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EFFECT OF CITRUS STUBBORN DISEASE ON NAVEL ORANGE PRODUCTION IN A COMMERCIAL ORCHARD IN CALIFORNIA

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SUMMARY

The impact of citrus stubborn disease (CSD), caused by *Spiroplasma citri*, on commercially cultivated citrus is not fully understood or quantified. Our objective was to measure these impacts on citrus production and assess bacterial distribution in trees having different symptom severities. *S. citri*-positive and adjacent healthy navel orange trees in a commercial grove in central California were evaluated. Measurements included canopy height and width, trunk diameter, fruit number and weight, and number of prematurely dropped fruit. Thirty fruit per tree were evaluated for color, size and sunburn. Juice was extracted, weighed, and total soluble solids and titratable acidity measured. Bacterial distribution in trees exhibiting mild or severe symptoms was assessed by q-PCR and spiroplasma culture. Fruit from *S. citri*-positive trees were smaller, and more often mis-shapen, than those from healthy trees. Significant fruit number reduction occurred only in severely symptomatic trees, in which *S. citri* was broadly distributed within the tree canopy. All other variables were statistically indistinguishable regardless of symptom severity or pathogen presence. The reduction in fruit weight, size and number in severely symptomatic trees validate the concern that CSD is a significant constraint to production and marketability in California.

Key words: *Spiroplasma citri*, mollicute, vascular pathogens, crop losses.

INTRODUCTION

Oranges, grapefruits, lemons and limes are among the most popular fruits in the United States, following only bananas and apples in fresh fruit consumption (Pollack, 2003). Fresh citrus fruit production in the United States is concentrated in California, where dry summers, hot days, and cool nights enable uniform fruit ripening among many cultivars (Walheim, 1996). Citrus stubborn disease (CSD) has been present in this state for many years; however, the effects on fruit yield and quality have not been quantified. The disease was attributed initially to a virus-like agent (Fawcett, 1946), but in 1972 the etiological agent was identified as a wall-less bacterium, *Spiroplasma citri* (Fudl-Allah *et al.*, 1972; Saglio *et al.*, 1973).

S. citri is a phloem-limited mollicute transmitted by several species of leafhoppers in a propagative manner or by grafting of infected plant material (Liu *et al.*, 1983; Oldfield *et al.*, 1977). The importance of citrus as a host of the primary insect vector of *S. citri*, *Circulifer tenellus* (Baker), is not fully understood, but the concentration of *S. citri* in infected trees near orchard edges suggests migration of inoculative insects from weeds to the commercial crops during the summer when the environment becomes unfavorable for annual weeds (Calavan and Bové, 1989).

Although CSD has been present in California since 1915 (Calavan and Oldfield, 1979), its effects in the San Joaquin Valley has had greater visibility in the past 5 years with more citrus growers reporting symptoms consistent with CSD including general stunting, short leaf internodes, leaf mottling, unseasonal blossoming, and lopsided fruit (Calavan and Oldfield, 1979). Bacterial culturing and PCR analyses from such trees (Mello *et al.*, 2008a; Yokomi *et al.*, 2008, 2010), have consistently tested positive for *S. citri*.

In the late 1960s, Calavan (1969) assessed the impact of CSD on the production and fruit quality of sweet oranges (cv. Valencia Frost) in a commercial orchard in California (Calavan, 1969). Yields of infected trees ranged from 44 to 74% lower than those of healthy trees, and fruit from diseased trees weighted 6 to 17% less than did those from *S. citri*-negative trees, depending on the root-stock used (Calavan, 1969). In Cyprus, natural infections of *S. citri* in cv. Frost Washington Navel trees decreased citrus production by 28%, and fruit produced by such trees were 20 to 38% lighter than those produced by *S. citri*-free trees. Fruit from *S. citri*-positive plants also were 8 to 15% smaller in diameter than those from *S. citri*-free trees, but no effects

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were observed on the amount or quality of juice (Kyriakou *et al.*, 1996). Plants inoculated artificially via grafts from infected tissues sustained greater impacts on fruit quality and yield than plants naturally inoculated; production and fruit size of *S. citri*-positive plants were, respectively, 92% and 7% lower than from *S. citri*-negative trees (Calavan and Christiansen, 1966).

