Improved Extraction of DNA of Ca. Liberibacter Species from Plants and Cultivated Cells Using Pressure Cycling Technology (PCT)

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ABSTRACT

Huanglongbing, one of the most destructive diseases of citrus, is caused by three species of Liberibacter. Diagnosis of the disease is reliant on real-time PCR (RT-PCR). Detection of the pathogen is complicated, especially from small survey samples, because of low titer and uneven distribution of the bacterium throughout the infected plant and the complex nature of the plant tissue from which it is extracted. Cultured cells of Liberibacter species are also difficult to disrupt for efficient DNA extraction. Pressure cycling technology (PCT) is a dynamic technique that can be used for the highly-efficient extraction of protein and nucleic acids from simple and complex samples. Here we compare DNA extraction methods on the three known citrus species of Liberibacter from infected plants and cultured cells using PCT with the PCT Shredder™ and NEP 2320 Barocycler™ (Pressure Biosciences Inc., South Easton, MA) and commercially available DNA extraction kits. Combinations of these techniques and methods were tested and the resulting samples were used with RT-PCR on the Applied Biosystems 7900 (Foster City, CA).

MATERIAL AND METHODS

Liberibacter samples. Source leaf material of Liberibacter asiaticus came from symptomatic citrus trees obtained from Dr. Robert Shatters from the USDA research station at Fort Pierce, FL. Cultures of *L. americanus* and *L. africanus* came from isolations of infected symptomatic trees growing in a Percival lighted dew chamber (model E 54-UDL, Perryville Iowa).

Plant Tissue. Three leaves from 12 samples of suspect infected leaves and one sample of healthy control leaves had their mid-veins removed, cut into 2cm sections, and surface sterilized in a 10% commercial bleach solution. The 2cm sections were divided into four groups and processed by mincing the tissue in 400ul of water with a sterile scalpel or placing the tissue into a PCT Shredder Tube™ (Pressure Biosciences Inc., South Easton, MA) and grinding it for 20s in 400ul of water in the Shredder Stand (Figure 1.) or placing the tissue into a PCT Shredder Tube™ and grinding it for 20s in 1ml of DNAzol® (Molecular Research Center, Cincinnati, OH) or frozen with liquid nitrogen and ground up in a mortar with a pestle. Both the minced and shredded tissue in water samples were allowed to stand for 15 minutes before the liquid (tissue extract) was removed and transferred to 1.5ml eppendorf tubes for further processing. The shredded tissue in DNAzol® was placed in the NEP 2320 barocycler at 40C and run for 40 cycles of 45,000psi for 25 seconds followed by ambient pressure for 10 seconds. Following pressure cycling, DNA was extracted from this sample according to the manufacturers' directions for DNAzol. The ground tissue was processed for DNA extraction with the Plant DNeasy Kit™ (Qiagen, Valencia, CA) according to the manufacturers' directions.

Figure 1. The PCT Shredder Tube™, Shredder Stand and tool for grinding tissue samples. (a) Tissue samples are loaded into the bottom of the tube (top in picture) and sealed in with a ram (right of tube). The extraction liquid is then added to the tube (bottom in picture) closed with the lid (left of tube). (b) The tube is seated in the PCT Shredder stand and ground using the grinding tool before being placed into (c) the NEP2320 Barocycler for pressure cycling

Cultures. Three sets of duplicate plates for each of *L. africanus* and *L. americanus* isolated from symptomatic inoculated leaves (Figure 2.) were washed from Liber G agar medium with 400ul of sterile distilled water. The plate washing was divided into three 100ul subsamples and processed by (A) extraction with the Qiagen DNeasy Kit™ (Valencia, CA) according to the manufacturers instructions for Gram negative bacteria, or (B) the addition of 900ul of DNAzol® and pressure cycling as above or (C) no processing. DNA was eluted in 100ul of sterile water. The concentration of DNA recovered from each sample was measured on the ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE).

Figure 2. Symptomatic leaf from a tree inoculated with *L. africanus* used as source material for isolations

Real-Time PCR. RT-PCR was run according to Schuenzel et al.[1] on the Applied Biosystems 7900 (Foster City, CA). The minced tissue and shredded tissue in water were tested directly (no additional processing) and after being boiled for 5 minutes. The DNA extracted by PCT in DNAzol® and the Plant DNeasy Kit™ were tested directly. Plate washings and DNA from cultures were tested directly.

RESULTS

Plant Tissue RT-PCR Results. Results of the individual samples are summarized in Table 1. The use of PCT with DNAzol® greatly increased the frequency of positive results from symptomatic tissue. Using pressure cycling together with The PCT Shredder™ and DNAzol® found an increase in RT-PCR positive samples of *L. asiaticus* by 17-42% from infected plants over other methods. Boiling tissue extracts instead of testing them directly increased the likelihood of detecting Ca. L. asiaticus by 8-25%. All samples were positive for RT-PCR for PCT with DNAzol® whereas all other methods resulted in 10 or fewer positive detections.

Table 1. RT-PCR Results from Plant Tissue

	Minced Direct	Minced with Boiling	Shredded Direct	Shredded with Boiling	Plant DNeasy	PCT with DNAzol
A	++	++	-	++	++	+++
B	+	++	-	-	+	++
C	-	+	++	++	-	++
D	+	++	+	++	-	+++
E	-	-	-	-	-	-
F	+	++	+	++	-	+++
G	-	-	-	-	-	+
H	++	++	-	++	-	+++
I	-	++	++	++	++	+++
J	+	-	+	-	+	+++
K	+	++	+	++	++	+++
L	-	+	+	++	+	+++
M	-	++	++	++	++	+++

Sample E was the healthy plant negative control.
- No Amplification. Amplification curve failed to cross the Cycle threshold (Ct) within 50 cycles,
+++ Ct Value between 20 and 30 cycles, ++ Ct Value between 30 and 40 cycles, + Ct Value between 40 and 50 cycles.

RT-PCR Results from Cultures. All three samples for both *L. africanus* and *L. americanus* were negative by RT-PCR for the direct and DNeasy samples; all three samples were positive for the PCT with DNAzol® extracted samples (Not Shown). The ND-1000 spectrophotometer failed to detect a measurable amount of DNA in the DNeasy samples; a range of 2.8-6.6ng/ul was found for the *L. africanus* cultures and 2.0-4.0ng/ul for the *L. americanus* cultures in the PCT with DNAzol® samples.

Discussion. Detection of HLB from plant samples and cultures requires sensitive and specific real-time primers and probe, but also requires a versatile and reliable method of extracting DNA from those samples. While a simple crude preparation of plant samples would be the easiest method, RT-PCR results from this technique are variable and amplification curves are jagged and limited by RT-PCR inhibitors. Boiling the sample preparations improves the shape of the curve and removes most of the inhibitors, however the results are still variable. Neither sampling method is reliable for detection of low concentrations of any of Liberibacter species tested. DNA extraction using a common commercial kit, resulted in lower number of RT-PCR positive results from plant material and no positive results from culture samples. The PCT Shredder Tube™ in combination with DNAzol® and PCT, provide a reliable and highly efficient method of DNA extraction from both plant and culture samples giving earlier Ct values and robust amplification curves for RT-PCR and a greater DNA yield and number of positive amplifications from cultures.

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Reference.

1. Schuenzel, E. L., Sechler, A., Wang, Z., Hu, H., Coletta-Filho, H., and Schaad, N. W. 2007. Duoplex real-time polymerase chain reaction protocol for the detection of *Candidatus* Liberibacter asiaticus and *Can.* L. americanus in citrus. Phytopathology 97:S106

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