Titors of ‘Ca. Libereibacter asiaticus’ in Murraya paniculata and Murraya-reared Diaphorina citri Are Much Lower Than in Citrus and Citrus-reared Psyllids

Abigail J. Walter1, YongPing Duan, and David G. Hall2
USDA-ARS Horticultural Research Laboratory, 2001 S. Rock Road, Fort Pierce, FL 34945

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Abstract. Huanglongbing, one of the most devastating diseases of citrus, is associated with the bacterium ‘Candidatus Liberibacter asiaticus’ vectored by the Asian citrus psyllid, Diaphorina citri, in North America. Murraya paniculata is a common ornamental plant that is an alternate host of both the psyllid and bacterium. We tested M. paniculata and Citrus sinensis grown together in the same field for their titer of ‘Ca. L. asiaticus’. We found the bacterium in both M. paniculata and C. sinensis, but the titer was four orders of magnitude lower in M. paniculata. We also assayed D. citri from laboratory colonies reared on either ‘Ca. L. asiaticus’-infected M. paniculata or infected Citrus spp. Psyllids reared on infected M. paniculata also carried bacterial titers five orders of magnitude lower than psyllids reared on infected Citrus spp. These observations imply resistance to huanglongbing in M. paniculata.

Huanglongbing (HLB) is one of the most devastating diseases of citrus worldwide. It is associated with the fastidious bacteria ‘Candidatus Liberibacter asiaticus’ (CLas), ‘Ca. L. africanus’, and ‘Ca. L. americana’. The bacteria are vectored by the Asian citrus psyllid, Diaphorina citri, in North America. Murraya paniculata is a common ornamental plant that is an alternate host of both the psyllid and bacterium. We tested M. paniculata and C. sinensis grown together in the same field for their titer of ‘Ca. L. asiaticus’. We found the bacterium in both M. paniculata and C. sinensis, but the titer was four orders of magnitude lower in M. paniculata. We also assayed D. citri from laboratory colonies reared on either ‘Ca. L. asiaticus’-infected M. paniculata or infected Citrus spp. Psyllids reared on infected M. paniculata also carried bacterial titers five orders of magnitude lower than psyllids reared on infected Citrus spp. These observations imply resistance to huanglongbing in M. paniculata.

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1To whom reprint requests should be addressed; e-mail David.Hall@ars.usda.gov.

1To whom reprint requests should be addressed; e-mail David.Hall@ars.usda.gov.

2To whom reprint requests should be addressed; e-mail David.Hall@ars.usda.gov.
maintained on infected Citrus spp. or infected *M. paniculata* under identical conditions in a growth chamber. All colonies were maintained at 25°C, 70% relative humidity, 14:10 h light:dark, under 400 W high-pressure sodium and metal halide growth light bulbs (Metalarc M59 M400/U and Lumalux S51 LU400 bulbs; Sylvania, Danvers, MA). The infection of *Citrus* plants originated from a field-collected shoot and was passed between plants by grafting or by placing an uninfected plant in a cage with an infected plant and an active ACP population. Colonies on *M.paniculata* were established in Dec. 2010 by placing several hundred adult ACP from an infected *Citrus* colony on uninfected *M. paniculata* plants for 7 d to transmit the pathogen, removing the adults, then adding a new population of uninfected adults to ensure that infected ACP taken from the colony originated from *M. paniculata*. All adult ACP sampled for this study had spent their entire life on the infected host plant. Samples of 21, 20, and 30 ACP were taken during Jan. 2011 from colonies reared in cages containing infected *Citrus* jambhiri Lush. (rough lemon), both infected *C.sinensis* and *Citrus paradisi* Macf. (grapefruit), or infected *M. paniculata*, respectively. An additional 24 adults were sampled from the *M. paniculata* colony in Feb. 2011. At the time of sampling, *Citrus* plants in the colonies had visual symptoms of HLB, and the *M. paniculata* were quantitative PCR-positive using the LJ900 primers (Morgan et al., 2012) with a maximum of 7678 hyv/hyvII copies per reaction. Crude extracts of individual adults were made as described in Walter et al. (2012), and PCR was performed using 2 μL of crude extract per reaction.

