3.2 Improved Detection of Low-titer, Non-lethal, Seed Transmitted *Candidatus* Liberibacter asiaticus in Citrus, Periwinkle and Dodder Using Nested PCR

Benyon L.S.[1], Zhou L.-J.[2], Duan Y.-P.[1], Powell C.A.[2], and Gottwald T.R.[1]

[1]USDA-ARS-USHRL, Fort Pierce, FL 34945, [2]University of Florida, IFAS-IRREC, Fort Pierce, FL 34945

Introduction:

Citrus Huanglongbing (HLB), a lethal disease of citrus, is widespread, occurring in most citrus producing areas, including Asia, Africa, and the Arabian Peninsula. It was most recently reported in Brazil and Florida. Since the initial report of the disease in Florida in August of 2005, HLB has spread throughout thirty of the citrus producing counties (2). The disease is associated with phloem-limited fastidious α-Proteobacteria in the genus Liberibacter. *Candidatus* Liberibacter asiaticus (Las) is the most widely-distributed species and the only species that has been detected in Florida to date. Las bacterium lives in the sieve tube cells of infected plants. The low concentration and uneven distribution of the bacterium in HLB-infected host plants make it difficult to detect consistently. Many detection methods have been developed, including biological indexing, light or electron microscopy, polymerase chain reaction (PCR), real-time PCR, and loop-mediated isothermal amplification. Currently conventional PCR and quantitative real-time PCR (qPCR) are widely used for HLB detection and as a confirmation assay. Due to the low titer of the HLB pathogen in certain HLB disease phenotypes, its insect vectors, and the progenies of the HLB-infected plants, it is necessary to develop an extremely sensitive detection technique to reduce false negative results. Conventional PCR has been found to be up to 1,000 fold less sensitive than qPCR (4,5) and nested PCR (5). Both nested and qPCR have been shown to have a detection limit of 10 Liberibacter cells per reaction. Here we report that by using nested PCR we were able to detect Las bacterium from samples that were considered negative by qPCR. Las can be transmitted via insect vector, the Asian citrus psyllid (*Diaphorina citri*), by grafting, or through dodder transmission (1,3,6). However, there is little information on the seed transmission of HLB. Determining if the Las bacterium is seed transmissible is crucial for HLB disease control. Periwinkle (*Catharanthus roseus*) and dodder (*Cuscuta campestris*) are two experimental host plants in which HLB bacteria can multiply well. We tested for seed-transmission in these two host plants along with trifoliate (*Poncirus trifoliata*, syn. *Citrus trifoliata*) and sweet orange (*Citrus sinensis*) seedlings. The main objective of this research was to determine if the Las bacterium can translocate from seeds to seedlings and cause HLB symptoms in host plants.

Methods:

Fresh dodder, periwinkle and citrus samples were collected, kept on ice, and DNA isolation was begun on the same day. The Qiagen DNeasy Plant mini protocol was used with modifications.

Primer pairs listed in Table 1 were used for conventional PCR, nested PCR and quantitative PCR. Selected PCR products were cloned into the TOPO TA cloning vector pCR2.1, and subjected to sequence analysis. Real-time PCR amplifications were performed using Applied Biosystems 7500 Real-Time PCR System, and data were analyzed using Applied Biosystems 7500 system SDS software version1.2.

Nested PCR was performed in a Peltier Thermal Cycler 200 (MJ Research) using different primer sets for the three genes, 16S rDNA, beta-operon and the outer membrane protein, with 40 cycles for first round PCR and 35 cycles for second round PCR.

Table 1. Las specific primer sequences.

Primer	**Target**	**Sequence (5'-3')**	**Annealing Temp**	**Citation**
OI1	16S rDNA	GCGCGTATGCAATACGAGCGGCA	64°C	Jagoueix, S. et al., 1996
OI2C	“ “	GCCTCGCGACTTCGCAACCCAT	64°C	Jagoueix, S. et al., 1996
LJ74f	“ “	CGGGCGATTAAGTTAGAGGT	54°C	Duan, Y.P. et al., 2008
CGO3f	16S rDNA	RGGGAAAGATTTTATTGGAG	53°C	Zhou, L.J. et al., 2007
CGO5r	‘’ ‘’	GAAAATAYCATCTCTGATATCGT	53°C	Zhou, L.J. et al., 2007
MHO317	β-operon	GTGTCTCTGATGGTCCGTTTGCTTCTTTTA	64°C	Hoy, M. et al., 2001
MHO319	‘’ ’’	GAACCTTCCACCATACGCATAGCCCCTTCA	64°C	Hoy, M. et al., 2001
Bf2	β-operon	GCGTTCATGTAGAAGTTGTG	53°C	Ding, F. et al., 2005
Br2	‘’ ’’	CCTACAGGTGGCTGACTCAT	53°C	Ding, F. et al., 2005
OMP6f	*omp*	CACCGTAGAAGGGCATATTGAT	59°C	This Study
OMP1r	""	CATGCGATTACCTATACGAAAACC	59°C	This Study
OMP3f	“ "	CCCTCAATTTCTATCCGCT	59°C	This Study
OMP2r	" "	TTATCTGACAMCAAACGGTAT	59°C	This Study
HLBas	16S rDNA	TCGAGCGCGTATGCAATACG	58°C	Li, W. et al, 2006
HLBr	" "	GCGTTATCCCGTAGAAAAAGGTAG	58°C	Li, W. et al, 2006
Probe				
HLBp		56-FAM/AGACGGGTGAGTAACGCG/3BHQ-1		Li, W. et al, 2006

