CALIFORNIA CITRUS NURSERY BOARD

Final Report: Early detection of Huanglongbing (HLB) in nursery trees following infestation with Asian citrus psyllid (ACP)

The overall objective of this project was to determine the time from when a nursery plant is exposed to an Asian citrus psyllid (ACP) infected with Candidatus Liberibacter asiaticus (CLas), the bacterium associated with Huanglongbing (HLB), can be detected in plants and to provide a management tool for nurserymen.

Proposed specific sub-objectives of the project were: 1) investigate the diagnostic reliability of testing nursery plant roots rather than leaves; and 2) comparison of the Smart-DART™ LAMP assay with the standard qPCR assay for detection of CLas. Due to several complications regarding use of the Smart-DART™ LAMP assay this component of the project was dropped, and research focused on the timing of detection of CLas infections using the standard qPCR protocol. During the course of this project we have made significant findings relevant to detection of CLas infections in nursery trees that have importance to California Citrus nurseries. Based on the findings it is possible to provide recommendations regarding monitoring nursery stock for infection with CLas.

Progress

During the course of this project we have refined our protocols for producing CLas-infected ACP with high CLas titer, for timing flush so that it is in the optimum stage for CLas feeding, egg laying and transmission of CLas to citrus, numbers of ACP needed to result in high levels of CLas transmission and subsequent development of HLB symptoms. The protocol that we have developed for producing plant material and CLas-infected ACP and exposure to ACP is illustrated in **Fig. 1**.

CLas titer in infected ACP used to inoculate nymphs resulting following feeding on CLas-free

Significant outcomes of the research demonstrate:

1) qPCR is sensitive enough to detect single CLas cells (**Fig. 2**). This finding is significant for two reasons. First, because it is possible to detect a single CLas cell with qPCR there is no assay that can be "more sensitive" than qPCR, in other words, if there is less than a single copy, there is nothing to detect, therefore it is impossible to be more sensitive. Second, qPCR is the approved USDA APHIS protocol for detection of CLas in citrus and ACP. Using the standard qPCR protocol for detection of CLas means that additional validation is not necessary. It is highly unlikely that the approved APHIS protocol will be changed.
2) A high incidence of CLas-infected ACP can be generated by allowing adult ACP to feed and lay eggs on CLas-infected citrons (**Fig. 3**)
3) CLas transmission can occur in as little as two days of exposure to CLas-infected ACP (**Fig. 4**). However, exposure durations of greater than seven days leads to a greater incidence of HLB symptom development. Exposure to CLas-infected ACP for two weeks results in ca. eighty percent of plants becoming infected.
4) CLas titer in ACP has a significant impact on the incidence of transmission to citrus and eventual development of HLB symptoms (**Fig. 5**).

5) When plants are infected with CLas by ACP transmission the appearance of HLB occurs within four months following exposure (**Fig. 6**).
6) Two weeks following exposure to CLas-inoculative ACP CLas was only detected in the shoot tips on which ACP had fed (**Fig. 7**)
7) The incidence of CLas detection and CLas titer were highest in stem tissue closest to the shoot tips and decreased with increasing distance from the tip (**Fig. 8**)
8) Sampling roots has much less diagnostic reliability than does sampling leaves and roots are more difficult to sample than are leaves (**Table 1**).
9) CLas detection in roots was affected rootstock dependent and CLas titer, when detected in roots was always lower than in CLas-infected shoots (**Table 2**).
10) Detection of CLas prior to the development of HLB symptoms predicts development of HLB disease (**Table 3**). However, the incidence of false negative (no CLas detected, HLB develops) was higher than the incidence of false positives. This is most likely due to sampling error when there are no visible HLB symptoms.
11) Significant differences in the incidence of CLas infection was seen among 'Lisbon' lemon, 'Tango' mandarin, and 'Washington' navel (**Fig. 9**). 'Lisbon' had the greatest incidence of infection, but did not reach titers as high as in 'Tango' and 'Washington'.
12) Expression of HLB symptoms are more severe in 'Tango' and 'Washington' than in 'Lisbon' (**Fig. 10**).

