Journal of Integrative Agriculture 2019, 18(0): 2–11

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RESEARCH ARTICLE

Limited Infection by '*Candidatus* Liberibacter asiaticus' in 'Valencia' sweet orange trees in the presence of *Citrus tristeza virus*

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Abstract

Huanglongbing (HLB) is the most destructive disease of citrus and is associated with '*Candidatus* Liberibacter asiaticus' (CLas), a member of the α-proteobacteria. *Citrus tristeza virus* (CTV) is another pathogen of citrus with very great historic as well as current importance. Both CLas and CTV are phloem-restricted pathogens. A severe CTV isolate, CTV-B6, and CLas-B232 induce a group of symptoms of phloem dysfunction that overlap, but the mild isolate CTV-B2 does not cause any loss to commercial trees. Prior inoculation and establishment of CLas-B232 did not affect subsequent establishment of either CTV-B2 or CTV-B6, while super infection by CLas-B232 was reduced by prior establishment of CTV-B2 and to a lesser extent by prior infection with CTV-B6. Trees co-infected with CTV-B6 and CLas-B232 developed more severe symptoms, typical of CTV-B6, than either of the two pathogens co-infected with CTV-B2. In this study, we confirmed that CLas established in the rootlets earlier and with higher concentration than in leaves. The distribution of CLas in the plant infected by CLas-B438 alone and with CTV-B2 fits a previously proposed model but CLas was more sporadically distributed in a plant co-infected by CLas and CTV-B2 than in a plant infected by CLas alone. These biological phenomena are aligned with previously analyzed transcriptome data and the study provides a novel idea that mild CTV strains may provide some protection against CLas by limiting its multiplication and spread. The protective effect may be due to opposite regulation of key host defense pathways in response to CTV-B2 and CLas-B438.

Keywords: huanglongbing, plant defense, plant protection

1. Introduction

Citrus greening or huanglongbing (HLB), is a devastating disease of citrus that has caused very tremendous damage to the citrus industry in Florida, Brazil, China and some other countries (Bové 2006; Gottwald 2010; Wang and Trivedi 2013; Zheng *et al*. 2018) and threatens the commercial viability of the citrus industry. Growers limit the damage

Received 21 September, 2018 Accepted 11 December, 2018
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doi: 10.1016/S2095-3119(19)62605-5

caused by HLB by identifying and removing infected trees, planting CLas free seedlings, providing rigorous control of the vector, *Diaphorina citri* (Kuwayama), and providing nutrition, often by foliar application (Rouse 2013). *Citrus tristeza virus* (CTV) nearly destroyed the citrus industry in the 1930's and 1940's due to sweet orange on sour orange decline worldwide (Bennett and Costa 1949; Moreno *et al*. 2008). Both pathogens are widespread globally and threaten the citrus industry everywhere. HLB is associated with a phloem-restricted, non-cultured member of the α-proteobacteria, '*Candidatus* Liberibacter asiaticus' (CLas) (Bové 2006) and *Citrus tristeza virus* (CTV), a *Closterovirus*, is also phloem-restricted (Bar-Joseph *et al*. 1979). There is great variation within the global population of CTV, and at least seven distinct CTV genotypes have been defined (Hilf *et al*. 2005), and these genotypes can exist in many different combinations in co-infected citrus trees (Roy *et al*. 2010) although superinfection of a tree by CTV of the same genotypic group is excluded (Folimonova *et al*. 2010; Bergua *et al*. 2016). Both pathogens are transmitted by phloem-feeding insects, the citrus psyllid, *Diaphorina citri*, in the case of CLas (Canale *et al*. 2016) and aphids, notably *Toxoptera citricida* (Kirkaldy), in the case of CTV (Halbert *et al*. 2004). Simultaneous infection of trees with both pathogens is common, and many foliar symptoms produced by both pathogens related to phloem dysfunction are similar. These include the production of new shoots that are uniformly yellow, chlorosis that is somewhat typical of zinc deficiency and leaves that are stiffened due to accumulation of starch with twisting caused by corking along the middle vein (Bové 2006). A blotchy mottle symptom of mature leaves and misshaped fruit with inverted color development and aborted seeds are not seen in CTV-infected trees and are used for presumptive diagnosis of HLB (Bové 2006).

Foliar symptoms of HLB are easily observed when infection is well established, and symptomatic leaves are usually submitted for confirmatory detection of CLas by PCR, transmission electron microscope (TEM) or immune tissue printing (Hocquellet *et al*. 1999; Folimonova *et al*. 2009; Ding *et al*. 2015). Recently attention has been drawn to the root system, with data that the density of fibrous roots of CLas-infected trees was greatly reduced (Graham *et al*. 2013; Wu *et al*. 2018) and the root system declined prior to the appearance of foliar symptoms. This is consistent with a model that phloem-limited bacteria move from the initial insect feeding site in new flush to the nutritional sink of the root system, and spread from there to new leaf flushes when they emerge and become nutritional sinks.