The impact of *S. citri* infection on yield and tree height was correlated with the severity of CSD symptoms on artificially inoculated plants (Calavan, 1969), but similar studies have not been done under California field conditions with infections resulting from natural vector transmission. The relationship between symptom severity and fruit yield and quality under orchard conditions has not been measured, although severity may be correlated with bacterial titer (Calavan and Bové, 1989) and/or strain virulence (Calavan, 1969). The objective of this study was to estimate the impact of *S. citri* on Navel sweet orange [*Citrus sinensis* (L.) Osb.] production in a commercial orchard in California and to determine pathogen distribution in trees with mild versus severe CSD symptoms. Some results were previously reported (Mello *et al.*, 2007, 2008b).

MATERIALS AND METHODS

Orchard. The study plot was done in a commercial orchard adjacent to the foothills of the San Joaquin Valley in northeastern Kern County, California. It contained approximately 1,800 sweet orange trees, cv. Thompson Improved Navel, grafted onto Carrizo citrange (*Citrus sinensis* Osb. x *Poncirus trifoliata* L. Raf.) rootstock. Trees were approximately 20-year-old with no history of pruning.

Sampling. Evaluations were done in 2006 and 2007. Four fruit from each of 380 trees were harvested randomly in May of 2006 and tested to confirm the presence of *S. citri*. Receptacles were processed using standard procedures (Bové *et al.*, 1983; Kyriakou *et al.*, 1996; Mello *et al.*, 2010) for spiroplasma cultivation in LD8 medium and fruit central axis were subjected to polymerase chain reaction (PCR) using spiralin and P-58 based primers (Yokomi *et al.*, 2008). From the initial 380 screened trees, 20 trees in 2006 and 32 trees in 2007 were selected for this study based on their proximity to one another. Half of the trees were *S. citri*-negative and half were positive by both culturing and PCR. *S. citri*-positive trees were classified as mildly or severely symptomatic. Trees designated mildly symptomatic were nearly asymptomatic with only a few branches showing abnormally short internodes and/or leaf mottling. Severely symptomatic trees were characterized by leaf mottling and short internodes on all branches, and many displayed off-season blooming. Trees testing *S. citri*-negative by culturing and PCR and *S. citri*-positive trees were selected and compared in adjacent pairs to minimize potential environmental effects caused by variations in soil fertility and/or soil texture. The presence/absence of *S. citri* was re-confirmed every six months by both spiroplasma culturing and PCR. To assure that the results were not skewed by the presence, in the evaluated trees, of other pathogens commonly found in California citrus orchards (Flint, 1991), all 32 trees evaluated were visually inspected for the presence of bark cracks with oozing sap, symptoms typical of infection by *Phytophthora spp*. Root samples were incubated in modified Seinhorst mist apparatus (mist chamber) (Barker, 1985) to assess the presence of parasitic nematodes; and leaf petioles were subjected to enzyme-linked immuno-sorbent assay (ELISA) to test for *Citrus tristeza virus* (CTV) (Nikolaeva, 1995).

Fruit yield and quality evaluations. Field and laboratory evaluations were performed in October 2006 and 2007. Tree height, tree canopy diameter at 0.5 m from the ground and trunk scion and rootstock (RS) diameters were measured 10 cm from the soil (rootstock) or 5 cm from scion/rootstock graft (scion). Fruit dropped prematurely under the tree canopy circumference were counted. The number of fruit produced was estimated with the aid of a 0.6 x 0.6 m pvc pipe frame (Hall and Albrigo, 2007). The frame was held by hand against the tree canopy at positions 0º, 90º, 180º and 270º around the tree circumference, on the upper, medium and lower canopy, for a total of 12 locations per tree. All fruit within the frame area, extending inward to the trunk, were counted. After these field evaluations, 30 fruit from each tree were harvested arbitrarily and transported to the ARS facility in Parlier, CA for laboratory analyses. Fruit were weighed, and the length and width of each fruit were measured with a digital caliper. The presence/absence of sunburn was recorded. Rind color was evaluated using a CR-300 Minolta (Osaka, Japan) digital colorimeter, using the parameters of light (L), chroma (C) and hue angle (H) with three readings per fruit. Juice was then extracted using a manual juicer (Sunkist, USA) and weighed. Aliquots of the juice were used to measure the content of soluble solids (Brix) using a digital Atago refractometer PR-101 (Tokyo, Japan), and the titratable acidity (TA) (citric acid equivalents) was determined using an automatic titration Radiometer TIM 850 (Copenhagen, Denmark) (Ting and Rouseff, 1986). Results were evaluated using PC SAS version 9.1 (SAS, Cary, USA). The data were analyzed assuming a randomized complete block design (PROC MIXED in SAS). Individual comparisons were made using the DIFF option in a LSMEANS statement.