Samples were assayed for the presence of CLas using two quantitative PCR primer sets. We assayed for the presence of the internal repeat sequence of the hyv/hyvII repeat pro- phage genes (Zhao et al., 2011) using the LJ900 primers (forward GCGCATTTAAC ACAAGAAGTGATATC, reverse ATAACATCAATTGCTGTAGTATCAGAC) as described by Morgan et al. (2012). This primer set is very sensitive to low-titer infections but is not ideal for absolute titer quantification because the primers target the nearly identical tandem repeats of two prophage genes with varying numbers of repeats in different CLas isolates (Zhou et al., 2011). We use the number of detected repeats in the samples, as determined by standard curves built using negative samples from each extraction method and the pLJ153.1 plasmid described by Morgan et al. (2012), as a relative measure of the number of bacterial genomes. For the LJ900 primers, quantitative PCR was performed in a 15 μL reaction using PerfeCTa SYBR Green FastMix 2× master mix (Quanta Biosciences, Inc., Gaithersburg, MD), a re- action concentration of 600 nM forward and 900 nM reverse primer, and nuclease-free water with a temperature program of 95°C for 5 min, then 50 cycles of 95°C for 3 s, followed by 62°C for 30 s, then one cycle of 95°C for 15 s, 62°C for 1 min, and a gradual ramp to 97°C for 15 s. All samples that amplified on those primers were rerun in triplicate. A sample was not considered positive unless at least two of the three triplicate samples amplified and had the correct melt profile. Samples were also tested for the presence of the 16S rDNA of the CLas genome using the HLBaspr primers (forward TCG AGCGGATGTCAATACG, reverse GCGGTATTCGCGTAAAGGTAG, probe AG ACCGGTGTAAACCGC with 6-FAM reporter dye and TAMRA quencher) developed by Li et al. (2006). This method is less sensitive than the LJ900 primers for low-titer samples. For the HLBaspr primers, quantitative PCR was performed in a 20 μL reaction using TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA), a re- action concentration of 0.4 mM of each primer, 500 nM probe, and nuclease-free water with a temperature program of 95°C for 5 min then 50 cycles of 95°C for 3 s followed by 60°C for 30 s. Each sample was run only once on the HLBaspr primers. A detection threshold of 0.02 ARn was used for all samples. All PCR was performed on an ABI 7500 Fast system (Applied Biosystems).

We tested the normality of the hyv/hyvII copy number using the Shapiro-Wilk statistic and compared the number of repeat copies detected in the plant or psyllid samples using the nonparametric Kruskal-Wallis test (Proc UNIVARIATE, Proc TTEST; SAS Institute, 2008).

**Results**

*Candidatus Liberibacter asiaticus* in plant material. Four of the 120 *M. paniculata* samples consistently amplified using the LJ900 primers, but the titer of bacteria in these plants was always low (Table 1). None of the *M. paniculata* samples that were negative using the LJ900 primers had detectable levels of CLas 16S rDNA; one of the LJ900-positive *M. paniculata* samples amplified on the HLBaspr primers with a Cq value of 32.8. Yellowing symptoms had been observed on most of the sampled *M. paniculata* and did not appear to be associated with CLas infection. All 10 of the *Citrus* samples had detectable CLas 16S rDNA (Cq range, 26.9 to 30.6). The hyv/hyvII copy numbers of the *C.sinensis* samples were non-normal (*W* = 0.80, *P* = 0.0139), so numbers of repeats were compared by the nonparametric Kruskal-Wallis test. The bacterial titer of *M. paniculata* was significantly lower than the titer found in *C.sinensis* (Murraya range, 39 to 6.0 × 10^1^ repeats per reaction; median, 811 repeats; *Citrus* range, 2.7 × 10^1^ to 3.1 × 10^3^ repeats per reaction; median, 6.8 × 10^2^ repeats; *χ^2^* = 8.00, df = 1, *P* = 0.0047 (Fig. 1). There was a 10-fold difference in the median values of CLas titers in ACP from colonies on Murraya and Citrus.