Conclusions

- The use of qPCR for detection of CLas in citrus nursery trees cannot be surpassed by other methods in terms of sensitivity and rapidity. Diagnostic samples can be assayed within 48 hours after collection and the qPCR method is capable of extremely high throughput, an essential characteristic of any effective survey method.
- CLas infections can be detected using qPCR within days following infection, well prior to the appearance of HLB symptoms.
- Most, if not all, CLas infections result when CLas-infected ACP adults feed on newly emerged citrus flush.
- Intergenerational transmission of CLas in ACP appears to require passage through citrus leaves.
- Although a high proportion of ACP nymphs acquire CLas when trees are fed on by CLas infected ACP adults, the proportion of trees that eventually develop HLB is much lower, suggesting there is a minimum infectious dose of CLas required to initiate the development of HLB.
- When CLas titer in ACP adults is less that about 100,000 (10^5) copies per insect, although transmission to ACP nymphs is high, the incidence of HLB disease development is low.
- For trees that are infected with CLas with high enough titer to induce HLB disease (ca. 10,000 copies per mg petiole), there is approximately a four month latency (incubation) period between infection of citrus with CLas via CLas-infected ACP and the appearance of HLB symptoms.
- Shoots are the most reliable tissue for diagnosis of CLas infection in citrus nursery tress.
    - The incidence of CLas detection is consistently greatest in leaves, intermediate in stems and lowest in root.
    - CLas titer is always highest in the leaves that develop from flush that was fed on by CLas-infected ACP.
    - CLas was never detected in roots unless it was also detected in shoots.
    - The incidence of infection in roots is correlated with CLas titer in shoots.

- There was no significant impact of rootstock / scion combination on detection of CLas infections in citrus nursery trees.

Recommendations for CLas detection in citrus nursery trees

1. Vigilant control of ACP is essential. Without ACP, there is can be no CLas transmission to citrus.
2. If ACP adults, eggs or nymphs are found in the citrus nursery they, along with the citrus tissue on which they are found, should be assayed immediately. Along with ACP, the citrus on which the ACP are found should also be assayed for CLas.
3. Although the LAMP assay has shown to be effective for detection of CLas in ACP, the assay has not yet been adapted for citrus tissues. In addition, the LAMP assay has not yet been optimized as a method to hand off to growers.
4. In the absence of ACP or suspicious HLB symptoms on citrus nursery trees, routine diagnostic sampling is not likely to identify infected trees. With no target, sampling is essentially random making the chance for detection minimal.
5. Recently expanded flush leaves should be sampled. This is the tissue where CLas transmission occurs and where there is the greatest probability of infection. Use of roots for diagnostic sampling offers no advantage, and several disadvantages when compared to roots.
6. There is only about a four month lag between CLas infection in citrus and development of HLB symptoms.
7. None of the data suggest that there are scion or rootstock impacts that affect the detection of CLas in infected citrus nursery stock, providing additional support for the validity of the standard qPCR assay for the detection of CLas.
8. In the absence of ACP in the citrus nursery, the only means for CLas infection to occur in citrus nursery stock is through CLas-infected budwood. For this reason, it is essential to protect and monitor budwood source trees for CLas infection.

Figure 1. Experimental approach.

Propagation of CLas-free plant material. Several hundred grafted citrus trees produced every six months. Trees are produced and maintained in an ACP exclusionary greenhouse and maintained insecticide free.

Production of CLas-infected ACP. Citrons are graft inoculated with CLas-infected budwood. At ca. four months after inoculation infection is systemic. Adult ACP are confined with infected citrons and allowed to reproduce. The adults that develop are used to inoculate experimental trees.

Exposure of experimental trees to Clas-infected ACP. Trees are pruned to promote production of uniform flush at the stag most attractive to ACP. One meter x one meter x 2 meter cage is used to contain trees and ACP.

Following exposure to Clas-infected ACP plants are treated with insecticide to eliminate ACP and then held in an ACP exclusionary greenhouse and monitored for Clas infection, titer and development of HlB symptoms.

