HortScience 46(6):854–857. 2011.

Levels of *Candidatus* Liberibacter asiaticus and *Xanthomonas citri* in Diverse Citrus Genotypes and Relevance to Potential Transmission from Pollinations

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Additional index words. breeding, citrus canker, huanglonging, HLB

Abstract. **The diseases huanglongbing [HLB, associated with *Candidatus* Liberibacter asiaticus (CLas)] and Asian citrus canker [ACC, caused by *Xanthomonas citri* (Xcc)] are widespread in Florida and many other citrus-growing areas, presenting unprecedented challenges for citrus breeding. Because HLB and ACC weaken trees and compromise cropping, breeding is much less efficient using seed parents that have been exposed to these diseases. Therefore, it would be highly desirable to use unique disease-exposed selections only as pollen parents with pollen applied to disease-free trees. However, there may be a risk of introducing these diseases using such pollen sources. To assess this potential, abundance of the pathogens associated with these diseases was assessed in anthers and flowers using quantitative polymerase chain reaction. Because CLas is systemic, levels on mature leaves from the flower source trees were assessed to see if the presence of CLas in flowers was associated with leaf levels. Disease-exposed trees were tested in 10 genotypes from each of three broad genotypic categories, which reflect different levels of susceptibility to the diseases associated with the pathogens studied: *Poncirus trifoliata* hybrids (most resistant to HLB), *Citrus maxima* and hybrids (susceptible to both diseases), and *C. reticulata* and hybrids (considerable resistance to ACC). Of the 30 samples of each tissue type analyzed for CLas, 88% of mature leaves, 69% of flowers, and 88% of anthers had one or more CLas bacterium per sample. The trifoliate genotypic group had significantly lower levels of CLas than the pummelo and mandarin groups in mature leaf samples, but CLas levels were more similar between groups in anther and flower samples, and the pathogen was present in most of the trifoliate hybrids tested. Mean numbers of CLas detected per nanogram nucleic acid were 100 to 800 times higher in mature leaf samples, most characteristic of HLB symptoms, compared with anther samples. Xcc DNA was detected in 30% of flower samples and 23% of anther samples. No significant differences in Xcc levels were found between tissue type or genotypic group. However, regressions between Xcc levels in flowers and percent of plant pedigree derived from mandarin had a negative correlation and an r^2 of 0.159 ($P = 0.029$). The biology of CLas is consistent with the pathogen being present in anthers from unopened flowers, whereas the ACC pathogen detected inside flowers was likely the result of contamination despite great care in sample collection and handling. Where exceptional diligence to exclude HLB and ACC is appropriate, results suggest that there may be a risk of spreading these pathogens through use of pollen from trees on infected farms.**

Two devastating citrus diseases have become widespread in Florida in the last 6 years and are providing unprecedented obstacles to citrus breeding. ACC (caused by Xcc) has been introduced into Florida on at least three occasions. Despite successful eradication after the first two introductions and substantial containment after the third introduction, the pathogen was widely spread by hurricanes in 2004 and ACC became endemic in Florida (Gottwald et al., 2002; Irey et al., 2006). HLB (associated with the bacterium CLas) was verified in Florida in 2005 (Gottwald et al., 2007) and is now present in all commercial citrus-growing counties. Development of resistant cultivars is needed, and there are sources of resistance to these diseases within the citrus gene pool, including advanced selections in the USDA/ARS citrus breeding program. Several advanced hybrids of trifoliate orange (*Poncirus trifoliata*) have fruit quality approaching commercial acceptance and appear to have some field resistance to HLB. Similarly, some mandarin and pummelo hybrids appear to offer resistance to ACC.

Received for publication 8 Apr. 2011. Accepted for publication 19 Apr. 2011.
The technical assistance of Scott Ciliento and Angela Ledger are gratefully acknowledged.
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HLB was found in the USDA Ft. Pierce, FL, farm in 2006, and infected trees are maintained to enhance research on HLB resistance and control. ACC was also confirmed on this farm in 2006 and is now widespread with numerous trees displaying lesions. In contrast, before starting this work, neither HLB nor ACC had been found at the USDA Leesburg, FL, farm, although HLB was recently confirmed at this site and will be controlled. Advanced breeding lines are present at both farms with mature trees of recently identified selections, suitable for crossing, usually present as single seedling-derived individual trees. Because HLB and ACC weaken trees and compromise cropping, breeding is much less efficient using seed parents affected by these diseases. In addition, moving pollen has previously permitted crosses between parents in widely separated collections, including different countries. Therefore, it would be highly advantageous if pollen could be carried from potentially infected trees to hybridize onto clean trees without significantly risking the spread of disease. Potentially affected breeding parents include genotypic groups that are significantly less susceptible to each disease with *Poncirus trifoliata* hybrids displaying considerable resistance to HLB (Folimonova et al., 2009) and *C. reticulata* and hybrids having considerable resistance to ACC (Hu, 2009; Sarkar et al., 2007).

