4.16 Isothermal Detection of Huanglongbing in Psyllids and Citrus Tree Samples

Russell, P.F., McGowen, N., Bohannon, R. Agdia, Inc., Elkhart, IN, USA

Huanglongbing (HLB) disease is found throughout Asia, in Brazil, Mexico, the USA, and parts of Africa and has seriously affected citrus production in many regions. Three species of the causative agent Candidatus Liberibacter, which is vectored by psyllids, have been identified. These are Candidatus Liberibacter asiaticus, Candidatus L. americanus, and Candidatus L. africanus.

We report here on two methods for detecting HLB using isothermal nucleic acid amplification. Specific detection and identification of the three species is possible using DNA purified from infected psyllids and citrus trees as well as using crude extracts prepared from the same samples. Plant samples tested include infected citrus trees from South Africa, Brazil, and the USA.

Materials and Methods

Psyllid extracts were kindly provided by Dr. John Hartung. Citrus leaves infected with L. asiaticus, L. americanus, or L. africanus were provided by Dr. John Hartung, Dr. Barry Manicom, and Dr. Marcelo Pedreira de Miranda.

Samples were initially processed using the Norgen Plant/Fungi DNA Isolation Kit (Catalog # 26200) to isolate pure DNA for use as comparison controls. Crude extracts were prepared by excising leaf midribs and grinding the samples at a 1:5 ratio in Extraction Buffer. Neat or diluted extract was then used in the AmplifyRP® test. Psyllid DNA extracts were used neat or diluted serially.

Detection of the target sequence with Agdia’s isothermal AmplifyRP® test was done according to the standard protocol, using primers and probes targeted to the region surrounding the protein chain elongation factor. A single microliter of target sample (purified DNA or crude extract) is used for each reaction. Two methods for detection are available: 1) the “exo” test uses real-time fluorometric measurement and is complete after 12-15 minutes, and 2) the “nfo” test is an endpoint assay utilizing a strip to detect the amplicon and provides results in 30 minutes.

Results

The exo and nfo AmplifyRP® assays were optimized using either psyllid DNA (L. asiaticus) or DNA isolated from infected plants (L. africanus and L. americanus). Multiple primer/probe combinations were tested to achieve the maximum sensitivity and specificity for each assay. Optimized assays were then tested using extracts prepared from infected plant tissue.
Figure 1, Graph A illustrates the specificity and sensitivity of the *L. asiaticus* exo test for diluted crude plant extracts. The test can detect HLB at a dilution of 1:1000 of the initial extract and evinces no cross-reactivity with either *L. americanus* or *L. africanus*.

Graph D compares the detection of *L. asiaticus* in psyllid DNA extracts that were previously tested using quantitative PCR. AmplifyRP® is able to detect HLB in samples with a wide range of Cq values.

Graphs B and C show the specificity and sensitivity for the *L. americanus* and *L. africanus* exo tests, respectively. The *L. americanus* assay is sensitive down to a 1:100 dilution of crude plant extract. It demonstrates no cross-reactivity with either DNA from either *L. asiaticus* or *L. africanus*. The *L. africanus* assay is not completely specific, as it recognizes *L. asiaticus*. However, its sensitivity for *L. africanus* is 10-fold higher than it is for *L. asiaticus*.

Figure 2 shows examples detection on strips of the AmplifyRP® nfo *L. asiaticus* and *L. americanus* tests. Panel A demonstrates the specificity and sensitivity of the *L. asiaticus* test. Panel B provides an example of the specificity of the *L. americanus* test. The AmplifyRP® nfo *L. africanus* test shows a similar cross-reactivity to *L. asiaticus* as the exo test, with sensitivity to *L. africanus* 10-fold greater than for *L. asiaticus*.

Discussion

Using AmplifyRP®, we can detect all three HLB varieties with a high degree of sensitivity. AmplifyRP® can also identify, either directly or through a process of elimination, those varieties. A summary of this can be found in Table 1. While more extensive testing needs to be done, we believe that AmplifyRP® is a fast and easy method for detection of HLB, either as a primary screen or for confirmation. The test can be performed using crude plant extracts and produces results in under 30 minutes total.

Acknowledgements

We would like to thank the following individuals and their labs for help with this project: Dr. Barry Manicom, ARC-ITSC, for providing plant material infected with *L. Africanus*; Dr. John Hartung, USDA-ARS, for providing psyllid DNA extracts containing *L. asiaticus* and plant material infected with *L. asiaticus, L. americanus*, and *L. africanus*; and Dr. Marcelo Pedreira de Miranda, FUNDECITRUS-Brazil, for providing plant material infected with *L. africanus*.

Reference

Fig. 1. **Graph A** illustrates the specificity and sensitivity of the *L. asiaticus* exo test for diluted crude plant extracts. **Graph D** compares the detection of *L. asiaticus* in psyllid DNA extracts that were previously tested using quantitative PCR. **Graphs B and C** show the specificity and sensitivity for the *L. americanus* and *L. africanus* exo tests, respectively.
Fig. 2. nfo detection of HLB plant samples. **Panel A:** *L. asiaticus* test on dilutions of crude extract. Strips 1-3 Las 1:125, 1:625, 1:3125. Strip 4 Lam neat, strip 5 Laf neat. **Panel B:** *L. americanus* test on 4 ng total plant DNA. Strip 1 Lam, strip 2 Laf, strip 3 Las.

<table>
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<th>Test</th>
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<th>Infected plant crude extract</th>
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<td></td>
<td>Cq 19.1-19.8</td>
<td>Cq 25.9-26.6</td>
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<tr>
<td><em>L. asiaticus</em></td>
<td>1:1000</td>
<td>1:100</td>
<td>ND</td>
</tr>
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<td><em>L. africanus</em></td>
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<td><em>L. americanus</em></td>
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Table 1. Summary of limits of detection for HLB tests.