Many CLas-infected field trees in Florida are also infected with CTV, and both mild and stem-pitting strains of CTV are common. Symptoms produced by CLas alone or CLas with CTV were similar when observed in Papua New Guinea (Davis *et al*. 2005). Research has been carried out previously to observe the interactions of the two pathogens. Interactive effects were not observed when trees were co-infected with CLas and one of several CTV strains (T36, T30, T68, T3 or VT) (Folimonova *et al*. 2009). However, another study found that CTV could play a role in infections caused by the related pathogen '*Ca*. Liberibacter africanus' (CLaf), as the percentage infection differed by 10% or more between trees on the same rootstock with different CTV isolates (Van Vuuren and Manicom 2005a). It was also reported that the presence of a CTV isolate (CD4) reduced the incidence of misshapen fruit on Valencia trees infected by CLaf (Van Vuuren *et al*. 2000) and reduced HLB incidence after pruning (Van Vuuren and Manicom 2005b), similar to the results reported by Su (1982) and Chen *et al*. (1972), in early research on HLB. These results may be affected by different strains of both CLas, CLaf and CTV, as well as different varieties of citrus.

Recently, many transcriptome studies have been performed on trees infected by either CLas or CTV alone (Cristofani-Yaly *et al*. 2007; Gandía *et al*. 2007; Liu *et al*. 2012; Aritua *et al*. 2013; Mafra *et al*. 2013; Martinelli *et al*. 2013; Yang *et al*. 2013). We investigated the transcriptome of sweet orange seedling trees infected with CLas and mild and severe CTV strains alone or in combination by graft inoculation (Fu *et al*. 2017a, b). In the course of that work we unexpectedly found that when CLas was inoculated simultaneously with CTV (mild or severe) strains, both mild and severe CTV strains became rapidly established in the trees but the incidence of CLas was low when assayed by qPCR using a standard method (Li *et al*. 2006). In order to characterize whether the interaction between the citrus host and CLas was affected by the presence of mild or severe strains of CTV, it was necessary to inoculate the trees first with CLas and then with CTV. In this study, we describe the interactions among mild and severe strains of CTV with CLas in sweet orange seedlings in a greenhouse setting.

2. Materials and methods

2.1. Inoculation of CTV and CLas

CTV-B2 (mild strain: T30 group, Florida), CTV-B6 (severe strain; complex genotype; received as SY568, California), CLas strain B232 (Thailand) and CLas strain B438 (Florida) are maintained *in planta* as part of the Exotic Pathogens of Citrus Collection (EPCC) at the USDA Beltsville Agricultural Research Center (BARC) in Beltsville, MD, USA. Both strains of CLas are virulent and are maintained in rough lemon in our greenhouse because they kill sweet orange trees. Seeding trees of 'Valencia' sweet orange were divided into three groups as follows:

In group I, CLas-B232 paired with CTV-B2 or CTV-B6, and CTV-B2 paired with CTV-B6 were inoculated into trees simultaneously by bud grafting with 10 replicates (designated B2/B232, B6/B232 and B2/B6), and 10 trees were mock-inoculated with healthy buds. Among these inoculated trees, those that were confirmed to be positive for CTV-B2 by RT-PCR but negative for CLas-B232 were re-inoculated with CLas-B232 36 weeks after the first inoculation.

In group II, CLas-B232 was pre-inoculated in 20 trees, and 3 months later, 10 trees were graft-inoculated with CTV-B2 or CTV-B6. Ten healthy trees were mock inoculated with healthy buds as a control. Eighteen months later, half (ten) of the mock inoculated healthy trees from group I and group II were graft-inoculated with buds from trees co-infected with CTV-B2 and CLas-B232. Ten more healthy trees were mock-inoculated.

In group III, 30 sweet orange seedlings were inoculated with CLas-B438 (Florida) 4 weeks after inoculation with CTV-B2, and 31 sweet orange seedlings were inoculated with CTV-B2 8 weeks after inoculation with CLas-B438 (Florida). Leaf pieces were used as CLas inoculum in this experiment. Nine trees were self-inoculated as controls. All trees were maintained in the same greenhouse under controlled variables for observation through 3 years.

2.2. Detection of CTV and CLas in leaf and root samples

DNA for detection of CLas was extracted with Plant DNeasy® Mini Kit (Qiagen, Valencia, CA) from leaf midribs or fibrous roots (about 0.1 g) of the inoculated and greenhouse grown 'Valencia' sweet orange trees. Both symptoms of HLB, and the amount of CLas present within individual leaves are known to vary widely within branches of a tree (Folimonova *et al*. 2009). Therefore, midribs from three leaves with symptoms (when present) were taken from new growth flushes and three rootlets were consistently sampled from each tree during the summer months when growth was most active. The grafting site was about 15 cm above the soil surface and the distance between the grafting site and leaves varied, depending on the site where the new flushes emerged. qPCR assays were performed as described (Li *et al*. 2006) with a Smartcycler® (Cepheid, Sunnyvale, CA).

