Assessment of Citrus Stubborn Disease Incidence in Citrus

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ABSTRACT. Citrus stubborn disease, caused by *Spiroplasma citri*, has occurred in California for over 90 yr; however, detection methods for estimating disease incidence have not been optimized. Two 8 ha commercial citrus plots were sampled in 2005 and 2006. Different tissues of sweet orange were tested as sources for spiroplasma cultivation and three sampling procedures for estimating disease incidence were compared using cultivation and PCR. Fruit receptacles and columellas yielded cultivable spiroplasmas more consistently than did leaves, midribs, petioles, or bark. Stat sampling, in which every fifth tree every fifth row was sampled, resulted in estimated incidences of 45.9% and 1.3% by cultivation in groves 1 and 2, respectively. Hierarchical sampling, in which every fourth quadrat was sampled, yielded non-transformed incidences of 71.4% and 3.6% in the same groves by culturing, and 73.3% and 3.6% by PCR. In every-tree sampling, all trees in six blocks of 64 trees in each grove, sampled individually, yielded incidences of 50% and 1.6% by culturing and 58.4% and 2.1% by PCR. Thus, stubborn incidence in grove 1 was confirmed as high and that of grove 2 low. In these tests, PCR was superior to culturing; it is relatively inexpensive, sensitive, and rapid, permitting analysis of a large number of samples.

Index words: epidemiology, bacterial cultivation, PCR, pathogen detection

Citrus stubborn disease (CSD), a vascular disease caused by the wall-less bacterium, *Spiroplasma citri*, has been reported in California citrus orchards since 1915 (8). Distribution of the pathogen within a citrus tree is often uneven, and severely affected trees usually are stunted with short internodes, small mottled leaves, unseasonal blossoms, lopsided fruits and premature fruit drop (6).

S. citri is transmitted naturally by several different species of leafhoppers (9, 13). The principal vector, the beet leafhopper (*Circulifer tenellus*) overwinters in several weeds common to the foothills of the San Joaquin Valley, California. During the spring, as the vegetation dries, the beet leafhoppers migrate back to the Valley floor and feed on citrus foliage, potentially transmitting *S. citri* as they migrate to preferred hosts (4, 5).

Although diagnosis of CSD is typically based on symptoms, the effects caused by *S. citri* in citrus are relatively unspecific and could be misidentified. Molecular detection techniques and culturing of the pathogen, although effective for diagnosis, have not been applied in large-scale field studies. Despite the significance of CSD in California, few evaluations have been done to assess the actual incidence and distribution of the disease in California orchards. The objectives of this study were to (i) assess the suitability of different citrus tissues as sources for spiroplasma cultures, and (ii) compare the ability of three sampling techniques to assess CSD incidence in two commercial citrus orchards in California.

MATERIALS AND METHODS

Plot locations. Two commercial orchards located 6 km apart in northeastern Kern Co., CA were selected for this study. Trees in both orchards were approximately 20 years old and the plots were each 8.1 ha in size. The first location (orchard 1) was planted to the cultivar Barnfield Navel sweet orange, grafted onto Carrizo rootstock. The second location (orchard 2) was planted to the cultivar Thompson Improved Navel sweet orange, grafted onto Carrizo citrange rootstock.

Suitability of different citrus tissues as sources for culturing spiroplasma. Since *S. citri* is a phloem sieve tube inhabitant, any citrus tissue that contains phloem sieve tubes potentially could yield *S. citri* in culture. To optimize the procedure for cultivation of *S. citri* from diseased citrus trees, various host tissues were compared for their suitability as sources. Sweet orange trees with characteristic CSD symptoms were evaluated in two commercial orchards in northeastern Kern Co., CA.

To optimize the procedure for cultivation of *S. citri* from diseased citrus trees, various host tissues were compared for their suitability as sources. Six to 11 sweet orange trees with characteristic CSD symptoms were evaluated.

From each tree sampled, three sets of tissue were collected, each consisting of columella, fruit receptacle (tissue between the fruit peduncle and columella), stem bark, leaf without mid-rib, leaf mid-ribs and leaf petiole (14). The three samples of each type from each tree were then combined; for example, the three columella samples from a single tree were processed together as a single columella repetition from that tree. Culturing was done in LD8 medium using standard procedures previously described (3, 12). This experiment was performed three times, once in 2005 and twice in 2006. Cultures were evaluated by dark-field microscopy using an Olympus BH-2 microscope (Olympus® Optical Co., Tokyo, Japan) (1200 x), 7-15 days after culturing, for the presence of typical spiroplasma cells (15).

