Validation and Comparison of a Hierarchal Sampling Plan for Estimating Incidence of Citrus Stubborn Disease

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ABSTRACT. Citrus stubborn disease (CSD) is a production-limiting disease caused by *Spiroplasma citri*, a culturable wall-less prokaryote. The pathogen is transmitted mainly by the beet leafhopper (BLH) in California. The objective of this study was to validate and compare two methods to estimate incidence of CSD in the San Joaquin Valley of California. To accomplish this, 100% of trees from 19 field plots located within 5 citrus groves were screened via real-time PCR to determine true incidence within each field plot. This data was used to simulate two hierarchical sampling plans. Both sampling plans divided groves into 4-tree quadrats (adjacent 2 trees x 2 rows) and collected samples from 25% of the quadrats. For the first sampling plan, all four trees in a quadrat were bulked and the proportion of sampled quadrats that contained one or more infected trees was determined. This value was used to estimate incidence assuming that infections were distributed randomly. The second sampling plan screened all four trees in a quadrat individually, with incidence calculated as the proportion of infected trees. To evaluate the accuracy of the two sampling plans at estimating incidence, a computer program simulated the two sampling plans using the field collected data. With disease incidence < 30%, accuracy of incidence estimates from both sampling plans was similar. However, with disease incidence >45%, screening trees individually was more accurate at estimating disease incidence than bulk screening samples.

Index words: Epidemiology, Spiroplasma citri, sampling

Citrus stubborn disease (CSD) is a production-limiting disease caused by *Spiroplasma citri*, a culturable wall-less prokaryote. In California, *S. citri* is transmitted mainly by the beet leafhopper (BLH), *Circulifer tenellus* (8). Symptoms of CSD vary in intensity with variety and include stunted growth, unseasonable growth flushes and blossoms, low yield and small lopsided fruit. All citrus are susceptible but sweet orange and grapefruit varieties are the most economically affected (1,4). Recently, an evaluation of the impact of CSD on sweet orange production in California was published by Mello et al. (7).

A polymerase chain reaction (PCR) assay developed for detection of *S. citri* was shown to be as effective as culturing (6,10). Yokomi et al. (9) reported incidence of CSD in central California citrus groves using PCR data and compared two sampling approaches. In the first approach, results were obtained for single trees. In the second approach, a hierarchical sampling (HS) plan was used that collected samples from 25% of quadrats and assayed samples individually. The purpose of this report was to validate and compare two HS plans to accurately estimate disease incidence and facilitate studies on the epidemiology of CSD.

MATERIALS AND METHODS

Field plots and estimates of CSD "true" incidence by real time quantitative (q) PCR. Incidence of CSD in 19 field plots located within five citrus groves from different regions of California was determined by sampling all trees within a plot (Table 1). Field plots ranged in size from 64 trees (16 quadrats) to 612 trees (153 quadrats) with a total of 2,903 trees subjected to qPCR screening to verify presence or absence of *S. citri* using the methods described below.

TABLE 1. NUMBER OF *SPIROPLASMA CITRI*-INFECTED TREES IN PLOTS USED TO VALIDATE HIERARCHICAL SAMPLING PLANS TO ESTIMATE INCIDENCE OF CITRUS STUBBORN DISEASE

Plot location	County	Variety	Plot dimensions	Tree age (yr)	Rep	No. infected/No. sampled	% "true" infection
Richgrove 1	Kern	TI Navel	8R x8T	20	1	0/63	0
					2	9/61	14.8
					3	7/61	11.5
					4	0/64	0
					5	0/64	0
					6	0/64	0
					Grove totals	**16/377**	**4.2**
Richgrove 2	Tulare	Barnfield Navel	8Rx8T	20	1	46/62	74.2
					2	31/62	50.0
					3	37/64	57.8
					4	41/64	64.1
					5	35/64	54.7
					6	37/64	57.8
					Grove totals	**227/380**	**59.7**
Ducor	Tulare	Spring Navel	16Rx16T	19	1	41/254	16.1
					2	50/256	19.5
					3	80/256	31.3
					Grove totals	**171/766**	**22.3**
Lindsay	Tulare	Powell Navel)	34Rx18T	12	**Grove totals**	**10/612**	**1.6**
Huron	Fresno	Powell Navel	16Rx16T	10	1	7/256	2.7
					2	8/256	3.1
					3	14/256	5.5
					Grove totals	**29/768**	**3.8**
					Overall totals	**453/2903**	**15.6**