Total RNA for detection of CTV was extracted with the Trizol reagent (Invitrogen, Carlsbad, CA). cDNA synthesis was done with the Thermoscript RT-PCR System for First-Strand cDNA Synthesis (Invitrogen, Carlsbad, CA). CTV cDNA (2 μL was amplified in a 25-μL reaction volume containing 10× reaction buffer, 10 mol L^{-1} dNTP, 25 mol L^{-1} $MgCl_2$, 10 μmol L^{-1} of each primer and 0.3 U of platinum *Taq* polymerase (Invitrogen, Carlsbad, CA). Conventional PCR amplification reactions were carried out with a PTC-200 (MJ Research, Waltham, MA) as follows: 94°C for 3 min, then 94°C for 30 s, 57°C for 40 s, and 72°C for 40 s for 30 cycles. The reactions were incubated an additional 10 min at 72°C prior to storage at 4°C. Amplification products were analyzed by electrophoresis in TAE buffer on 1.5% agarose gels, and stained with Gel Red (Biotium, Fremont, CA). Generic and strain-specific primers were used to detect CTV-B2 and CTV-B6 (Table 1). Detection of CLas by qPCR or CTV by RT-PCR started at 4-week intervals after inoculation.

2.3. Statistics

Pair-wise comparisons of groups of inoculated plants were carried out to determine whether the presence of one pathogen in a plant effected the ability of another pathogen to establish. The effect of one pathogen's presence on another pathogen's subsequent establishment was evaluated by Fisher's exact test of Chi-square analysis using SPSS16.0 (IBM). The establishment of CLas-B232 in roots and leaves was compared within plant inoculation group II with one- and two-tailed *t*-tests, assuming unequal variances.

2.4. Immune tissue prints

CLas was also assayed following infection by CLas-B438 only and by CLas-B438 in combination with CTV-B2 by

Table 1 Primers used for PCR in this study.

Primer name	Primer sequence (5′→3′)	References
Generic CTV (+)	ATGGACGACGARACAAAGAAATTGAAGA	Roy *et al.* (2010)
Generic CTV (–)	TCAACGTGTGTTGAATTTCCCAAGCT	
CTV-B2 (+)	TGTTGCGAAACTAGTTGACCCTACTG	
CTV-B2 (–)	TAGTGGGCACAGTGCCAAAAGAGAT	
CTV-B6 (+)	TTTGAAAATGGTGATGATTTCGCCGTCA	
CTV-B (–)	GACACCGGAACTGCYTGAACAGAAT	
CLas f	TCGAGCGCGTATGCAATACG	Li *et al.* (2006)
CLas r	GCGTTATCCCGTAGAAAAAGGTAG	
CLas p	AGACGGGTGAGTAACGCG	

immune tissue printing with rabbit anti-OmpA polyclonal antibody (Ding *et al*. 2015, 2016). Three branches were detached from trees with established infections of CLas-B438 alone or with CTV-B2, as well as from trees that were mock inoculated. A series of freshly cut surfaces were pressed on nitrocellulose membranes (Whatman, 0.45 μm pore size; Cat. No. 88018) with 0.5 mm between each cut. The membranes were processed as described (Ding *et al*. 2015).

3. Results

3.1. Establishment of CTV and CLas

Group I Both strains of CTV established rapidly in the inoculated sweet orange seedlings, with 70–80% infection by 12 weeks post-inoculation (wpi) and 90–100% infection by 16 wpi. In contrast, CLas-B232 established more slowly, with only 5% (1/20) infection in week 12 and only 30–40% infection at week 32. There was no interaction between the two strains of CTV, as both strains established at 90–100% by week 16 when they were inoculated simultaneously (Fig. 1).

Group II Twenty sweet orange seedlings were inoculated with CLas-B232 alone at the beginning of the experiment. In contrast to the results seen in the co-inoculated trees in group I, CLas-B232 established rapidly in the sweet orange trees in the absence of CTV, with 85% (17/20) of the trees positive for CLas-B232 by week 12 (Fig. 2). These positive results were primarily from root samples, and compared with only 5% (1/20) positive for CLas-B232 at week 12 when the trees were co-inoculated with CTV (Fig. 1). Only 45% (9/20) of leaf samples were positive for CLas-B232 by 16 wpi (Fig. 2). The mean Ct values for root and leaf samples at week 16 (Fig. 2) were 20.5±13.2 and 22.3±1.6, respectively. These differences were not significantly different with a two-tail *t*-test (*P* (0.07)>0.05) but significantly different with a one-tail *t*-test (*P* (0.035)<0.05) (Appendix A), consistent with rapid movement to roots and larger populations of CLas in roots than in leaves.