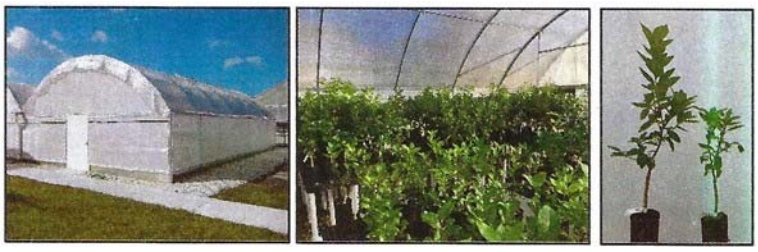


Figure 2. Estimates of CLas copy numbers based on qPCR. Dilutions of a plasmid containing the CLas 16S target sequence were assayed by qPCR. The X axis represents estimated CLas Log Copy Numbers (CLas Log CN) based on mass of the plasmid as determined by spectrophotometry in each assay. A regression equation was developed (CLas Log CN = 11.5- (0.3*Ct) which indicated 100% (+/- 5%) amplification efficiency and an r^2 value of 0.99. This indicates that the standard qPCR assay has an end point of 38 which is equivalent to single copy of CLas target DNA. The Y axis represents Log CN estimates based on Ct values. Note the perfect agreement between the two methods. The regression line intercept is zero, which is equal to a single copy on a log scale.

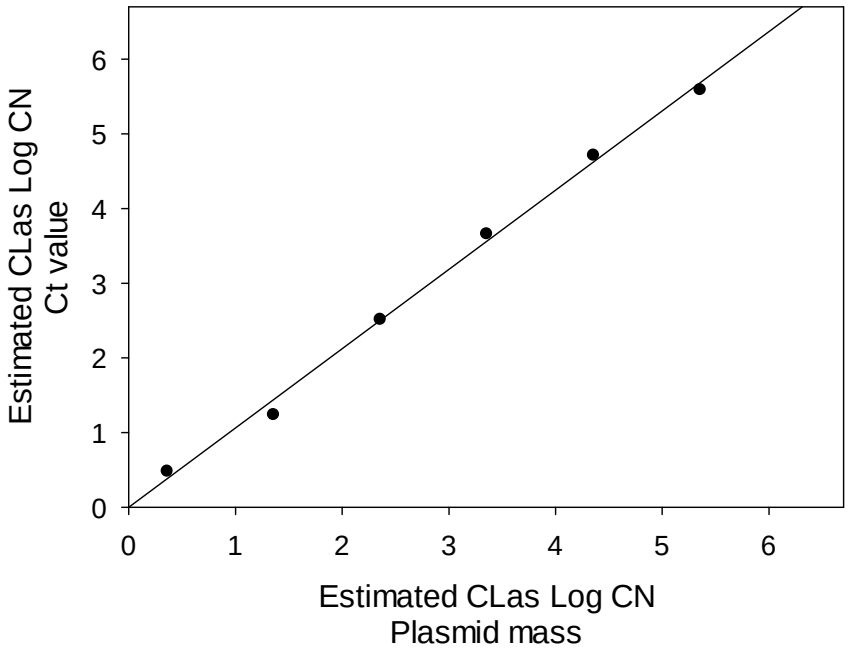
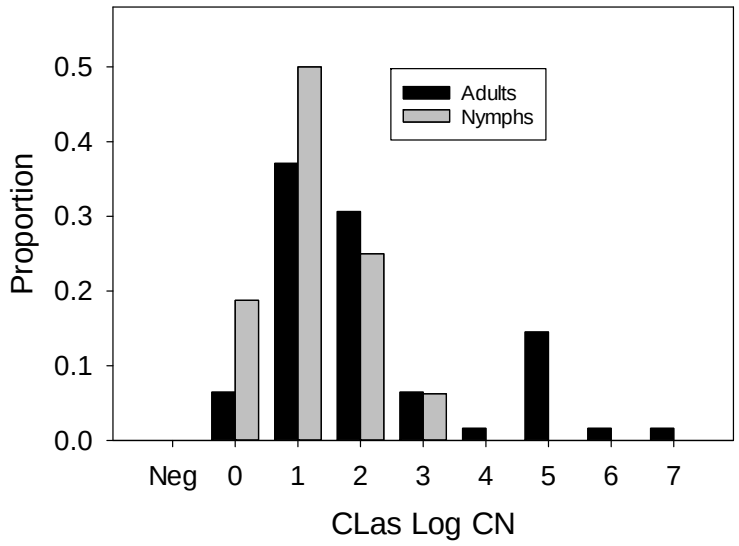