[1]R = Continuous rows; T = Continuous trees

[2]Infection by stubborn disease was based on a positive result of real time PCR assay to detect presence of *Spiroplasma citri* DNA in fruit columella or leaf petiole samples. PCR results were confirmed by testing random negative and positive plus all questionable results by culturing of *S. citri* in LD8 medium (10).

Two hundred mg of fresh fruit columella tissue from three fruits or ten leaf midribs per tree were excised and homogenized in 5 ml modified cetyltrimethylammonium bromide (CTAB) buffer (2) using a Homex 6 homogenizer (BioReba. AG, Reinach, Switzerland). One microliter containing 100 to 150 ng of DNA was used to perform the qPCR assay (10). Quantitative real-time PCR primers for *S. citri* detection were designed from sequences within the spiralin gene (Accession U13998) (3) using Primer Express (Version 3.0, Applied Biosystems, Foster City, CA) (Table 2). Reactions consisted of 0.8 µM of each reverse and forward primer, 1 µl of plant DNA extract or 1µl of *S. citri* cell culture, in a total volume

of 25 µl of 1X iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Reactions were performed on an iQ5 Real-Time PCR System (BioRad). The amplification profile was 95° C for 5 min, followed by 38 cycles at 95 °C for 15 s and 60° C for 45 s. Control samples in each run included DNA extracts from infected and healthy plants, *S. citri* culture and non-template DNA control. The melt curve of the product was used to confirm PCR products as *S. citri* DNA. Conventional PCR was periodically performed with an aliquot of the same extract to verify product size and sequenced if necessary. To further validate positives identified by qPCR, portions of fruit columellae from positive and negative samples were spot checked by bacterial isolation in LD8 broth medium and cultivated at 30°C. After 3 to 14 days, 10 µl of culture medium was examined by dark field microscopy at 400x for presence of motile, spiral spiroplasma cells (10).

TABLE 2
PRIMERS FOR REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) ASSAY FOR DETECTION OF *SPIROPLASMA CITRI* BASED ON SEQUENCES FROM THE SPIRALIN GENE.

Primer	**Primer sequence (5' to 3')**	**Position[1]**	**Expected amplicon size (bp)**
SP209f	AAGCAGTGCAAGGAGTTGTAAAAA	209-232	79
SP288r	TGATGTACCTTTGTTGTCTTGATAAACA	261-288	

[1]Nucleotide position refers to the GenBank accession number U13998 (3).

Hierarchical sampling plans validation. The accuracy of two HS plans for estimating incidence was evaluated. Both sampling plans divided citrus groves into 4-tree quadrats (adjacent 2 trees x 2 rows) and collected samples from trees in 25% of quadrats.

The first sampling plan bulk screened the four trees from each quadrat, providing data on the proportion of sampled quadrats with one or more infected trees. With bulk screening, incidence was estimated as Hughes and Gottwald (5):

$$\text{estimated incidence} = 1 - \left(1 - \frac{\text{Number of quadrats with one or more infected trees}}{\text{Number of quadrats}}\right)^{0.25}$$

The second sampling plan individually screened the four trees from each quadrat. With individually screening trees, incidence was estimated by determining the proportion of infected trees.