Relationship between occurrence of misshapen fruit and isolation of *S. citri*. Because *S. citri* infection impacts citrus fruit formation, (9) the presence of misshapen fruits (lopsided or "acorn" shaped) can be a predictor of *S. citri* infection. To assess the correlation between the occurrence of misshapen fruits and the ability to isolate *S. citri*, 356 trees in orchard 1 were selected randomly and the receptacles of three fruits from each tree were processed for spiroplasma cultivation. The impact of the presence of zero, one, two or three misshapen fruits per tree on the isolation of *S. citri* was assessed by a chi-square test using SAS software.

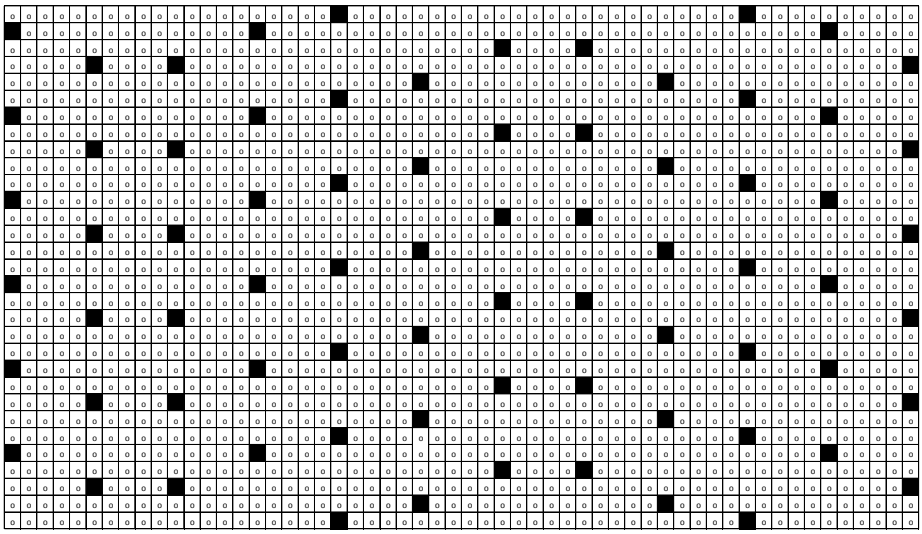
PCR. For polymerase chain reaction (PCR) analysis, samples consisted of columellas from the same fruits used for cultivation. One hundred mg of lyophilized columella tissue was homogenized using a MiniBeadBeater-96 (Bio-Spec Product, Bartlesville, OK), and the DNA was extracted by the CTAB method (7). PCR was performed using primers designed for the gene for the putative adhesin P89 and the adhesion putative multigene P58 (1, 16).

Estimation of citrus stubborn incidence using three sampling techniques. To estimate CSD incidence in selected California orchards, and to evaluate the suitability of several previously reported sampling design strategies, the two orchards described above were evaluated using three different techniques.

Stat sampling. Stat sampling, a technique in which every fifth tree in every fifth row is sampled (Fig. 1A), was used by the Central California Tristeza Eradication Agency (CCTEA) before the development of a hierarchical sampling technique. In this work, from each sampled tree, one fruit was harvested from each of the four canopy quadrants. When present, misshapen fruits were preferentially selected. The fruit receptacles were processed for *S. citri* cultivation and presence of spiroplasmas in culture tubes was considered diagnostic for CSD.

Hierarchical sampling (HS). In this method, four trees (two on the right side of the row and the next two on the left side of

A.



B.