Figure 3. Frequency distributions of CLas titers in ACP adults used to inoculate CLas-free 'Valencia' orange nursery trees and the first generation of ACP nymphs that developed on those plants. In this experiment one hundred percent of the ACP adults used to inoculate were infected with CLas and one hundred percent of the first generation nymphs were infected with CLas.



Adults n = 62 individuals
Nymphs n = 16 pools of 5

Fig. 4. Effects of exposure duration and post-exposure incubation duration on detection of CLas in 'Valencia' orange. Trees were exposed to ca. 5 CLas-inoculative ACP for two, three, four or seven days. Trees were assayed for CLas following four, eight and sixteen days post-exposure incubations.

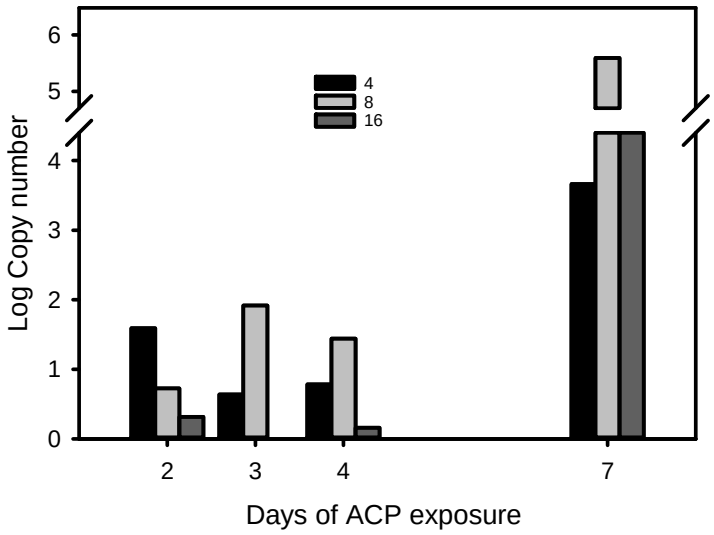


Fig. 5. Effects of CLas titer in ACP adults on the incidence of CLas infection in 'Valencia' orange. Trees were exposed to ca. five CLas-inoculative ACP adults for two weeks. At the end of the exposure, the ACP were adults and nymphs were removed and assayed for CLas. CLas was assayed in citrus trees at two, four and six weeks post-exposure.

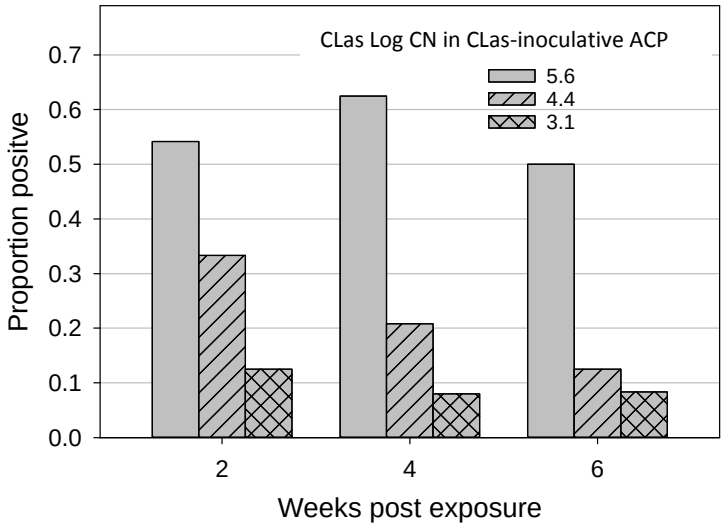


Figure 6. Appearance of Valencia/Swingle at four months after exposure to CLas infected ACP adults for two weeks. The tree on the left tested CLas negative at six weeks and four months following exposure to ACP; the tree on the right tested CLas positive at six weeks and four months after exposure to ACP.