To compare the accuracy of incidence estimates from the two sampling plans, a computer program simulated the sampling plans using field collected data and compared estimated values of incidence to true incidence. For each plot, the two sampling plans were simulated by dividing the plot into quadrats of four trees and used a computer program to randomly draw 25% of quadrats. Using the randomly selected quadrats, incidence was estimated assuming that trees in each quadrat were bulk or individually screened. This process was repeated 2,000 times for each plot. For each plot, the accuracy of each sampling plans estimate of incidence was assessed by determining the average deviation of estimates from "true" incidence for each of the 2,000 random draws by:

$$\text{Mean deviation from true incidence} = \sqrt{\frac{\sum_{i=1}^{i=2000}\left(\text{estimated incidence}_i - \text{true incidence}\right)^2}{2000}}$$

RESULTS AND DISCUSSION

Incidence of CSD in field plots ranged from 0 to 74% (Table 1). Field plots that had no infected trees were exclude from the simulation analysis (4 plots). With disease incidence <30%, the mean deviation of estimates of incidence for both sampling plans was similar (Fig. 1). However, with disease incidence >45%, estimates of disease incidence were more accurate with trees tested for *S. citri* individually compared to bulk testing trees in each quadrat (Fig. 1).

Field diagnosis of CSD is difficult and often inaccurate. Symptoms can be confused with those of other pathogens and with nutritional or horticultural problems (4). Diagnosis is further complicated by seasonal variation in symptom expression, with symptoms clear during hot summer months but absent in spring as new growth is developing. Isolation and cultivation to detect infection by *S. citri* is cumbersome, expensive, requires microbiological skills; all of which is not conducive for epidemiological studies. In contrast, PCR assays designed to detect *S. citri* are highly sensitive, require only a small amount of material and can be conducted reliably in a high throughput manner (10). Regardless, it is critical to assess the effects of sample collection technique and laboratory assay procedure on estimation of incidence of infected trees.

In this study, the HS sampling pattern as described by Hughes and Gottwald (5) was shown to provide reasonable estimates of disease incidence provided that incidence was < 30% (Fig. 1). Further, with low incidence, bulking of samples collected from a quadrat provide as accurate a measure of incidence as did assaying each tree singly. With disease incidence > 45%, testing all trees from a quadrat provided a more accurate estimate of incidence than bulking samples. While testing trees individually requires screening 4X as many samples as bulk testing, high-throughput processing and decreased costs of reagents makes this option viable. Further, the most expensive part of surveying orchards is often the time required to travel and collect samples, which is unlikely to be affected by keeping individual tree samples separate.