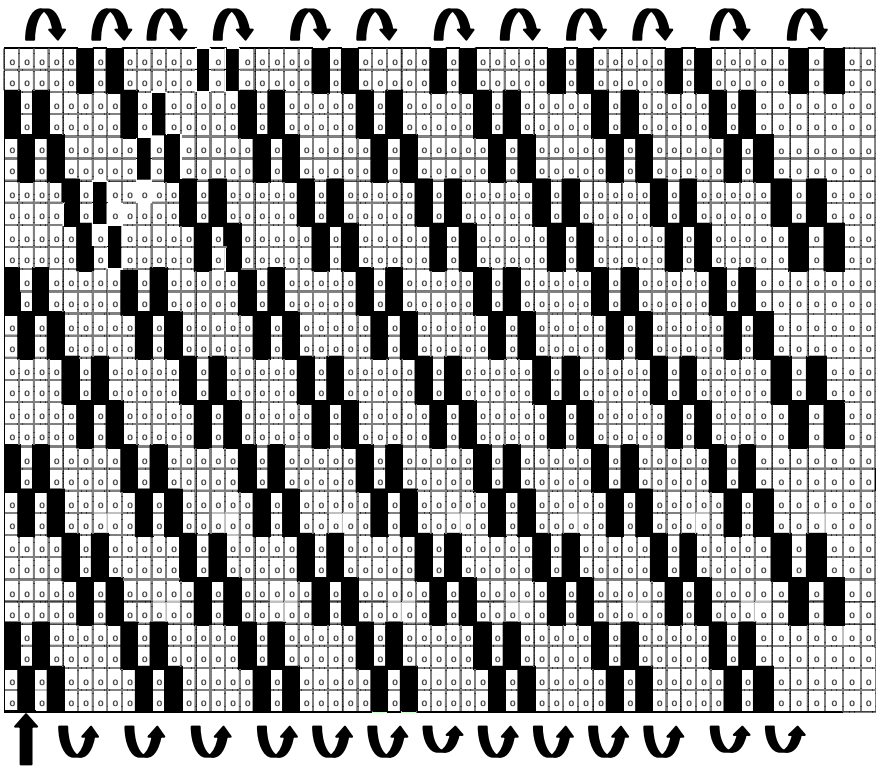


Fig. 1. Field sampling techniques used to estimate citrus stubborn disease in two commercial sweet orange orchards in Kern County., CA. A. Stat sampling: every fifth tree in every fifth row was sampled; each black square represents one sampled tree; B. Hierarchical sampling (HS), each group of 4 black squares represents 4 trees pooled as a single sample (11) arrows show sampling direction.

the row) were sampled. Each group of four trees was considered a quadrat and considered one sample (11). Two fruits harvested from opposite sides of each tree canopy were pooled together with the other fruits of the quadrat, for a total of eight fruits per sample. After the sampling of the first quadrat the next four trees of row were by-passed and than a new quadrat were sampled, (Fig. 1B), hence 25% of the orchard trees weresampled. When present, misshapen fruits were preferentially selected. Infection was assessed by cultivation from fruit receptacles in LD8 broth and by PCR.

Every-tree block sampling (ETBS). In the third sampling strategy six blocks of 8 by 8 trees comprised the sampling unit. Because stat and HS sampling had already indicated high incidence and homogenous distribution of CSD in orchard 1, the six blocks were selected in the four corners and in the center of the plot (16). In contrast, since stat and HS results from orchard 2 had indicated an aggregated distribution of infected plants, the 8 by 8 blocks were selected in areas with both major and minor distribution of CSD (16). Three fruits were harvested from different canopy sectors from each of the 768 trees in the two orchards. When present, misshapen fruits were preferentially selected. Fruit receptacles were used for *S. citri* cultivation and columellas were lyophilized and processed for PCR as described above.

Sampling for all experiments was done from June through August, 2006. All sampling for a given replication was completed on the same day (stat and HS) or within one week (every-tree sampling). Disease incidences were calculated as the number of infected samples divided by the total number of samples, multiplied by 100.

RESULTS

Suitability of different citrus tissues as sources for spiroplasma cultures. In the three different evaluations performed, citrus fruit columellas and receptacles consistently yielded higher percentages of spiroplasma cultivation than did the other tissues tested. The percentage of citrus stubborn-symptomatic trees yielding spiroplasma cultures from receptacles and columellas ranged from 63.6 to 100%, while the presence of *S. citri* in other citrus tissues varied from 0 to 50% (Table 1).

Relationship between the occurrence of misshapen fruits and isolation of *S. citri*. The percentage of fruits that were misshapen, among harvested citrus samples, was significantly correlated with number of positive cultures resulting from those fruits (data not shown). Samples containing one, two or three misshapen fruits were culture-positive 67.3, 70.6, and 75% of the time, respectively. Chi-square analysis resulted in a *P*-value of 0.01, indicating that the presence of misshapen fruit is a useful predictor of successful cultivation of *S. citri*.

Estimation of citrus stubborn incidence using three different sampling techniques. The two commercial citrus orchards sampled had significantly different incidences of CSD, regardless of the sampling strategy used (Table 2). Using the results of spiroplasma cultivation to determine whether a tree was infected, stat sampling indicated 45.9% disease incidence in orchard 1 and 1.3% in orchard 2 (Table 2). HS indicated incidences of 71.4 and 3.6%, respectively, in orchards 1 and 2. Results from the ETBS sampling (six blocks of 64 trees) were similar to those obtained by stat sampling, yielding 50 and

1.6% incidence in orchards 1 and 2, respectively.