Figure 7. Distribution of CLas in Valencia orange nursery tree following two weeks of exposure to CLas-infected ACP. Each leaf, stem and root section (section boarders are indicated by cross hatched lines) was assayed for CLas using qPCR. CLas was detected only in the leaves shaded in purple. Ct values for CLas-positive leaves are also presented.

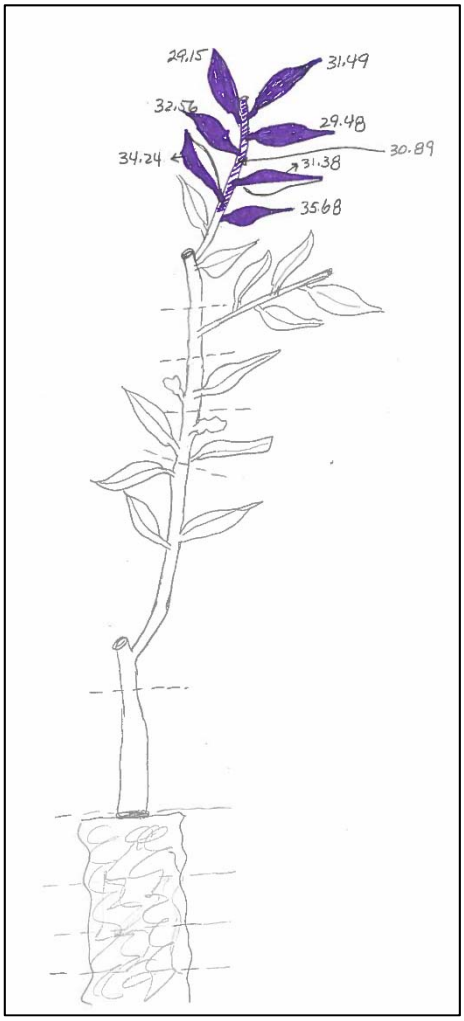


Figure 8. CLas detection in Valencia orange nursery tree shoot tips following two weeks exposure to CLas-inoculative ACP. The distance from tip (acropetal) to base (basipetal) of the shoot was ca. 10 cm.

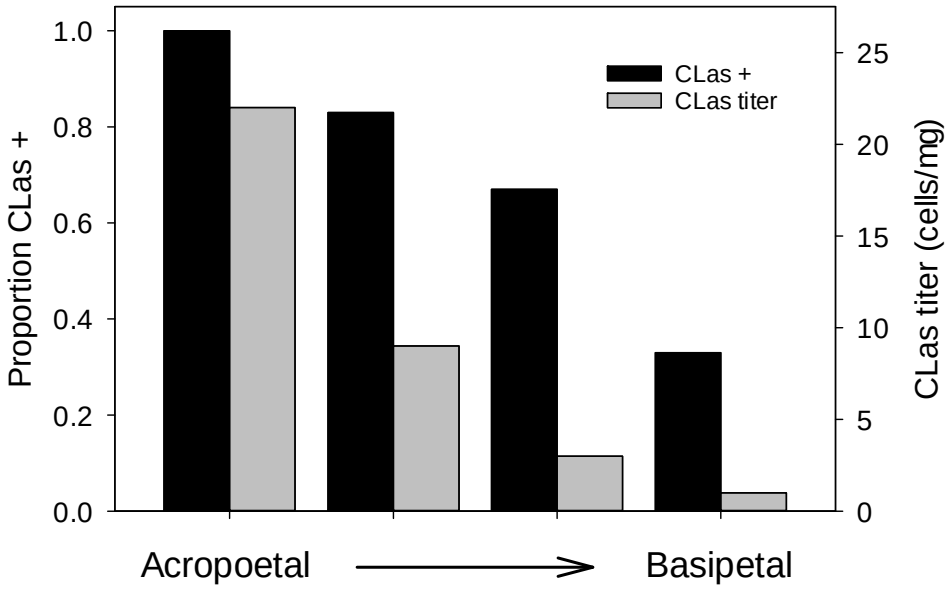


Table 1. Incidence of CLas infection in shoots and roots of inoculated 'Marsh' grapefruit.

