Short communication

Elimination of *Candidatus Liberibacter asiaticus* (CLas), the bacterium associated with citrus Huanglongbing in citrus trees by heat treatment

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Summary

The Huanglongbing (HLB, Citrus greening disease) is a bacterial disease and causes significant economic and production loss in citrus industry worldwide. Our objective was to establish a heat treatment method that can directly affect not only the above ground parts including leaves and stems, but also the roots of infected trees to eliminate HLB bacterium. We carried out heat treatment in water to eliminate the HLB bacterium by immersing HLB-diseased grapefruit trees in water heated to 45°C in water-tub for 12 and 24 h. In result of quantitative polymerase chain reaction (qPCR) assay, at 30 days after heat treatment in water (DAH), the bacterium was detected in stems of both of samples, untreated and heat treated in water, with Ct value 22.9 to 33.7. At 240 d after heat treatment in water, the bacterium was detected in leaves, stems, roots and trunks samples of untreated trees but not in the water-heat treated grapefruit trees. It is consistent with HLB symptoms on untreated grapefruit trees, blotchy mottle, yellow vein and cork. No symptoms on heat treated trees. This suggested that the heat treatment in water was effective in removing HLB bacterium from infected citrus potted seedling. Thus, we expect that the results of this study will provide important information for the citrus industry to produce citrus nursery stock.

Keywords

bacterial disease, citrus industry, heat treatment, Huanglongbing, nursery stock

Significance of this study

What is already known on this subject?

- HLB bacterial titers are influenced by seasonal temperature variation, and are at the lowest in the spring-summer season, during the high temperature period. Some thermotherapy studies have reported that the bacterial titers were reduced or eliminated by increasing the ambient temperature surrounding the HLB infected tree by more than 40°C.

What are the new findings?

- Heat treatment in water which can directly affect the entire plant including naked roots of the infected potted citrus seedling; it is effective against CLas. Both HLB symptoms and the bacterium were not detected at 240 days post-treatment.

What is the expected impact on horticulture?

- To protect high-quality citrus fruits production against HLB and citrus viruses, the information derived from these results can be used in producing disease-free citrus nursery stock.

Introduction

The Huanglongbing (HLB, Citrus greening disease) is a most destructive citrus disease which threatens citrus production worldwide. It is caused by three *Candidatus Liberibacter* spp., phloem-limited, unculturable and fastidious α -Proteobacteria: *Candidatus Liberibacter asiaticus* (CLas), *Candidatus Liberibacter americanus* (CLam) and *Candidatus Liberibacter africanus* (CLaf) (Gottwald, 2010). HLB-associated bacterium causes phloem collapse and phloem plugging, resulting in impairment of phloem transport of citrus, ultimately leading to metabolism imbalance and tree decline (Etcheberria et al., 2009; Fan et al., 2013; Koh et al., 2012).

HLB symptoms development is influenced by seasonal temperatures variation (Sauer et al., 2015; Zhang et al., 2013). It has been confirmed in Florida and Brazil that bac-

terial titers were the highest in autumn-early winter, and the lowest in spring-summer, coinciding with HLB symptoms in the field (Sauer et al., 2015; Zhang et al., 2013). *Candidatus Liberibacter* spp. possesses different temperature sensitivity depending on the area where citrus are produced (Bové et al., 1974; Lopes et al., 2009; Jagoueix et al., 1994). Among them, CLas is heat-tolerant, with high titers at 30–35°C, but drastically reduced at above 38°C (Lopes et al., 2009).

For the sake of reducing or eliminating the bacterium titer associated with HLB, various methods such as chemotherapy, including antibiotics, and thermotherapy have been studied. Many antibiotics such as ampicillin, carbenicillin, penicillin, and rifampicin have been found to be effective against CLas (Zhang et al., 2013, 2015) and direct injection of antibiotics into the trunk of an infected tree has been reported to significantly to reduce CLas titer (Shin et al., 2016). Despite the effectiveness of antibiotic use, it has been strictly regulated due to various environmental issues. Thermotherapy studies have been conducted by increasing ambient temperature surrounding infected trees with growth chamber in range of 40 to 42°C for 48 h or with portable houses for 7 or 9 d (Hoffman et al., 2013; Doud et al., 2017).

In this study, we elected a heat treatment method which

can directly affect not only the parts above ground such as leaves and stems, but also the roots of infected trees so as to eliminate HLB bacterium. After soil attached to roots was washed off, heat treatment in water was carried out by immersing HLB-diseased grapefruit trees in water heated to 45°C for 12 and 24 h, respectively, following which there was an evaluation of the effectiveness of the treatment to remove the HLB bacterium.