We analyzed the rate of infection by CLas-B232 in the presence or absence of CTV. When the two groups of plants were compared, 9 of 10 plants inoculated with CLas-B232 in the absence of CTV-B2 became infected by CLas within 12 weeks (Fig. 3-A), but only 3 of 10 plants became positive for CLas-B232 when simultaneously inoculated with CTV-B2 (Fig. 1-A; Appendix B). A contingency table was set up and Fisher's exact test (two-tailed *t*; *P*(0.02)<0.05) (Appendix C) indicated that the presence of CTV-B2 had a significant effect on the subsequent establishment of CLas-B232, but the pre-establishment of CLas-B232 did not affect further establishment of CTV-B2. 8 of 10 trees became infected when inoculated with CLas-B232 only (Fig. 3-B), while only 4 of 10 trees became infected by CLas-B232 when

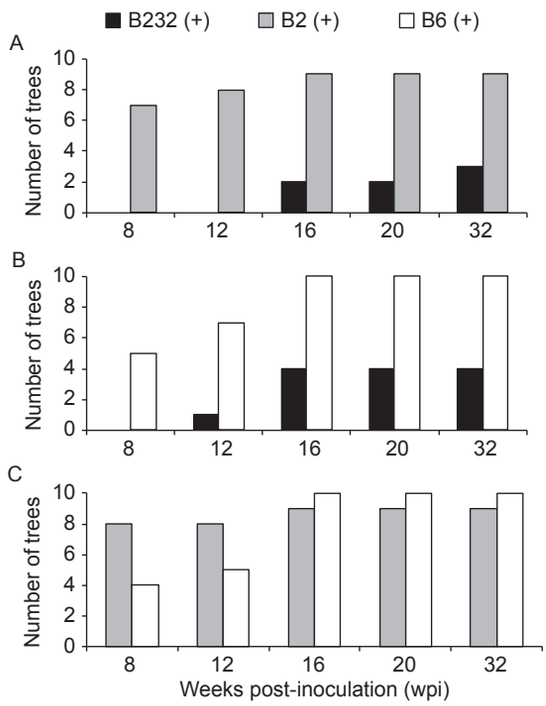

Fig. 1 Time course of infection of 'Valencia' sweet orange by *Citrus tristeza virus* (CTV) and '*Candidatus* Liberibacter asiaticus' (CLas). The pathogens indicated were graft-inoculated in sweet orange seedlings simultaneously, 10 replicates for each combination. The presence of CTV and CLas was assayed by RT-PCR and qPCR, respectively every 4 weeks post-inocultation (wpi). A, trees were co-inoculated with CTV-B2 and CLas-B232. B, trees were co-inoculated with CTV-B6 and CLas-B32. C, trees were co-inoculated with CTV-B2 and CTV-B6. The colored bars show the number of trees in which the pathogen was detected (*Y*-axis) at weeks post-inoculation (*X*-axis).

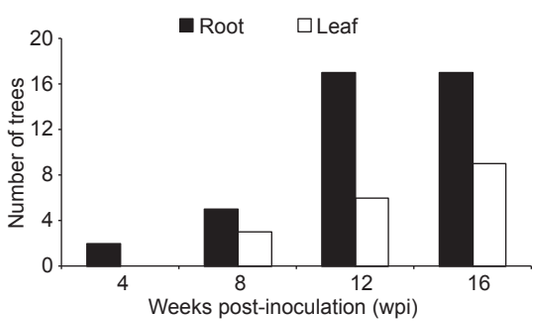

Fig. 2 Time course for detection of '*Candidatus* Liberibacter asiaticus' (CLas) in sweet orange roots and leaves. '*Ca*. Liberibacter asiaticus' B232 (Thailand) was bud-inoculated in 20 'Valencia' sweet orange seedlings. The bars show the number of root or leaf samples that were positive for CLas by qPCR (*Y*-axis) at the indicated number of weeks post-inoculation (*X*-axis).