When PCR was compared with cultivation to detect infection in trees sampled by HS and ETBS, PCR revealed slightly higher *S. citri* incidences than did cultivation when both were used to test the same samples (Table 2). The comparison side by side of the techniques showed that 31 and 13 samples were positive only by PCR and four and 12 samples were positive only by culturing in orchard 1, when it was evaluated by ETBS and HS respectively. In orchard 2, HS positive samples were identical regardless of the detection technique, while in the ETBS evaluation 4 PCR positive samples were negative by culturing and 1 that was positive by culturing was negative by PCR. The overall improvement provided by PCR in the detection of *S. citri*, in comparison with cultivation, ranged from 2.59 to 23 %. Since PCR is able to detect non-viable *S. citri* DNA it is important to also use culturing when an initial assessment is done in a commercial orchard to assure that the bacteria are active at that site.

TABLE 1
EVALUATION OF DIFFERENT CITRUS TISSUES AS SOURCES FOR CULTIVATION OF *SPIROPLASMA CITRI*

Tissue	# Positive samples[1]/Evaluations (dates)		
	1st evaluation (11/2005)	2nd evaluation (06/2006)	3rd evaluation (10/2006)
Leaves[2]	2/6	0/7	0/11
Leaf mid rib	0/6	0/7	0/11
Bark	2/6	2/7	0/11
Leaf Petiole	3/6	1/7	0/11
Columella	6/6	6/7	7/11
Receptacle	ND[3]	6/7	7/11

[1] (Number of positive samples/Total number of samples)
[2] Without mid ribs
[3] ND= not done

TABLE 2
INCIDENCE OF CITRUS STUBBORN IN TWO CALIFORNIA SWEET ORANGE COMMERCIAL ORCHARDS EVALUATED BY STAT, HIERARCHICAL AND EVERY-TREE BLOCK SAMPLE TECHNIQUES

Sampling method	Stat[a]	Hierarchical*			Every-tree block		
Detection method	Culturing	Culturing	PCR	Total[b]	Culturing	PCR	Total[b]
			Orchard 1				
Total number of samples	74	105	105	105	382	382	382
Number of positive samples	34	75	77	89	191	223	225
Incidence (%)	45.9	71.4	73.3	84.8	50	58.4	58.9
			Orchard 2				
Total number of samples	78	112	112	112	377	377	377
Number of positive samples	1	4	4	4	6	8	9
Incidence (%)	1.3	3.6	3.6	3.6	1.6	2.1	2.4

[a] Samples not evaluated by PCR
[b]Sum of samples positive by culturing and PCR

DISCUSSION

The symptoms of stubborn disease are relatively non-specific, with chlorosis and stunting resulting from phloem dysfunction due to spiroplasma habitation (6). Symptoms in citrus plants are intensified by high temperatures (2) typical in the summer in California. Symptoms can also vary in intensity in different sectors of a tree canopy. Such inconsistencies hamper accurate diagnosis of stubborn disease. We sought to develop a sampling and diagnostic strategy that would combine reliability with relative convenience, and that could be applied to various epidemiological studies of stubborn disease in orchard settings.

Comparisons of the three sampling approaches, stat, HS and ETBS, revealed that the first and the last provided very similar disease incidence data. This was seen regardless of whether the orchard had a high (orchard 1) or low (orchard 2) CSD incidence. HS estimated a higher incidence of CSD than did the other two methods, although this was seen much more in orchard 1 than in orchard 2, likely due to the pooling of samples from four trees in the former but not the latter.

From the different tree tissues used as sources for cultivation, spiroplasma cultures were obtained from greater percentages of fruit receptacles and columellas than from stem bark, leaves without midribs, leaf midribs, or leaf petioles of the same trees. Whether this finding reflects a higher pathogen titer in receptacles and columellas was not investigated in this study, but since spiroplasmas translocate with the flow of photosynthates to "sink" tissues in rapidly growing or storage tissues (10) their accumulation in these two phloem-rich fruit tissues would not be surprising.

Our data support the finding of Yokomi et al. (16) that PCR is more effective than spiroplasma cultivation to confirm *S. citri* infection. To be sure no false positives were recorded, they cloned and sequenced the amplicon and found 100% identity to the P58 sequence reported for *S. citri* (17). They also showed results of

melting curves from real time PCR assays with SYBR-green. Furthermore, the diagnosis of fewer CSD trees in Orchard 1 illustrates the difficulty and limitation of using cultivation as the sole diagnosis of infection. Not surprisingly, the combination of both PCR and cultivation provide results more reliable than those provided by either test alone. The fact that stat and ETBS estimates were somewhat lower than those obtained by HS was not unexpected since the latter method did not consider individual samples from the block of four trees tested in HS. In related work, Yokomi et al. (17) observed that adding evaluations of the individual trees in a bulk sample can provide a more complete picture of the overall disease incidence than does testing only the bulked samples. However, the goal of this specific research was to assess the incidence by three current sampling techniques, as they were developed for studying other citrus diseases. Our work confirms the utility of the methods for important applications related to disease epidemiology and pathogen biology.

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