Materials and methods

Plant material

The typical HLB symptoms, blotchy mottle, yellow vein and cork, of grapefruits (*Citrus paradise* 'Ruby Red') trees potted were observed (Figure 1A) and collected in the green house of the Indian River Research and Education Center (IRREC, Fort Pierce, FL). Prior to heat treatment, the leaves, stems and roots of the HLB-diseased grapefruit tree used in this study were sampled and confirmed in the detection of HLB-bacterium with Ct values 21.3 to 34.3 (Table 1).

Heat treatment

Heat treatment in water was carried out at 45°C for 12 and 24 h, respectively, with HLB-diseased grapefruit trees (Figure 1). Water in the water-tub was heated to 45°C with

portable water heater and checked every 30 min, using four thermometers (Figure 1C). After soil attached to the roots was washed off, whole trees including the roots were immersed in heated water on water-tub for 12 and 24 h, respectively (Figures 1B and 1C). Three plants were used for each of the treatments, as control, 12 and 24 h heat treatment in water. Following heat treatment, one of trees treated for 12 h and two for 24 h were observed to be dead respectively. After heat treatment, while the leaves and stems turned brown, trunks were still green (not brown). Also soon all the leaves on the heated trees fell off (Figure 1D). All treated trees were covered with mesh to protect HLB-infection against psyllid insect vectors (Figure 1E). Within three weeks, emerging new flushes were observed.

Genomic DNA extraction

Total genomic DNA was extracted using a modified method of the Qiagen DNeasy Plant mini kit protocol (Qiagen, Germantown, MD) reported previously (Hoffman et al., 2013). Leaves, stems, trunks and roots of HLB diseased grapefruit tree were collected before heat treatment in water and 240 d after heat treatment in water. Only the stems were sampled at 30 d following heat treatment. The midrib of leaf samples were excised. Barks were stripped from stems and trunks samples that are dark green, not brown. Fibrous roots were

FIGURE 1. Heat treatment in water process for removing HLB-bacterium in infected grapefruit trees. (A) Typical HLB symptoms on grapefruit. Red arrow indicates blotchy mottle, green; cork, blue; yellow vein. (B) Grapefruit tree before heat treatment in water. Soil attached to roots was washed off. (C) Heat treatment in water. Four red arrows are thermometers that checked every 30 min during the water-heat treating. (D) Grapefruit tree after heat treatment in water. (E) Grapefruit covered with mesh. After treated, trees were covered with mesh to protect HLB-infection against psyllid, insect vectors. (F) Grapefruit tree at 240 d after heat treatment in water. The red arrow shows where the trunks of trees were sampled.

TABLE 1. Result of detection HLB bacterium in infected-grapefruit tree before and after heat treatment in water.

	Treatment ^a	Before treatment	30 DAH ^b	240 DAH ^c
Leaves	Control	22.3 ± 0.19	–	22.8 ± 0.10
	Control	25.1 ± 0.22	–	20.9 ± 0.21
	Control	22.5 ± 0.12	–	29.3 ± 0.15
	45°C, 12 h	23.8 ± 0.36	–	ND
	45°C, 12 h	21.3 ± 0.21	–	ND
	45°C, 24 h	24.2 ± 0.12	–	ND
Stems	Control	25.9 ± 0.27	22.9 ± 0.10	22.6 ± 0.14
	Control	27.4 ± 1.47	24.4 ± 0.12	27.0 ± 0.25
	Control	24.1 ± 0.31	31.5 ± 0.14	25.8 ± 0.21
	45°C, 12 h	22.5 ± 0.18	33.1 ± 0.15	ND
	45°C, 12 h	26.6 ± 0.30	33.7 ± 0.33	ND
	45°C, 24 h	27.1 ± 0.20	23.2 ± 0.13	ND
Trunks	Control	–	–	29.1 ± 0.03
	Control	–	–	24.4 ± 0.86
	Control	–	–	28.2 ± 0.11
	45°C, 12 h	–	–	ND
	45°C, 12 h	–	–	ND
	45°C, 24 h	–	–	ND
Roots	Control	33.3 ± 1.08	–	34.6 ± 0.17
	Control	25.0 ± 0.33	–	31.8 ± 0.15
	Control	34.3 ± 2.71	–	32.3 ± 0.21
	45°C, 12 h	33.4 ± 0.55	–	ND
	45°C, 12 h	32.5 ± 0.82	–	ND
	45°C, 24 h	32.5 ± 0.83	–	ND
NC		ND	ND	ND

^a: No treatment and heat treatment in water at 45°C for 12 and 24 h.

^b: 30 days after heat treatment in water.

^c: 240 days after heat treatment in water.

ND: Not detected, samples were considered as negative.

