology of conidia

To confirm the Diplodia infection in HLBs fruit, fungi were isolated from 6 AS and 6 HLBs fruit abscission zone tissues by following the procedure that has been used in isolation of endophytes from inside plant tissues (Guo et al., 2000). The surface-sterilization was effective, as was indicated by the absence of any microbial growth on the medium after spreading and incubation for 2 weeks at room temperature of the final rinse water in the surface-sterilization procedure. The isolates from fruit calyx abscission zones were analyzed by qPCR for Diplodia identity. And the qPCR results indicated that Diplodia was isolated from 4 out of the 6 HLB samples; but no Diplodia was isolated from the 6 AS samples. The Diplodia positive fungi as verified by qPCR were then inoculated on autoclaved citrus twigs to induce characteristic conidia (Fig. 3). The conidia were observed under a light microscope. Figure 4 shows the mature Diplodia conidia.
induced in isolate from HLB fruit calyx abscission zone. The mature conidia are brown-colored, dark-walled and one-septate. They are highly consistent with the features and dimensions of the Diplodia conidia documented in the literature (Phillips et al., 2013). In conclusion, higher incidence of Diplodia stem-end rot was found in HLB symptomatic than in asymptomatic sweet orange fruit (Zhao et al., 2015).

**Literature Cited**