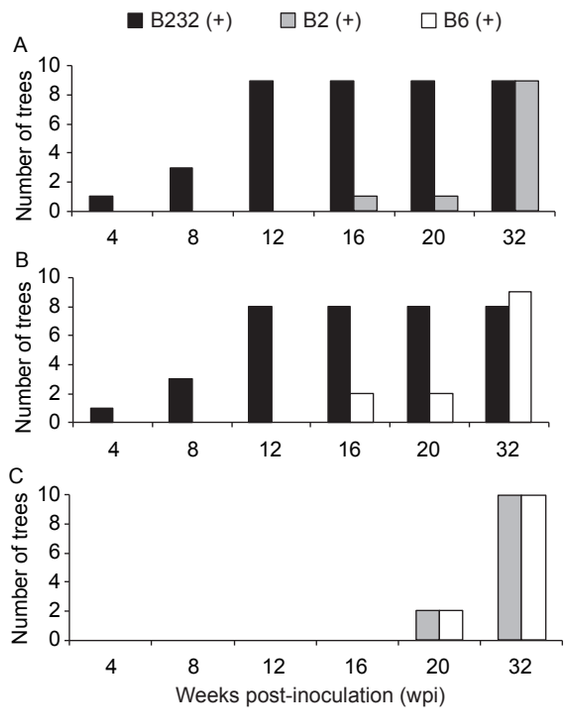

Fig. 3 Time course for detection of pathogens inoculated in 'Valencia' sweet orange seedlings. '*Candidatus* Liberibacter asiaticus' (CLas) was inoculated in 20 seedlings at the beginning of the experiment. CTV-B2 or CTV-B6 was separately inoculated into 10 of these trees after infection by CLas was confirmed by qPCR at 12 weeks. At 12 weeks, 10 trees were inoculated with CTV-B2 and CTV-B6. A, CLas-B232 followed by CTV-B2. B, CLas-B32 followed by CTV-B6. C, CTV-B2 and CTV-B6 together. The bars show the number of trees in which the pathogen was detected (*Y*-axis) at indicated number of weeks post-inoculation (wpi; *X*-axis). CTV, *Citrus tristeza virus*.

simultaneously inoculated with CTV-B6 (Fig. 1-B; Appendix B). The presence of CTV-B6 may have some effect on the establishment of CLas-B232, but it was not significant (two-tailed *t*; *P* (0.17)>0.05) (Appendix D). The presence of CLas-B232 did not affect the establishment of CTV-B6.

Group III We did not regularly monitor the establishment of the pathogens in group III by qPCR, but we counted living and dead trees 36 months after inoculation (Table 2). Among this group, 26 of 30 plants in which CTV-B2 was inoculated before CLas-B438 were alive and 4 trees were dead. Among the plants inoculated with CLas-B438 before they were inoculated with CTV-B2, 16 were alive and 15 were dead. All mock-inoculated trees were all alive. Fisher's exact test (*P*(0.005)<0.05) (Appendix E) verified the early presence of CTV-B2 had significantly affected the subsequent establishment and further invasion of CLas.

3.2. Observation of symptoms

All plants infected by any two of the three pathogens together were smaller by canopy size and height and less vigorous than mock-inoculated trees, regardless of whether the comparisons were among the trees with the most severe (Fig. 4-A and C) or the least severe symptoms (Fig. 4-B and D) among the 10 trees in each co-inoculation treatment. When CTV-B6 (severe strain) and CLas-B232 were inoculated together into sweet orange a severe decline was observed with defoliation (Fig. 4-A, b). The trees were also much smaller in size (Fig. 4-B-D, b). Symptoms in trees inoculated with CTV-B6 and CTV-B2 ranged from equal to CTV-B6 and CLas B232 (Fig. 4-A, b and c) to less severe (Fig. 4-B-D, b and c). The reduction of symptom severity in the presence of CTV-B2 was also evident in close examination of the leaves (Fig. 5). The presence of CTV-B2 dramatically reduced the appearance of chlorosis and blotchy mottle symptom induced by CLas-B232 (Fig. 5-A, B, and G). Trees co-inoculated with both mild and severe CTV strains showed typical symptoms of the severe strain CTV-B6, including leaf cupping and stiffening (Fig. 5-E and F). CLas-B232 was not detected either in leaf or root samples of the 6 trees inoculated with CLas-B232 in the presence of an established CTV-B2 infection (Fig. 6-A) nor in trees graft-inoculated with buds from trees co-infected by CTV-B2 and CLas-B232 (Fig. 6-B). No HLB symptoms were observed during 4 years of monitoring of these trees (Fig. 6).

Table 2 Status of 'Valencia' sweet orange trees 36-months after inoculation with CTV-B2 and CLas[1)]

Status	CTV-B2/CLas-B438	CLas-B438/CTV-B2	H
Tree alive	26	16	9
Tree dead	4	15	0

[1)] CTV-B2/CLas-B438, trees were inoculated with CLas-B438 4 weeks after inoculation with CTV-B2; CLas-B438/CTV-B2, trees were inoculated with CTV-B2 8 weeks after inoculation with CLas-B438; H, mock-inoculated trees.

Trees co-infected by CLas-B438 and CTV-B2 in group III were used for immunoassay. CLas-B438 was detected in these trees by qPCR 20 to 30 weeks after the inoculation of CLas-B438 in the presence of CTV-B2. Trees infected by CLas-B438 alone showed blotchy mottle symptoms, whereas trees co-infected by CLas-B438 and CTV-B2 did not show any symptoms. In consecutive tissue prints of stems, the pattern of distribution of CLas was generally similar in the phloem vessels whether the trees were infected by CLas-B438 alone or with CTV-B2 (Fig. 7). However, CLas-B438 showed more extensive lateral colonization of phloem cells in the plants infected by CLas-B438 only (Fig. 7-A–D), than in the presence of CTV-B2 (Fig. 7-E–H).