Distribution of *S. citri* in mildly and severely symptomatic citrus trees. *S. citri* distribution in mildly and se-

Table 1. Comparison of physical features of *Spiroplasma citri*-positive and *S. citri*-negative sweet orange trees from a commercial citrus grove in Kern County, California.

2006									
Variables	*S. citri*-positive vs *S. citri* negative[a]			Mild vs *S. citri* negative[b]			Severe vs *S. citri* negative[c]		
	S. citri-positive	*S. citri*-negative	P value	Mild	*S. citri*-negative	P value	Severe	*S. citri*-negative	P value
Tree height (m)	2.4	2.5	0.06	2.7	2.8	0.14	2.0	2.3	0.22
Tree width (m)	2.6	2.7	0.41	2.7	2.9	0.34	2.4	2.6	0.84
Root- stock diameter (cm)	19.4	19.7	0.71	21.6	21.1	0.67	17.3	18.3	0.36
Scion diameter (cm)	21.2	17.4	0.06	15.5	16.5	0.10	25.6	21.2	0.24
Fruit drop[d]	8.6	3.3	0.07	3.8	2.2	0.68	13.4	4.4	0.03
Fruit number [e]	5.9	7.9	0.02	7.1	6.0	0.37	4.7	9.9	<0.01
2007									
Variables	*S. citri*-positive vs *S. citri* negative [a]			Mild vs *S. citri* negative[b]			Severe vs *S. citri* negative[c]		
	S. citri-positive	*S. citri*-negative	P value	Mild	*S. citri*-negative	P value	Severe	*S. citri*-negative	P value
Tree height (m)	1.9	2.2	0.02	2.3	2.3	0.99	1.6	2.2	<0.01
Tree width (m)	3.2	3.4	0.04	3.5	3.4	0.91	2.9	3.3	<0.01
Root- stock diameter (cm)	19.6	20.1	0.65	21.3	20.8	0.72	17.9	19.3	0.32
Scion diameter (cm)	19.8	18.2	0.32	18.9	17.8	0.63	20.6	18.5	0.35
Fruit drop[d]	26.9	7.4	<0.01	20.0	7.5	0.05	33.7	7.2	<0.01
Fruit number [e]	7.8	11.5	<0.01	9.2	11.1	0.15	6.5	11.9	<0.01

Data compared by analysis of variance in a split plot arrangement using pairwise t-test comparisons.
[a] Includes 10 pairs (2006) or 16 pairs (2007) of *S. citri*-negative and *S. citri*-positive trees (mild and severe pooled together)
[b] Includes 5 (2006) or 8 (2007) mildly symptomatic trees and their corresponding *S. citri*-negative counterparts
[c] Includes 5 (2006) or 8 (2007) severely symptomatic trees and their corresponding *S. citri*-negative counterparts
[d] Number of fruit dropped within the boundaries of the tree canopy
[e] Average number of fruit from 4 sides of trees within the area of a 0.6 x 0.6 m PVC frame

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verely symptomatic trees was assessed by harvesting 10 fruit at random from each of ten CSD affected trees (5 mildly and 5 severely symptomatic) in August 2007. Receptacles were processed for spiroplasma cultivation in LD8 broth (Bové *et al.*, 1983; Kyriakou *et al.*, 1996; Mello *et al.*, 2010). Cultures were evaluated daily for turbidity and spiroplasma growth was confirmed by dark-field microscopy using an Olympus BH-2 microscope (Olympus Optical Co., Japan) (1200 x). Non-turbid samples were evaluated weekly by dark-field microscopy for 60 days. Relative spiroplasma titer in receptacles was measured as the time elapsed from isolation to the first microscopic visualization of *S. citri* cells. Due to issues resulting in heterogeneity of variance, the culturing data were transformed using an arcsine square root function. To assure that the time from cultivation to microscopic visualization of *S. citri* cells was correlated only with S. *citri* titer, and not due to differential adaptation of *S. citri* isolates to the broth, 5 cultures each from severely and mildly symptomatic trees were sub-cultured in LD8 broth with the same initial titer ($5x10^6$ cells/ml) and their growth rates assessed by direct counting under dark-field microscopy after 24 and 48 h.