Several hypotheses related to use of pollen from HLB- and ACC-exposed trees were tested in this study: 1) that anthers from unopened flowers would be free of CLas and Xcc; 2) that more resistant genotypes would have markedly reduced levels of CLas and/or Xcc; and 3) that levels of CLas in anthers would be correlated with CLas levels in disease-diagnostic samples of mature leaves. To test these hypotheses, anthers, flowers, and leaves were collected from trees on the farm where ACC and HLB are widespread and were tested for levels of the bacteria associated with these diseases.

Materials and Methods

Plant material. Individual trees of 10 genotypes from each of three broad genotypic categories were selected from the variety collection at the USDA Ft. Pierce, FL, farm (Table 1). The categories were *Poncirus trifoliata* hybrids (none of which were deciduous), *Citrus maxima* and hybrids, and *C. reticulata* and hybrids, reflecting major USDA breeding emphases and wide variation in reported susceptibility to ACC and HLB. On each tree at two sampling times, the following were collected: six flowers at the late popcorn stage (typical of flowers used for controlled pollinations, just before petals open) and mature leaves with appearance most characteristic of HLB infection or from similar tree position and shoot age when no symptoms were observed. Care was taken to collect flower samples before leaf samples and with cleaning of instruments between samples to minimize cross-contamination.

Table 1. Analyses were conducted of *Candidatus* Liberibacter asiaticus (CLas) levels by quantitative polymerase chain reaction (qPCR) of 16S rDNA gene and of *Xanthomonas citri* (Xcc) levels through qPCR of *pthA* gene from indicated tissues of listed genotypes maintained in close plantings at the USDA ARS Ft. Pierce, FL, farm.[z]

		CLas genomes/ng nucleic acid			Xcc genomes/ng nucleic acid		Pedigree proportion estimation		
Genotype	Genotypic group	Anther samples	Flower samples	Leaf samples	Anther samples	Flower samples	From mandarin	From pummelo	From trifoliate
Mean	Mandarin B[y]	6.5 C[x]	7.68 C	3340 D	0.224 G[u]	0.003 G	0.9	0.1	0.0
Mean	Pummelo B	2.6 C	6.94 C	1551 D	0.010 G	0.136 G	0.3	0.7	0.0
Mean	Trifoliate A	1.7 C	0.85 C[v]	167 C	1.470 G	0.140 G	0.3	0.2	0.5
Mean	Combined groups	3.6 E[w]	5.2 E	1686 F	0.567	0.088	0.5	0.3	0.2
Fairchild	Mandarin	0.0	0.01	508	0.003	0.000	0.84	0.16	0.00
Fremont	Mandarin	1.7	1.10	7407	0.172	0.000	1.00	0.00	0.00
Kinnow	Mandarin	0.0	0.00	2921	0.003	0.000	0.94	0.06	0.00
Minneola	Mandarin	4.7	0.01	1918	0.003	0.000	0.69	0.31	0.00
Monreal Clementine	Mandarin	22.9	0.07	9990	0.452	0.002	1.00	0.00	0.00
Murcott	Mandarin	0.0	0.00	4505	0.003	0.000	0.88	0.13	0.00
Murcott seedless	Mandarin	35.4	0.02	640	0.003	0.000	0.88	0.13	0.00
Ortanique	Mandarin	2.4	75.30	772	0.014	0.000	0.88	0.13	0.00
Osceola	Mandarin	0.2	0.00	4647	0.089	0.000	0.84	0.16	0.00
Wilking	Mandarin	0.0	0.01	924	1.500	0.025	0.94	0.06	0.00
Chironja	Pummelo	0.0	0.01	0.1	0.004	0.001	0.38	0.63	0.00
Hirado	Pummelo	9.3	2.06	2032	0.003	0.000	0.00	1.00	0.00
Hybrid 9-24-26	Pummelo	9.6	2.03	277	0.025	0.415	0.00	1.00	0.00
Jackson	Pummelo	0.0	0.13	3088	0.022	0.001	0.56	0.44	0.00
Melogold	Pummelo	0.8	30.44	2891	0.022	0.000	0.13	0.88	0.00
Oroblanco	Pummelo	1.0	1.57	1026	0.003	0.536	0.13	0.88	0.00
Pearl	Pummelo	1.5	0.26	1085	0.003	0.000	0.69	0.31	0.00
Royal	Pummelo	0.2	29.45	1576	0.003	0.274	0.56	0.44	0.00
Triumph	Pummelo	0.1	0.28	2170	0.003	0.000	0.56	0.44	0.00
US Seedless Surprise	Pummelo	0.0	3.43	530	0.003	0.000	0.34	0.66	0.00
2610	Trifoliate	0.2	0.94	434	0.003	0.005	0.19	0.31	0.50
4717	Trifoliate	0.0	0.01	0.0	0.003	1.382	0.00	0.50	0.50
C-35	Trifoliate	3.9	0.34	42	0.003	0.004	0.38	0.13	0.50
Carrizo	Trifoliate	4.4	5.13	1009	4.672	0.003	0.38	0.13	0.50
Glen citrangedin	Trifoliate	0.0	0.02	0.0	0.003	0.000	0.44	0.06	0.25
Norton	Trifoliate	0.0	0.00	0.0	0.018	0.000	0.38	0.13	0.50
Swingle	Trifoliate	7.6	0.29	65	9.682	0.001	0.19	0.31	0.50
Swingle 4n	Trifoliate	0.0	1.68	14	0.030	0.000	0.19	0.31	0.50
US-119	Trifoliate	0.6	0.02	103	0.248	0.001	0.47	0.28	0.25
US-812	Trifoliate	0.2	0.01	0.0	0.003	0.001	0.50	0.00	0.50