–: Samples could not be collected.

sampled. Hereafter, all of the samples were chopped finely. Two hundred milligrams of finely chopped samples were placed in a sterile 2-mL tube with silicone-carbide shards and, 2.3-mm chrome-steel beads. Then then 800 µL AP1 extraction buffer and 80 µL of 20% SDS were added. Samples were homogenized with a Fast Prep-24 homogenizer (MP Biomedical, Solon, OH) at a speed of 6.5 for 100 s. Eight µL of RNase A was added and samples were incubated for 30 min at 65°C. Next, 400 µL of AP2 buffer was added and samples were put on ice for 5 min. All subsequent processes were the same as the standard protocol. Finally, genomic DNA samples were eluted in 100 µL of elution buffer and stored at -30°C.

qPCR assay

To evaluate the effect of eliminating the HLB-bacterium via heat treatment, qPCR assay was conducted with LJ900 primers and probe (forward: GCCGTTTAAACACAAAAGATGAAT-ATC, reverse: ATAAATCAATTGTCTAGTTTACGAC, probe: ACATCT-TTCGTTTGAGTAGCTAGATCATTGA) targeting tandem repeat in *LasA_I* and *LasA_{II}* genes of *CLas*, as previously studied (Zhou et al., 2011). The qPCR was performed using CFX96 Touch™ Real-Time PCR Detection System (Bio-rad Laboratories, Inc., Hercules, CA) and used in final volume 20 µL of reaction mixture containing 2 µL template DNA, 1× qPCR probe master mix (MGmed, Republic of Korea), 250 nM forward/reverse primers, and 250 nM taqman probe. All genomic DNA samples were assayed in three replicates and

sterilized water was used as negative. The qPCR reaction protocol was pre-denaturated at 95°C for 3 min, which followed by 40 cycles at 95°C for 10 s and then 60°C for 30 s. All reactions were performed in duplicate. A detected Ct value is considered as positive and undetected as negative.

Results and discussion

In result of qPCR assay, before treatment, HLB bacterium was detected in the leaves, stems and roots sampled from grapefruit tree used in this study with Ct value 21.3 to 34.3 (Table 1). At 30 d following heat treatment in water, only the stems of untreated and water-heat treated trees were sampled and the bacterium was still detected at Ct value 22.9 to 33.7 (Table 1). At 240 d after heat treatment in water, asymmetrical blotchy mottle, the most characteristic symptom of HLB, on the leaves of untreated grapefruit, still remained (Figures 2A and 2B) but this was not so far heat treated trees (Figures 2C and 2D). The genomic DNA extracted from the leaves, stems, roots and trunks of treated and untreated trees were assayed qPCR. The bacterium was detected in the leaves, stems, roots and trunks of untreated trees, but not in the water-heat treated grapefruit trees (Table 1).

Given these results, the heat treatment used in this study may have been effective in eliminating HLB bacterium, or the pathogen may not have been detected due to a lack of sufficient time to reach a new flush because HLB bacterium moves slowly in host plants after infection (Shokrollah et

FIGURE 2. Grapefruit at 240 d after untreated and heat-treatment in water. (A) and (B) HLB symptoms of untreated grapefruit. Red arrows indicate asymmetrical blotchy mottle of leaves, most characteristic symptom of HLB, which is not the same on opposite sides of the leaf mid-rib. (C) Grapefruit 240 d after heat treatment in water for 12 and (D) 24 h.

al., 2009). However, as a result of assaying, including of the trunks and roots, it was confirmed that no HLB bacterium was detected in the heat-treated HLB-diseased trees. At 30 d following heat treatment, some of the dead HLB bacteria remained after heat treatment. Thus it is thought that such bacterium was detected by qPCR. At 240 d after heat treatment, in all heat-treated tissues of grapefruit, no HLB bacterium was detected and no symptoms were observed. This suggested that the heat treatment in water was effective in removing HLB bacterium from infected citrus potted trees. At 240 d after heat treatment, in all heat-treated tissues of grapefruit, no HLB bacterium was detected and no symptoms were observed. This suggests that the heat treatment in water was effective in removing HLB bacterium from infected citrus potted trees.

Previous studies have been carried out to reduce or remove HLB bacterium by increasing the ambient temperature of citrus potted seedling in growth chamber, or by utilizing heat from solar radiation on trees in field with portable green-houses (Hoffman et al., 2013; Doud et al., 2017). We performed a heat treatment that was able to directly affect the roots as well as the leaves and stems, and confirmed that neither HLB symptoms nor the bacterium was detected at 240 d post-treatment. This suggests that the heat treatment in water was effective in removing HLB bacterium from infected citrus potted trees.

Conclusion

This heat treatment method, by immersing HLB-diseased grapefruit trees with washed roots in 45°C of water for at least 12 h, was effective in terms of removing HLB bacterium, resulting in no HLB bacterium detected and no symptoms at 240 d following heat treatment. Although this method is not applicable to trees under field condition, it can be used on potted seedlings. As well, the information derived from these results can be used to produce disease-free citrus nursery stock.

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