To assess whether *S. citri* distribution was broader within the canopy of CSD severely symptomatic trees than in mildly symptomatic trees, a second set of experiments was conduced in October 2007. Fruit and leaves from the same 10 trees used in the previous study were harvested from the following specific tree locations: two canopy aspects (east and west) and three canopy tiers (top, middle and base), for a total of 18 samples per tissue (fruit central axis or petiole) per tree. *S. citri* presence was assessed by q-PCR, using *S. citri* P-58 gene-based primers, on DNA extracted separately from fruit central axis and leaf petioles (Yokomi *et al*., 2008, 2010). The presence or absence of *S. citri* in fruit central axis and leaf petiole samples was analyzed by a chi-square test using SAS software 9.1 and PROC FREQ (SAS, Cary, USA). Data were sorted first by disease status (mild vs. severe) and canopy aspect and then by status alone. The suitability of fruit central axis and leaf petiole as sources of DNA template also was compared by a chi-square analysis.

RESULTS

Fruit yield and quality evaluations. No evidence of parasitic nematodes or *Phytophthora spp.* was seen in any of the sampled trees, and all samples were ELISA negative for CTV (data not shown). In both 2006 and 2007, *S. citri*-positive trees produced fewer fruit, and fruit were of lower quality (smaller and misshapen) than those from spiroplasma-free trees.

S. citri-positive trees produced 25 and 32% lower number of fruit than those of spiroplasma-free trees in years 2006 and 2007, respectively (Table 1). Fruit production of severely symptomatic trees were negatively impacted in comparison with those of their *S. citri*-negative counterparts (52 and 45% lower in 2006 and 2007, respectively, $p<0.01$). The number of fruit produced by CSD mildly symptomatic trees were not statistically different from its *S. citri*-negative pairs ($p=0.37$ and 0.15, respectively, in 2006 and 2007) (Table 1). In both years severely symptomatic trees had significantly greater fruit drop than did spiroplasma-free trees; while fruit drop from mildly symptomatic trees significantly exceeded that of *S. citri*-negative trees only in 2007 (Table 1).

No difference in tree size (height and canopy width) was observed in 2006, but high data variability and lower sample number (5 compared to 8) influenced the analyses. However, in 2007 trees harboring *S. citri* were approximately 14% shorter and 6% smaller in canopy width than were the *S. citri*-negative trees. Severely symptomatic trees sustained greater differences in tree size than did mildly symptomatic trees ($p<0.01$) (Table 1).

Fruit from *S. citri*-positive trees were lighter in weight and smaller than those from *S. citri*-negative trees. Significant differences in levels of fruit sunburn, which results from the lack of leaf shading due to shorter leaves internodes and/or defoliation on *S. citri*-positive plants, which dries the juice vesicles, occurred only in 2007 (Table 2). *S. citri*-positive trees had approximately 8% more misshapen fruit than did *S. citri*-negative trees. This difference was even greater (15.4% more) when the comparison was restricted to *S. citri*-negative vs. severely symptomatic trees (Table 2). Of the three variables measured by the digital colorimeter (light, chroma, and hue angle), only chroma was different, and was lower in *S. citri*-positive trees than *S. citri*-negative trees during the 2007 evaluation. This difference was much greater in severely symptomatic trees ($p=0.04$) than in mildly symptomatic ones (Table 2). The only significant difference in juice quality was observed in juice weight in 2006 in the comparison *S. citri*-positive mildly symptomatic trees vs. *S. citri*-free trees (Table 3).

Distribution of *S. citri* in mildly and severely affected citrus trees. Receptacles of fruit from severely symptomatic trees yielded *S. citri* cultures almost twice as frequently as did those from mildly symptomatic trees when fruit samples were harvested randomly within the tree canopy ($p=0.02$) (Fig. 1A). The average time required to reach log phase was longer for *S. citri* cultures obtained from mildly symptomatic trees (27 days) than for those from severely symptomatic trees (19 days) ($p=0.07$) (Fig. 1B). Sub-cultures of *S. citri* isolates obtained from mildly and severely symptomatic trees multiplied at similar rates in LD8 broth when the initial cell concentration was the same (Fig. 2)

Real-time PCR evaluations using primers designed to amplify the P-58 multi-copy gene demonstrated that the

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Table 2. Comparison of fruit features from *Spiroplasma citri*-free and *S. citri*-positive sweet orange trees from a commercial citrus grove in Kern County, California.