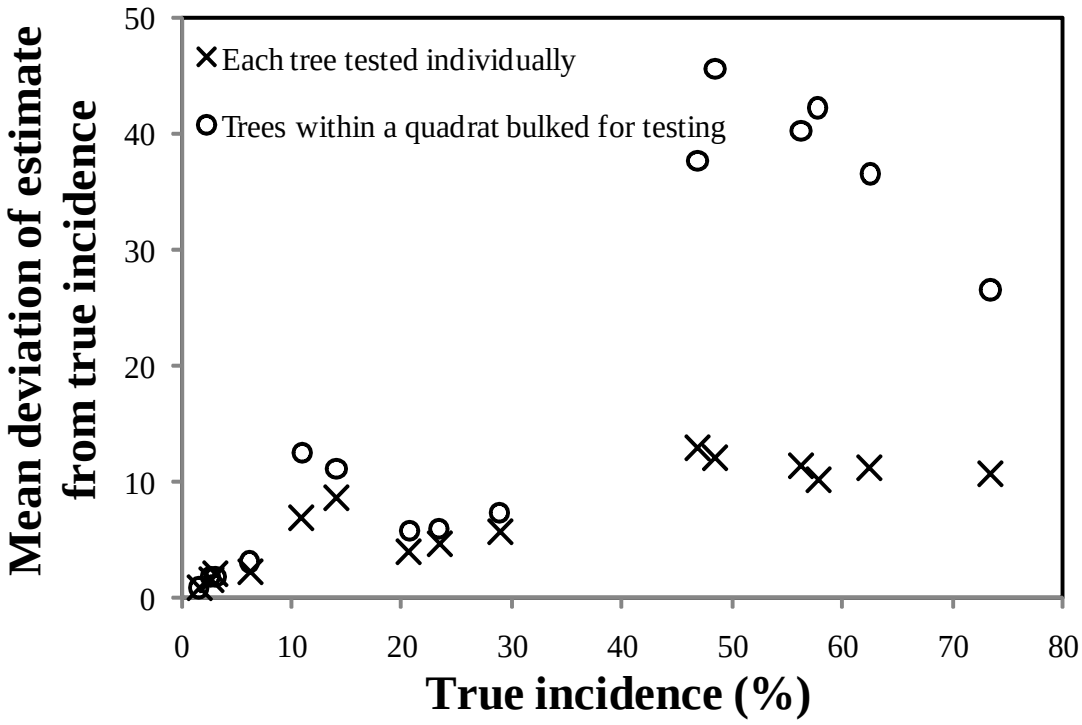

Fig. 1. Mean deviation of estimates of *Spiroplasma citri* incidence from true incidence of *S. citri* at 15 field plots. For each plot, 25% of quadrats were randomly selected and incidence was estimated assuming that samples within a quadrat were bulk tested or tested individually. Estimates of incidence were then compared to the value of true incidence based on screening all plants within the plot and this process repeated 2,000 times.

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LITERATURE CITED

1. Bové, J. M. and M. Garnier
2000. Stubborn. In: *Compendium of Citrus Diseases*. L. W. Timmer, S. M. Garnsey, and J. H. Graham (eds.), 48-50. APS Press, St. Paul, MN
2. Doyle, J. J.
1991. DNA protocols for plants. In: *Molecular Techniques in Taxonomy*. G. Hewitt, A. W. B. Johnson, and J. P. W. Young (eds.), 283-293. NATO ASI Series H, Cell Biology Vol. 57.

3. Foissac, X., C. Saillard, J. Gandar, L. Zreik, and J. M. Bové
1996. Spiralin polymorphism in strains of *Spiroplasma citri* is not due to differences in posttranslational palmitoylation. J. Bacteriol. 178:2934-2940.
4. Gumpf, D. J. and E. C. Calavan
1981. Stubborn disease of citrus. In: *Mycoplasma Diseases of Trees and Shrubs.* K. Maramorosch, K. and S. P. Raychaudhuri (eds.), 97-134. Academic Press, New York.
5. Hughes, G., and T. R. Gottwald
1998. Survey methods of citrus tristeza virus assessment. Phytopathology 88: 715-723.
6. Mello, A. F. S., R. K. Yokomi, U. Melcher, J. Chen, E. Civerolo, A. Wayadande, and J. Fletcher
2010a. New perspectives on the epidemiology of citrus stubborn disease in California orchards. Plant Health Progr. Doi:10.1094/PHP-2010-0526-04-SY
7. Mello, A. F. S., R. K. Yokomi, M. E. Payton, and J. Fletcher
2010b. Effect of citrus stubborn disease on navel orange production in a commercial orchard in California. J. Plant Pathol. 92: 429-438.
8. Oldfield, G. N., D. A. Sullivan, and E. C. Calavan
1984. Inoculativity of leafhopper vectors of stubborn disease in California. In: *Proc. 9th Conf. IOCV*, 125-130. IOCV, Riverside, CA.
9. Yokomi, R. K., A. F. S. Mello, J. Fletcher, and M. Saponari
2010. Estimation of citrus stubborn disease incidence in citrus groves by real-time PCR. In: *Proc. 17th Conf. IOCV*, 131-141. IOCV, Riverside, CA.
10. Yokomi, R. K., A. F. S. Mello, M. Saponari, and J. Fletcher
2008. PCR-based detection of *Spiroplasma citri* associated with citrus stubborn disease. Plant Dis. 92: 253-260.