4. Discussion

CTV established in the trees faster than CLas, regardless of whether CLas was inoculated along with either mild or

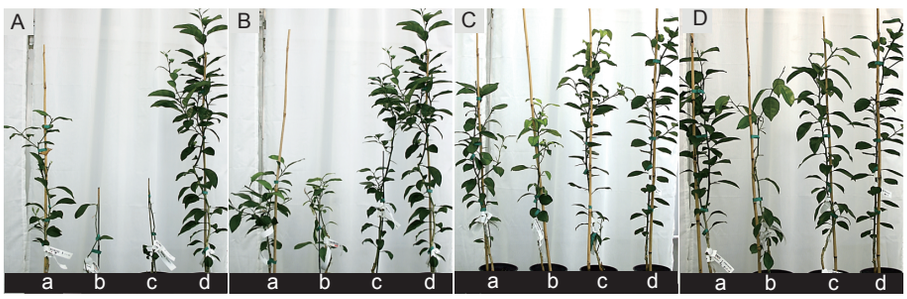

Fig. 4 Symptoms of 'Valencia' sweet oranges seedling co-infected with '*Candidatus* Liberibacter asiaticus' (CLas) and *Citrus tristeza virus* (CTV). Panels A and B are comprised of trees that displayed the most or least severe disease symptoms among the 10 trees in group I, in which two pathogens were inoculated simultaneously. The pictures were taken 18 months after inoculation. Panels C and D are comprised of trees that displayed the most or least severe disease symptoms among the 10 trees in group II, in which CTV-B2 or CTV-B6 were inoculated 12 weeks post-inoculation of CLas. The pictures were taken 18 months after the first inoculation. a–c, trees were co-infected with CTV-B2/CLas-B232, CTV-B6/CLas-B232 and CTV-B2/CTV-B6, respectively; d, mock-inoculated healthy tree.

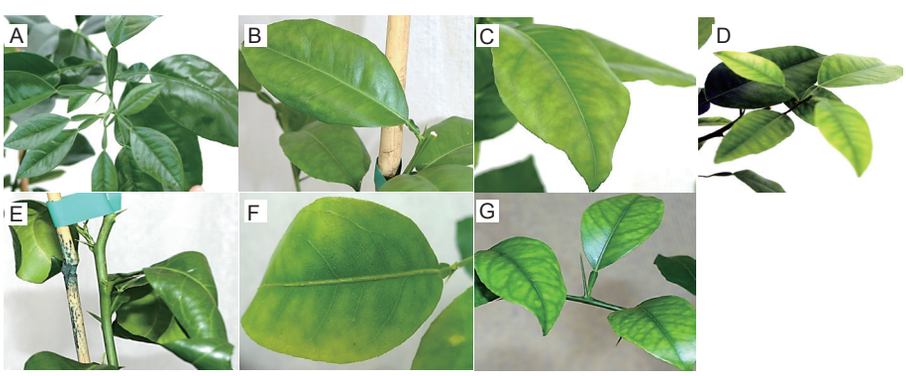

Fig. 5 Foliar symptoms on 'Valencia' sweet orange. A and B, foliar symptoms from trees co-infected with CTV-B2 and CLas-B232. C and D, foliar symptoms from trees co-infected with CTV-B6 and CLas-B232. E and F, foliar symptoms from trees co-infected with CTV-B2 and CTV-B6. G, chlorosis symptom caused by CLas-B232 alone. CTV, *Citrus tristeza virus*; CLas, '*Candidatus* Liberibacter asiaticus'.