2006										
Variables		*S. citri*-positive vs *S. citri* negative[a]			Mild vs *S. citri* negative[b]			Severe vs *S. citri* negative[c]		
		S. citri-positive	*S. citri*-negative	P value	Mild	*S. citri*-negative	P value	Severe	*S. citri*-negative	P value
Weight (Kg)		3.5	4.2	0.01	3.7	5.0	<0.01	3.2	3.5	0.48
Length (L) (mm)		47.0	54.8	<0.01	49.3	57.1	0.02	44.6	52.2	0.02
Width (W) (mm)		48.3	52.2	0.02	49.8	56.4	0.11	46.9	48.0	0.58
Ratio (L/W)		0.9	1.0	0.01	1.0	1.0	0.53	0.9	1.1	<0.01
Sunburn (%)		4.7	1.7	0.09	4.7	1.3	0.22	4.7	2.0	0.32
	L	50.3	50.2	0.91	48.6	47.6	0.63	52.1	52.8	0.74
Peel color[d]	C	38.8	39.2	0.34	31.0	36.5	0.28	40.7	42.0	0.80
	H	117.8	118.2	0.06	119.8	120.7	0.66	115.9	115.7	0.92
2007										
Variables		*S. citri*-positive vs *S. citri* negative[a]			Mild vs *S. citri* negative[b]			Severe vs *S. citri* negative[c]		
		S. citri-positive	*S. citri*-negative	P value	Mild	*S. citri*-negative	P value	Severe	*S. citri*-negative	P value
Weight (Kg)		4.5	5.0	0.05	5.2	5.2	0.92	3.8	4.9	0.01
Length (L) (mm)		65.3	71.0	<0.01	70.4	72.0	0.43	60.3	70.1	<0.01
Width (W) (mm)		64.6	68.3	0.03	69.2	69.1	0.94	60.0	67.6	<0.01
Ratio (L/W)		1.0	1.0	0.02	1.0	1.0	0.07	1.0	1.0	0.11
Sunburn (%)		6.0	1.9	<0.01	2.5	1.2	0.40	9.6	2.5	<0.01
	L	65.4	68.2	0.10	66.8	68.3	0.52	64.0	68.1	0.09
Peel color[d]	C	58.1	63.0	0.05	60.8	63.2	0.49	55.4	62.9	0.04
	H	94.6	90.4	0.11	92.7	90.4	0.53	96.5	90.3	0.10

Data compared by analysis of variance in a split plot arrangement using pairwise t-test comparisons. Evaluations performed in 2006 and 2007

[a] Includes 600 fruit (2006 evaluation) or 960 fruit (2007 evaluation) harvested from pairs of *S. citri*-negative and *S. citri*-positive trees (mild and severe pooled together)

[b] Includes 300 fruit (2006 evaluation) or 480 fruit (2007 evaluation) harvested from mildly symptomatic trees and their corresponding *S. citri*-negative pairs

[c] Includes 300 fruit (2006 evaluation) or 480 fruit (2007 evaluation) harvested from severely symptomatic trees and their corresponding *S. citri*-negative pairs

[d] Peel color parameter L, light; C, chroma and H, hue angle.

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Table 3. Juice quality characteristics from *Spiroplasma citri*-free and *S. citri*-positive sweet orange trees from a commercial citrus grove in Kern County, California.

2006									
Variables	*S. citri*-positive vs *S. citri* negative[a]			Mild vs *S. citri* negative[b]			Severe vs *S. citri* negative[c]		
	S. citri-positive	*S. citri*-negative	P value	Mild	*S. citri*-negative	P value	Severe	*S. citri*-negative	P value
Weight (Kg)	1.0	1.2	0.11	1.0	1.4	0.01	1.0	1.0	0.81
Brix (%)[d]	10.5	10.4	0.26	10.0	10.3	0.63	10.9	11.5	0.27
TA (%)[e]	1.4	1.5	0.92	1.2	1.3	0.24	1.5	1.5	0.19
Ratio (Brix/TA)	7.6	7.9	0.28	8.2	80.	0.61	7.0	7.8	0.06
2007									
Variables	*S. citri*-positive vs *S. citri* negative[a]			Mild vs *S. citri*-negative[b]			Severe vs Healthy[c]		
	S. citri-positive	Healthy	P value	Mild	*S. citri*-negative	P value	Severe	*S. citri*-negative	P value
Weight (Kg)	1.9	2.0	0.24	2.1	2.1	0.81	1.6	2.0	0.07
Brix (%)[d]	12.9	13.5	0.37	13.1	13.3	0.75	12.8	13.6	0.34
TA (%)[e]	1.3	1.3	0.96	1.3	1.3	0.58	1.4	1.4	0.54
Ratio (Brix/TA)	9.6	10.0	0.47	9.9	10.5	0.21	9.4	9.5	0.80