[z]Equations used to convert Ct to copy number (CN) were $CN_{(CLas\ 16s\ rDNA)} = 10^{(40.6\text{-}Ct)/3.72}$ and $CN_{(Xcc\ pthA)} = 10^{(38.3\text{-}Ct)/3.56}$. For CLas, CN is equal to one-third genome equivalents and for Xcc CN is equal to 1/40 genome equivalents. The estimated pedigree derived from each of three primary species was calculated based on known or proposed parentage and used for regressions in Table 3. CLas and Xcc genomes for individual genotype trees are means of three measurements: no hypotheses can be tested regarding differences between individual genotypes.

[y]Within this column, mean CLas genomes (per nanogram nucleic acids) composited across tissues followed by the same letter are not different by Kruskal-Wallis test at $P = 0.05$.

[x]Within this box, mean CLas genomes (per nanogram nucleic acids) composited for tissue type in genotypic groups followed by the same letter are not different by Kruskal-Wallis test at $P = 0.05$.

[w]Within this box, mean CLas genomes/ng nucleic acids composited across all genotypic groups followed by the same letter are not different by Kruskal-Wallis test at $P = 0.05$.

[v]Clas genomes are less in trifoliate flowers than flowers of other genotypic groups at $P = 0.057$.

[u]Within this box, mean Xcc genomes/ng nucleic acids composited for tissue type in genotypic groups followed by the same letter are not different by Kruskal-Wallis test at $P = 0.05$.

The first sample collection occurred on 30 and 31 Mar. 2010, early during the bloom period. A second collection occurred from the same trees on 13 Apr. 2010, but ultimately was not tested, because initial results displayed significant bacterial levels and permitted resolution of the experimental hypotheses.

On the afternoon after collection, three flowers from each tree were carefully dissected to remove the anthers, again with care taken to minimize contamination, including using freshly cleaned tools between flowers. All samples were frozen for subsequent nucleic acid extraction.

Total nucleic acid isolation and quantitative polymerase chain reaction. Total nucleic acid was extracted from 100 mg fresh weight of each tissue using Qiagen DNeasy kits (Carlsbad, CA) following the manufacturer's protocol. Total nucleic acid in the extracts was determined by spectrophotometry; 100 ng of total nucleic acid per sample was used as a template for quantitative polymerase chain reaction (qPCR). CLas was amplified using 16S rDNA primers and probe following Li et al. (2006) protocols. Primers targeting the *pthA* gene were used for quantification of Xcc (McCollum et al., 2010). For both pathogens, each extract was tested in triplicate; positive, negative, and no-template controls were also run for each analysis. Results from the first harvest date were deemed sufficient to omit analyses from samples collected on the second harvest date.