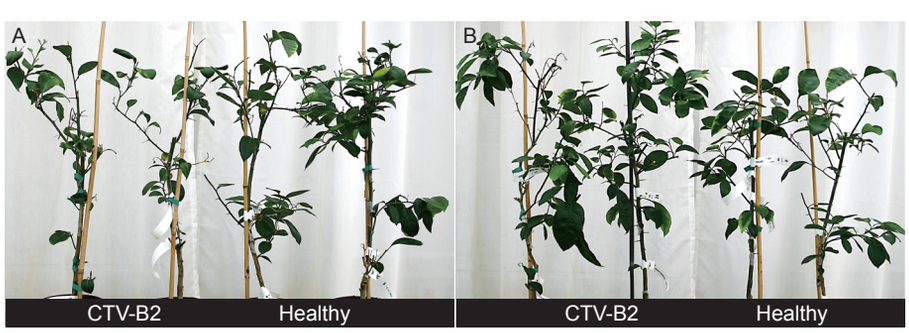

Fig. 6 CLas-B232 failed to establish in trees in the presence of CTV-B2. A, 'Valencia' sweet orange trees were simultaneously inoculated with CLas-B232 and CTV-B2 at the beginning. Eighteen months post-inoculation (mpi) , 6 trees infected by CTV-B2 but not CLas-B232 were re-inoculated with CLas-B232 by grafting. B, 10 mock-inoculated trees from group I and group II were inoculated with buds from trees co-infected with CTV-B2 and CLas-B232. CLas-B232 was not detected in these trees by the time that pictures were taken 36 months after the second inoculation. CTV, *Citrus tristeza virus*; CLas, '*Candidatus* Liberibacter asiaticus'.

severe strains of CTV. The prior establishment of CTV in the phloem cells affected the further establishment and invasion of CLas. This phenomenon was confirmed with a third set of plants, in which prior inoculation of trees

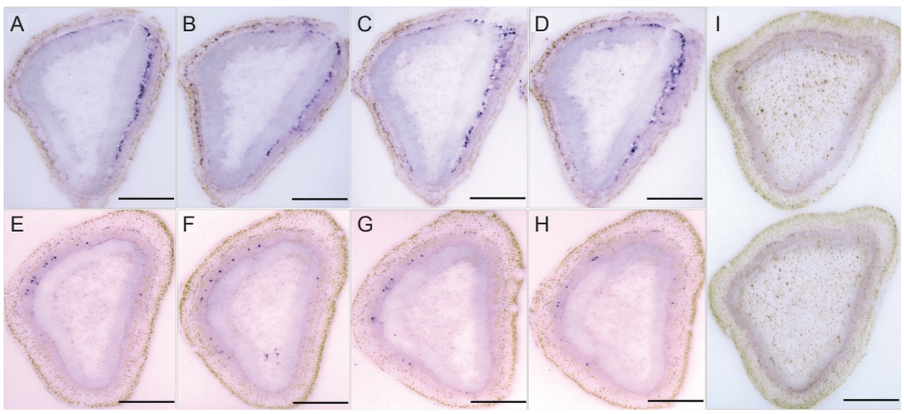

Fig. 7 Distribution of '*Candidatus* Liberibacter asiaticus' (CLas) in phloem sieve cells in consecutive stem tissue prints of 'Valencia' sweet orange infected by CLas-B438 alone and in co-infection with CTV-B2. A-D, consecutive stem sections of 'Valencia' sweet orange infected by CLas-B438 alone and showing blotchy mottle symptom. E–H, consecutive stems sections of symptomless 'Valencia' sweet orange co-infected by CLas-B438 and CTV-B2. The trees were positive by PCR for both pathogens. I, immune tissue prints stem from a non-inoculated plant. Black bars=1 mm. CTV, *Citrus tristeza virus*,

with CTV-B2 had a protective effect against subsequent inoculation by CLas. When previous researchers observed protective interactions between some strains of CTV and '*Ca*. Liberibacter africanus' (CLaf), they speculated that there may be chemical alterations and protective substances produced in the plant that inhibit the multiplication of CLaf (Rossetti *et al*. 1980; Van Vuuren and Manicom 2005a).

The host responses to infection induced by inoculation of small numbers of CLas into citrus by psyllids induce a protracted 'cryptic phase' in which the pathogen cannot be detected, and indeed many CLas infections following inoculation by infectious psyllids do not lead to HLB (Gottwald and McCollum 2017). This may be due to basal defense responses that are known to differ between susceptible and tolerant varieties of citrus (Wang *et al*. 2016). It is likely that plant defense responses, such as callose deposition at sieve plates or cell wall modifications, were triggered by the prior establishment of CTV-B2 (Fu *et al*. 2016, 2017b). These may suppress the establishment and spread of CLas, and the development of symptoms of HLB. Of particular interest, genes encoding zinc transporter precursors (ZIPs) and phloem proteins (PPs) were dramatically down-regulated by CTV-B2 alone, but were up-regulated by CLas-B232 alone (Fu *et al*. 2016). These genes were slightly up-regulated by co-infection of CTV-B2 and CLas-B232 (Fu *et al*. 2017b) at early stages of coinfection. The balancing effects of opposite regulation of genes in response to CTV-B2 and CLas-B232 may have reduced phloem damage and symptom development caused by CLas-B232 in response to graft inoculations and may also explain why CLas-B232 was not detected from the trees after graft-inoculations into established infections of CTV-B2 in inoculation groups I and II (Fig. 6). This is consistent with biological evidence that trees co-infected with CTV-B2 and CLas-B232 developed less severe symptoms and survived much longer (Figs. 4, 5-A and B). The low infection rate and mild symptoms of CaLas-B232 in the presence of CTV-B2 were confirmed by inoculation with CLas-B438 in plant group III. Tree survival is the relevant biological test following the inoculations, and increased rate of survival of trees inoculated with CLas-B438 in the presence of CTV-B2 was highly significant. The experiments were carried out concurrently with matched trees in the same greenhouse, eliminating environmental factors that might affect the results. The significantly greater survival of the trees following inoculation with CaLas-B438 in this experiment is consistent with the pre-established infection of CTV-B2 activating defense pathways that had a protective effect against CTV-B438.