Results:

Most HLB-infected periwinkle plants died within 6 month after inoculation, but seed transmitted Las-positive periwinkle plants survived for over one year. The periwinkle progenies from HLB-infected plants did not show blotchy-mottling but did exhibited atypical HLB symptoms, denoted by vein yellowing only when they were stressed by nutrient deficiency (Figure 1). Symptoms disappeared after the stress was removed. These results suggest that although Las was seed transmitted, it was either not the form that caused severe HLB symptoms and death, or a second, undescribed component of an HLB disease complex was not transmitted.

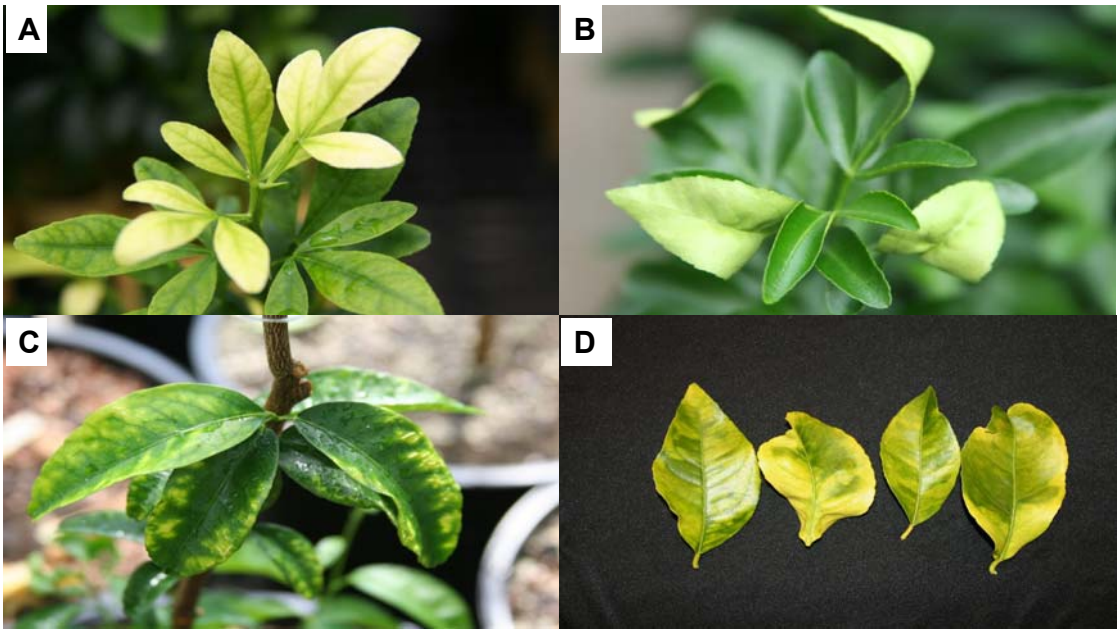
Figure 1 Phenotypic variations of Huanglongbing in periwinkle and their association with Las bacterial titer. **A**. HLB affected periwinkle leaves with typical blotchy mottle and a high titer of the Las bacterium. **B**. Vein yellowing periwinkle leaves with an extremely low titer of the Las bacterium.

The increased sensitivity of nested PCR allowed us to detect the Las bacterium in 14% more seed-transmitted periwinkle samples than conventional PCR and in 20% more samples than qPCR (Table 2).

Table 2. Detection of seed-transmitted Las in Dodder and Periwinkle samples by conventional PCR, nested PCR and qPCR

	Conv. PCR	**Nested PCR**	**qPCR**
Dodder	0/34	27/34	NT
Periwinkle (1)	22/82	58/82	NT
Periwinkle (2)	NT	77/99	57/99

Seed transmitted citrus seedlings did not display typical HLB associated blotchy mottle but did show other symptoms such as leaf yellowing, stunting, leaf curling and vein yellowing (Figure 2).



The increased sensitivity of nested PCR allowed us to detect the Las bacterium in 17% more seed-transmitted citrus samples than conventional PCR and in 10% more samples than qPCR (Table 3).

Table 3. Detection of seed-transmitted Las in citrus samples by conventional PCR, nested PCR and qPCR

	Conv. PCR	**Nested PCR**	**qPCR**
Citrus	25/170	54/170	7/32

In this work we evaluated and developed sets of primers useful for nested PCR detection of citrus Huanglongbing. Using these we were able to detect the Las bacterium in low-titer, seed transmitted host plants that were undetectable by conventional PCR or qPCR. Increasing the sensitivity of Las detection assays is of critical importance for the early discovery of the disease and for pathogen-free nursery maintenance.

Although nested PCR, with high affinity primers, is highly sensitive, false negatives will still occur if the template DNA falls near the detection threshold. Therefore sample duplication is necessary. Also due to more manipulations with nested PCR, extra caution has to be taken to avoid cross contamination.

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