**Discussion**

We have observed that the titer of CLas in *M. paniculata* was four orders of magnitude lower than the titer observed in diseased *C. sinensis*. Our observations are similar to findings from a quarantine laboratory study where CLas titers in *M. paniculata* inoculated with bacteria from *Citrus* were generally low and which subsequently declined or became undetectable over a period of 32 months (Damsteegt et al., 2010). Other reports support that titers of CLas are low in field-collected *M. paniculata* (Deng et al., 2007a; Walter et al., 2012; Zhou et al., 2007). These results are valid even if the number of hyv/hyvII repeats varies between CLas in the two plant species as a result of the magnitude of the difference we describe. The lower titer found in *M. paniculata* relative to *Citrus* suggests that *M. paniculata* is resistant to CLas. However, a large proportion of the CLas present in infected citrus is non-viable (Foliomova and Achor, 2010; Trivedi et al., 2009), so it is possible that the results from *Citrus* are artificially inflated as a result of the sampling of dead bacteria.

More importantly, we found that the titer of CLas in adult *D. citri* reared from infected *M. paniculata* was also four orders of magnitude lower than the CLas titer in psyllids reared from infected *Citrus* based on differences in

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**Table 1.** Date sampled, number tested, positive results by two quantitative polymerase chain reaction methods, and median copy number of hyvI/hyvII repeats from *Citrus sinensis* and *Murraya paniculata* growing in the same field and Asian citrus psyllid (ACP) cultured on infected *Citrus* spp. and *M. paniculata* under identical conditions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Date tested</th>
<th>Positive samples by HLBaspr</th>
<th>Positive samples by hyvI/hyvII</th>
<th>Median copy number detected by hyvI/hyvII</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. paniculata</em></td>
<td>Aug. to Sept. 2010</td>
<td>120</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>May 2010</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Jan. to Feb. 2011</td>
<td>54</td>
<td>16</td>
<td>24</td>
</tr>
<tr>
<td>ACP feeding on <em>M. paniculata</em></td>
<td>Jan. 2011</td>
<td>41</td>
<td>40</td>
<td>41</td>
</tr>
</tbody>
</table>

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It is not known whether the low CLas titers that we observed in ACP from *M. paniculata* are the result of differences in CLas load in the gut (for example, ACP feeding on infected *M. paniculata* may acquire less CLas than when they feed on infected *Citrus*) or a consequence of biological differences in CLas obtained from *Citrus* vs. *M. paniculata*. Although CLas was reported to replicate in ACP (Hung et al., 2004; Inoue et al., 2009), the low titers of CLas in ACP that acquired the bacterium from infected *M. paniculata* show that this replication does not overcome the titer differences resulting from a different amount of bacteria being acquired by ACP on the different hosts or possible differences in the rate of replication of CLas acquired from different plants. ACP reared from infected *M. paniculata* have been shown to transmit detectable levels of CLas to *Citrus* (Damsteegt et al., 2010). Whether the CLas transmitted from *M. paniculata* to ACP by ACP induce symptoms of HLB is currently under investigation.

CLas occurred at much lower titers in both *Murraya paniculata* and ACP reared from infected *M. paniculata* than in both diseased *Citrus sinensis* and ACP reared from infected *Citrus* sp. Further work on *M. paniculata* may be useful on two fronts. First, we need to understand whether ACP that acquires a CLas bacterium from *M. paniculata* is capable of transmitting CLas that will cause typical HLB disease in *Citrus*. Second, *M. paniculata* may not support CLas multiplication, which implies a potential resource for cloning resistance gene(s). The answers to both of these questions may yield new approaches for management of HLB in citrus.

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