Amplicons generated using either the CLas 16S rDNA primers or the Xcc *pthA* primers were cloned into the pGEM plasmid and each cloned amplicon was sequenced to confirm identity. Standard curves for quantification of each pathogen were developed by serially diluting plasmid containing the target amplicons in citrus DNA and using the dilutions as a template for qPCR. There was a highly significant linear relationship ($r^2 > 0.99$) between Ct and log copy number (CN) for each pathogen. Equations used to convert Ct to CN were $CN_{(CLas\ 16s\ rDNA)} = 10^{(40.6\text{-}Ct)/3.72}$ and $CN_{(Xcc\ pthA)} = 10^{(38.3\text{-}Ct)/3.56}$. Three copies of the 16s rDNA gene are present in the CLas (Duan et al., 2009); therefore, CN is equal to one-third CLas genome equivalents per reaction. The number of *pthA* genes per Xcc cell has been estimated to be 40 (Mavrodivea et al., 2004); therefore, CN is equal to 1/40 Xcc cells.

Data analysis. Data were analyzed using SAS (Cary, NC). Means were calculated for each genotype and tissue sampled. No standard data transformation resulted in

Ct-derived data satisfying assumptions for analysis of variance, so means were compared using Kruskal-Wallis nonparametric analyses.

Results and Discussion

Candidatus Liberibacter asiaticus. Of the 90 samples of each tissue type analyzed for the presence of CLas, 88% of mature leaves, 69% of flowers, and 88% of anthers were positive for CLas (we used Ct less than 38.9 for this level of confidence, indicating one or more CLas bacterium/sample). Nonparametric statistical analysis of Ct values indicate that the trifoliate genotypic group had significantly lower levels of CLas than the pummelo and mandarin groups across all tissue types ($P = 0.0399$). Tissue type ($P < 0.0001$) and interaction between genotypic group and tissue type ($P < 0.0001$) also displayed significant differences in CLas (Table 1), but CLas was nonetheless present in most of the hybrids tested, including most trifoliates. Several reports indicate that trifoliate orange and many of its hybrids are significantly more tolerant of or even resistant to HLB than are members of the genus *Citrus* (Folimonova et al., 2009; Stover et al., 2010), and mean CLas genomes per nanogram nucleic acids were 243 times greater in pummelo genotypic group leaves and 949 times higher in the mandarin genotypic group leaves compared with those from the trifoliate genotypic group; however, CLas levels across genotypic groups were much more similar when compared in anthers and flowers.

Numbers of CLas genomes in anthers were 0.15% to 1.0% of the levels detected in the mature leaves with leaf samples selected to be most characteristic of HLB infection (Table 1). Leaf levels of CLas were significantly higher than other tissues across all genotypes tested ($P < 0.0001$). The hypothesis that anther and flower CLas levels would correlate with CLas levels in leaf diagnostic samples was supported across all genotypic groups (Table 2) although r^2 values were less than 0.16. When correlations were assessed within each genotypic group, only the trifoliate group displayed significant relationships with levels of CLas showing r^2 of 0.664 between flowers and diagnostic leaves and the r^2 was 0.459 between anthers and diagnostic leaves. Mandarin hybrids had the highest levels of CLas (500 to 10,000 genomes per nanogram nucleic acids) in all leaf samples tested. 'Chironja', a pummelo hybrid, along with the trifoliate hybrids 'Glen', 'Norton', and '4717' had fewer than 0.1 CLas genomes per nanogram sample nucleic acid in all samples analyzed (Table 1).

Regressions between Ct values for CLas in the tested tissues and percent pedigree among the three parental species (Table 3) showed that leaf CLas levels were significantly negatively correlated with percent trifoliate pedigree ($P = 0.0004$, $r^2 = 0.366$) and positively correlated with percent mandarin pedigree ($P = 0.037$, $r^2 = 0.15$). Flower CLas levels were significantly positively correlated with percent pummelo pedigree ($P = 0.013$, $r^2 = 0.201$).