	Number CLas Positive/15 trees	
Rootstock	Shoots	Roots
US-942	14	6
Swingle	15	12
Sour	15	9

Table 2. CLas titer in 'Marsh' grapefruit shoots and roots.

	Log CN (Ct)		
		Rootstock	
Tissue	US-942	Swingle	Sour orange
Shoots	4.8	4.5	4.5
	(22)	(23)	(23)
Roots	Negative	2.0	2.0
	(>38)	(31.6)	(31.6)

Figure 9. Incidence of CLas titer in 'Lisbon' lemon, 'Tango' mandarin, and 'Washington' navel nursery trees following two week exposure to CLas infected ACP plus a six week incubation period. For each scion type the number of experimental trees was forty three.

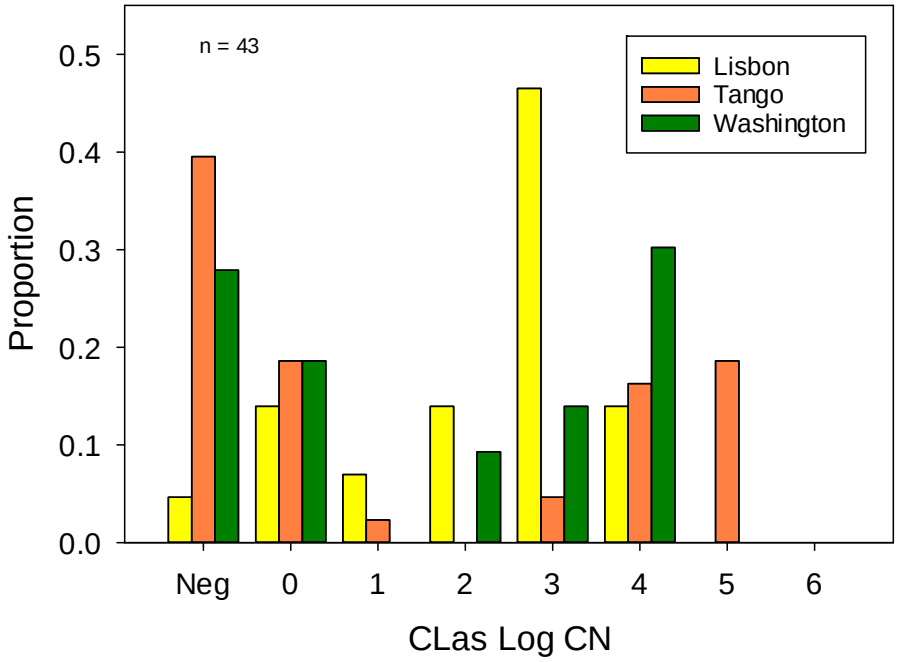


Table 3. Incidence of CLas detection in 'Lisbon' lemon, 'Tango' mandarin, and 'Washington' navel. Trees classified as CLas+ had Ct values less than or equal to 38. HLB symptoms were rated visually and classified as either not symptomatic or symptomatic. Samples for CLas assay were collected six weeks following exposure to CLas-infected ACP for three weeks. HLB symptoms were assessed at four months following exposure to CLas-infected ACP.

	Incidence			
Scion	CLas +	HLB	FN[z]	FP[y]
Lisbon	0.93	0.93	2	1
Tango	0.53	0.63	6	0
Washington	0.63	0.70	5	3

[z]FN (False Negative) - samples that tested initially CLas negative, but subsequently developed HLB symptoms

[y]FP (False Positive) - trees that tested CLas positive but HLB symptoms were not apparent at the time of rating.

Figure 10. Appearance of HLB symptoms in 'Lisbon' lemon, 'Tango' mandarin, 'Washington' navel trees. Trees were exposed to CLas-infected ACP (ca. five ACP per tree) for two weeks. Photographs were taken nine months following exposure. For each variety, the tree on the left consistently tested CLas negative and the tree on the right tested consistently CLas positive.

Lisbon

Tango

Washington