Studies have documented the uneven distribution of CLas in all phloem-containing tissues (Tatineni *et al*. 2008; Folimonova *et al*. 2009; Li *et al*. 2009). In our study, CLas was consistently detected at higher concentration in the rootlets than in the leaves soon after the inoculations, suggesting that CLas moved with the phloem flow soon after graft-inoculation to roots and then spread from rootlets to new flushes. This is consistent with the general pattern of phloem-limited bacteria, first observed with the aster yellows phytoplasma (Kunkel 1938) and as previously reported for CLas in trees in a commercial grove (Johnson *et al*. 2014; Wu *et al*. 2018). The initial infection of roots leads to their death (Johnson *et al*. 2014) and weakens the trees systemically by reducing the capacity for water and nutrient acquisition. This explains why micronutrient deficiency symptoms, typically zinc and iron, are present on new flush of HLB-affected trees (Johnson *et al*. 2014), and why plant vigor and reduced HLB disease severity could

be maintained through the foliar application of nutrients and several trace elements (Rouse 2013). This also can explain why many genes were differentially regulated in young leaves prior to symptom development (Fu *et al*. 2016, 2017b). Physiological changes in roots caused by the initial establishment of CLas could also elicit shifts in the composition of rhizosphere microbial community (Trivedi *et al*. 2011) and contribute to further root decline.

CLas preferentially colonized phloem in the vertical rather than the horizontal direction, regardless of whether the trees were infected by CLas alone or co-infected with CTV-B2, fitting the previously proposed model (submitted, 2018). However, it is notable that the colonization of phloem cells by CLas was less extensive in the phloem cells of trees pre-inoculated with CTV-B2 than in trees infected by CLas alone (Fig. 7). Although early ultrastructure work suggested that CLas and CTV were rarely present in the same cells of a plant (Chen *et al*. 1972), changes in gene expression induced by CTV-B2 are systemic and so may limit the multiplication and spread of CLas throughout the plant.

In contrast, inoculation with CTV-B6 led to severe disease symptoms (Bar-Joseph and Lee 1989; Brlansky and Lee 1990; Rocha-Pena *et al*. 1995; Soler *et al*. 2012). The principle symptoms observed in the co-inoculated trees were more typical of CTV-B6. These were so severe that symptoms of HLB were not apparent. It is likely that CTV-B6 became the dominant player in the co-infected trees because CTV established itself more rapidly and is more evenly distributed and in higher concentration than CLas (Bové 2006).

In contrast to pre-established infection by CTV, pre-establishment of CLas did not affect the subsequent establishment of either CTV strains. This may be attributed to the overall low concentration, slow movement and long latency of CLas (Bové 2006; Johnson *et al*. 2014) and differential activation of defense genes by the pathogens (Fu *et al*. 2016). Simultaneous inoculation of both CTV strains did not affect the establishment of either strain in our study, but trees were overwhelmingly dominated by CTV-B6 based on both biological and transcriptomic evidence (Fu *et al*. 2017a).

Conclusion

HLB is an existential threat to the citrus industry. Mild CTV strains have been extensively applied to cross protect citrus against severe CTV strains (Lee and Keremane 2013) and field-grown trees are commonly co-infected by CTV and CLas (Folimonova *et al*. 2009). The complexity of HLB symptoms in the field could result from complex interactions with other pathogens rather than from CLas alone (Graham *et al*. 2013). Nonetheless, CTV and CLas are typically studied in single infection models, rather than in co-infection models. Some co-infection studies have been attempted but the results are not consistent (Folimonova *et al*. 2009; Van Vuuren *et al*. 2000; Van Vuuren and Da Graça 2000; Van Vuuren and Manicom 2005b). This could be due to different mild CTV strains, CLas isolates, and citrus varieties used. The presence and the length of establishment of the mild CTV prior to inoculation with CLas is also important. Although our sample size was small, the biological and cellular results are consistent with the hypothesis that HLB incidence and symptoms were reduced by the presence of CTV-B2.

Acknowledgements

This research was funded by the National Natural Sciences Foundation of China (31671992), the National Key R&D Program of China (2018YFD0201500), the Fundamental Research Funds for the Central Universities of China (XDJK2018C032), the China Postdoctoral Fund (2018M633307), and also supported by the United State Department of Agriculture, Agricultural Research Serves (USDA-ARS).

Appendices associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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