CLas is known to be systemic within the phloem of infected citrus (e.g., Bove, 2006) and so it is not surprising that CLas is sometimes present even within anthers of unopened flowers. A panel reporting on HLB risk in Florida indicated that spread of CLas by pollen is "not known to occur" (APHIS, 2006). Tatineni et al. (2008) reported that CLas was found in flower parts, including petals, pistils, and stamens, though the stage of flower development and source plant species were not specified. Some viruses and viroids are known to be pollen-borne and contribute significantly to plant disease (reviewed in Mink, 1993). *Erwinia amylovora* has been found on pollen carried by bees (Alexandrova et al., 2003), but we find no report that bacterial pathogens can be pollen-borne as a result of systemic acquisition. However, because CLas is detectable in anthers, where exceptional diligence to exclude HLB is appropriate, our results suggest that there may be a risk of spreading HLB through pollinations.

Xanthomonas citri. Of the 90 samples of each tissue type analyzed for presence of Xcc, 30% of flower samples and 23% of anther samples (Table 1) were positive for Xcc (Ct less than 32.5, indicating one or more Xcc bacterium/100 ng nucleic acid sample). Numbers of Xcc bacteria detected in flowers were much lower than the numbers of CLas bacteria detected. Kruskal-Wallis analysis (Table 1) revealed no significant differences between Xcc levels by tissue type or genotypic group, although mandarin hybrids are notably less susceptible to ACC than the other two genotypic groups. This was reflected in regressions between Xcc levels in flowers and percent of plant pedigree derived from mandarin (Table 3), which had a negative correlation and an r^2 of 0.159 ($P = 0.029$) ACC is primarily a disease of foliage and fruit and is spread by wind-driven rain and casual contact transfer (review by Gottwald et al., 2002). The biology of this pathogen makes it unlikely that the Xcc bacterium would normally be present in the anthers of unopened flowers, which are used as pollen sources for citrus breeding. However, if great care to reduce contamination still results in detectable Xcc, it appears that the risk exists of transferring Xcc to ACC-free areas through pollinations from plant material in infected areas.

Table 2. Analyses were conducted of *Candidatus* Liberibacter asiaticus (CLas) levels by quantitative polymerase chain reaction of 16S rDNA gene on indicated tissues of 10 genotypes each (Table 1) of three genotypic groups maintained in close plantings at the USDA ARS Ft. Pierce, FL, farm.[z]

	Genotypes analyzed							
	All 3 genotypic groups		Mandarin genotypic group		Pummelo genotypic group		Trifoliate genotypic group	
Ct regressions	Correlation (r^2)	P value	Correlation (r^2)	P value	Correlation (r^2)	P value	Correlation (r^2)	P value
Anthers versus diagnostic leaves	0.158	0.029	0.011	0.767	0.133	0.299	0.459	0.031
Flowers versus diagnostic leaves	0.131	0.049	0.007	0.816	0.229	0.162	0.664	0.004
Anthers versus flowers	0.157	0.030	0.259	0.133	0.028	0.643	0.203	0.191

[z]Regression of CLas levels (as Ct values) were conducted between tissue types across all genotypic groups and in each group separately. All significant correlations are positive.

Table 3. Analyses were conducted of *Candidatus* Liberibacter asiaticus (CLas) levels by quantitative polymerase chain reaction (qPCR) of 16S rDNA gene and of *Xanthomonas citri* (Xcc) levels through qPCR of *pthA* gene from indicated tissues of 10 genotypes each (listed in Table 1) of three genotypic groups maintained in close plantings at the USDA ARS Ft. Pierce, FL, farm.[z]

	Versus percent mandarin pedigree		Versus percent pummelo pedigree		Versus percent trifoliate pedigree	
Ct regressions	Correlation (r^2)	P value	Correlation (r^2)	P value	Correlation (r^2)	P value
CLas Ct in anthers	0.001	0.892	0.021	0.446	0.014	0.539
CLas Ct in flowers	0.125	0.056	0.201	0.013	0.004	0.726
CLas Ct in diagnostic leaves	0.147	0.037	0.010	0.592	0.366	0.000
Xcc Ct in anthers	0.010	0.600	0.060	0.190	0.045	0.260
Xcc Ct in flowers	0.159	0.029	0.096	0.096	0.033	0.335

[z]Regression of CLas and Xcc levels were conducted against the estimated pedigree derived from each of three primary species. Pedigree was calculated based on known or proposed parentage and values used are listed in Table 1. Positive correlations between percent pedigree derived from each species and bacterial titers (negative correlations with Ct) are shaded and negative correlations are bordered in black.

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