Project Concluded
Final Report, December 31, 2009

Application of Real Time PCR for Detection of *Spiroplasma citri* In Citrus Nurseries and Budwood Trees

Raymond K. Yokomi
USDA, ARS
Parlier, CA

Jacqueline Fletcher
Entomology & Plant Pathology Dept.
Oklahoma State Univ., Stillwater, OK

We have completed the second and final year of this CCNB-funded project to develop data to support CDFA certification of the polymerase chain reaction (PCR) and real-time quantitative (q) PCR assays for detection of *Spiroplasma citri*, causal agent of citrus stubborn disease (CSD), in citrus nurseries.

Executive summary. With wide scientific acceptance of polymerase chain reaction (PCR) assays for specific and sensitive detection of plant pathogens (viral, virus-like, bacterial, and fungal), use of PCR to detect *S. citri* in young citrus propagations provides a powerful tool for the citrus nursery industry to test and certify its inventory as *S. citri*-free. We previously developed, validated and published reliable and sensitive PCR methods to detect *S. citri* in field trees (3). These methods were used in epidemiology studies where CSD was chronic (1,2). However, detection of *S. citri* in young plants growing in a citrus nursery in the CSD-endemic area is a different matter. Early reports with culturing suggested that the long latent period and low pathogen titer in newly infected trees makes *S. citri* detection extremely difficult or impossible in the citrus before propagations are sold. In our CCNB-funded project, we tested detection of S. *citri* in graft-inoculated Madam Vinous seedlings in the greenhouse and screenhouse. We detected presence of *S. citri* DNA within 33 days post inoculation with real-time quantitative (q) PCR; whereas cultivation required a minimum of 42 days to obtain positive test results. We used qPCR because it is more economical and user-friendly than conventional PCR. Using cultivation as the gold standard, qPCR was 100% effective in detecting *S. citri*-infected citrus in our greenhouse and screenhouse. The most effective (highest Ct value) DNA template was obtained using cetyltrimethylammonium bromide (CTAB) compared with sodium dodecyl sulfate (SDS)/potassium acetate (KOAc) for DNA extraction.. Although the detectable titer of *S. citri* was consistently and statistically higher when the DNA source was columella vs. leaf midrib ($P < 0.001$) from field trees, field , leaf midribs were also effective in all our greenhouse tests (Table 1, Fig. 1). Thus, we conclude that infection by *S. citri*, whether by graft or vector transmission, is detectable in a citrus nursery and that qPCR is a valid and appropriate method to detect the pathogen in citrus propagations and budwood source trees.

Results:

Objective 1. Under greenhouse and screenhouse conditions, determine the number of days post inoculation when S. citri is first detectable by real-time PCR. We conducted periodic inoculations from

known *S. citr*i-infected Spring Navel trees from a plot in Ducor (Tulare Co.) CA from Feb. 2008 to Dec. 2009. Data is summarized in Table 1.

- Highest transmission rates occurred in August and no transmission occurred in February, possibly in relation to pathogen activity based on titer, which is higher in summer heat and lower in winter cold (Fig. 1A, 1B).
- Pathogen DNA was detected as early as 33 days post inoculation in side-grafted Madam Vinous sweet orange seedlings in the greenhouse. whereas detection by culturing required an average of 71.5 days. The earliest positive culture was obtained after 42 days (Table 1).
- The latent period before detection was 9 days shorter by qPCR than by cultivation. Please note that these times are relative since successful transmission is a function of quantity and quality of the inoculum.
- Graft transmissions conducted in March resulted in 12% transmission and required 78 days for detection; vs. transmissions conducted June through September had higher rates of transmission (20 to 100%) and required only 33 to 46 days for detection.
- Higher rates of transmission were obtained with side vs. bud grafts, however, the stubborn pathogen was readily transmitted from both of these inoculum sources in the greenhouse (Table 1). No transmission occurred when leaf pieces were used as inoculum.
- A number of inoculation attempts using beet leafhoppers, fed on cultured *S. citri* in D10 medium from feeding sachets covered by stretched parafilm membranes, failed to achieve transmission. Although insect transmissibility can be lost after repeated subculture, the *S. citri* culture used in this experiment had been obtained recently from a field tree..

Objective 2. Under field conditions using young and mature sweet orange trees with known *S. citri* infections, determine the seasonal within tree distribution and relative duration of *S. citri* detection by real-time PCR. Stubborn source trees for these experiments were Spring Navel/Carrizo, planted in 1991 in a commercial orchard near Ducor, CA. Data is summarized in Fig. 1.

- Seasonal titer of *S. citri* DNA in tree canopies was highest in August; lowest in February-March. A decrease in titer was observed in fall but this may be a consequence of pathogenicity caused by high summer pathogen titer followed by a migration of spiroplasma cells down into the roots when cold weather arrives (Fig. 1B
- Pathogen DNA concentration was consistently higher in extracts from fruit columellae vs. leaf petioles ($P < 0.001$). However, pathogen detection from leaf petiole extracts of mature field samples was still good in spring-summer. Our data suggest that leaf extracts are still acceptable for *S. citri* detection in fall-winter as long as qPCR conditions are optimized and appropriate controls are included. The possibility of obtaining false positives or false negatives is always a concern whenever qPCR is conducted.
- Cycle threshold (Ct) values from real time PCR assays were converted to number of *S. citri* cells to show relative quantity of pathogen in tissue extracted. We estimated columella had levels of *S. citri* cells >6x greater than those in leaf petioles in infected field trees when values were averaged over the entire season (Fig. 1B).
- Extraction of pathogen DNA using a modified CTAB method resulted in higher Ct values than did the use of KOAc (4) (data not shown).

We will prepare a manuscript using this data for publication in a refereed journal, as requested by CDFA, to expedite their certification of qPCR for detection of *S. citri* in citrus.

References

1) Mello, A.F.S., Yokomi, R. K., and Fletcher, J. (In press). Assessment of citrus stubborn disease incidence in citrus. In: Proc. 17th Conf. Intern. Org. Citrus Virol., IOCV, Riverside, CA. Accepted for publication Apr. 29, 2008.
2) Yokomi, R. K., Mello, A.F.S., Fletcher, J. and Saponari, M. (In press). Estimation of citrus stubborn disease incidence in citrus groves by real-time PCR. In: Proc. 17th Conf. Intern. Org. Citrus Virol., IOCV, Riverside, CA. Accepted for publication Aug. 11, 2008.
3) Yokomi, R.K., Mello, A.F.S., Saponari, M., and Fletcher, J. 2008. PCR-based detection of *Spiroplasma citri* associated with citrus stubborn disease. Plant Disease 92: 253-260.
4) Yokomi, R.K., M. Saponari, P. J. Sieburth, H. Lin, and E.L. Civerolo. Submitted. Detection of tristeza, stubborn and huanglongbing pathogens in citrus by multiplex real-time polymerase chain reaction assays. Plant Disease.

Table 1. Comparison of real time polymerase chain reaction (qPCR) assays vs. culturing for the detection of *Spiroplasma citri* (stubborn) in Madam Vinous and Duncan grapefruit seedlings graft-inoculated with the stubborn pathogen from a source plant in different seasons. DNA extracted from leaf midribs.

Date	Tissue	No. pos./no. tested	% transmission	Avg. days to positive	
				qPCR	Culturing
2/22/08[2]	Side graft	0/2	0	n/a[1]*	n/a[1]
7/29/08[2]	Side graft	1/4	25	61	91
8/22/08[2]	Side graft	9/10	90	38	67
03/20/09[2]	Side graft	1/8	12.5	78.0	139.0
06/26/09[2]	Side graft	4/5	80.0	46.0	52.8
07/22/09[2]	Side graft	4/6	66.7	38.5	47.0
08/10/09[3]	Side graft	1/1	100.0	33.0	67.0
08/10/09[3]	Buds	9/15	60.0	43.3	65.9
09/24/09[3]	Side graft	3/15	20.0	33.0	42.0
08/10/09[3]	Leaf piece	0/15	0.0	n/a[1]	n/a[1]

[1]n/a = no transmission
[2]Grafted receptor plant was Madam Vinous sweet orange
[3]Grafted receptor plant was Duncan grapefruit

Fig. 1. Seasonal titer of *Spiroplasma citri* in leaf petiole vs. fruit columella in 18-y-old Spring Navel/Carrizo, Ducor (Tulare County), CA. Data per sample date is average of six trees. A) Average cycle threshold (Ct) values from real time polymerase chain reaction (qPCR) assays of *S. citri* DNA. B) Relative number *S. citri* cells in field samples after conversion of qPCR Ct values using regression analysis of a standard dilution of DNA from *S. citri* grown in culture.