Data compared by analysis of variance in a split plot arrangement using pairwise t-test comparisons. Evaluations performed in 2006 and 2007

[a] Includes juice extracted from 600 fruit (2006 evaluation) or 960 fruit (2007 evaluation) harvested from pairs of *S. citri*-negative and *S. citri*-positive trees (mild and severe pooled together)

[b] Includes juice extracted from 300 fruit (2006 evaluation) or 480 fruit (2007 evaluation) harvested from mildly symptomatic trees and their corresponding healthy counterparts

[c] Includes juice extracted from 300 fruit (2006 evaluation) or 480 fruit (2007 evaluation) harvested from severely symptomatic trees and their corresponding healthy counterparts

[d] Brix, measurement of dissolved sugar-to-water mass ratio

[e] TA, titration acidity assay using citric acid equivalents

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Table 4. Presence of *Spiroplasma citri* in different canopy tiers and aspects of Navel sweet orange trees, based on quantitative PCR analyses of fruit central axis and leaf petiole tissues from mildly and severely symptomatic trees, data obtained in 2007.

Status	Fruit central axis			Petiole		
	Mild symptoms	Severe symptoms	P value	Mild symptoms	Severe symptoms	P value
Number of positive samples (%)[a]	24.4	56.7	<0.01	4.4	21.1	<0.01
Number of positive samples from east side (%)[b]	28.9	53.3	0.02	6.7	22.2	0.03
Number of positive samples from west side (%)[b]	20.0	60.0	<0.01	2.2	20.0	0.01
Number of positive samples from lower canopy (%)[c]	20.0	46.7	0.03	6.7	20.0	0.13
Number of positive samples from mid canopy (%)[c]	26.7	63.3	<0.01	0.0	16.7	0.02
Number of positive samples from upper canopy (%)[c]	26.7	60.0	0.01	6.7	26.7	0.04

The presence or absence of *S. citri* in fruit central axis and leaf petiole samples was analyzed by a chi-square test using SAS software 9.1 and PROC FREQ. Data were sorted first by disease status (mild vs. severe) and canopy aspect and then by status alone. Percentage means number of samples *S. citri* positive by q-PCR divided by the total number of samples harvested multiplied by 100.

[a]Includes fruit central axis or petioles from 5 mildly or 5 severely symptomatic trees (90 samples from each tree status)

[b]Includes fruit central axis or petioles from 5 mildly or 5 severely symptomatic trees (45 samples from each tree status; 9 from lower canopy, 9 from mid canopy and 9 from upper cannopy)

[c]Includes fruit central axis or petioles from 5 mildly or 5 severely symptomatic trees (30 samples from each tree status)

Fig. 1. Percentage of sweet orange receptacle fruit from mildly or severely symptomatic citrus trees affected by citrus stubborn disease that yielded *Spiroplasma citri* cultures p=0.02 (A). Time required by *S. citri* cultures to achieve the log phase p=0.07 (B). Bars represent standard error.

Fig. 2. Number of *Spiroplasma citri* cells 24 and 48 h after an initial sub-culture in LD8 broth. Isolates cultivated from receptacles of sweet orange fruit obtained from mildly or severely symptomatic citrus trees affected by citrus stubborn disease. Different letters represent a p-value lower than 5%. Bars represent standard error.

presence of *S. citri* was not related to either canopy aspect (east vs. west) or tier (lower, medium and upper part of tree canopy), regardless of whether the DNA template source was fruit central axis or petiole tissue (data not shown). Tree disease status (mildly or severely symptomatic), on the other hand, had a significant effect on *S. citri* distribution as determined by real-time PCR analyses (Table 4). Severely symptomatic trees had twice as many spiroplasma-positive sites within the tree canopy than did mildly symptomatic trees when the DNA template came from the fruit central axis (Table 4). Use of leaf petiole tissue resulted in fewer positives than did fruit central axis, but petiole samples from severely symptomatic trees also had a greater number of positive real-time PCR results than did samples from mildly symptomatic trees (Table 4).

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DISCUSSION

Although CSD has been present in California orchards since 1915, disease diagnosis has been based, until recently, exclusively on symptoms, spiroplasma culturing and to a limited extent on serological tests (Yokomi *et al.*, 2008). CSD symptoms can be confused with nutritional deficiencies, other plant diseases, or environmental impacts (Bové *et al.*, 1974; Olson and Rogers, 1969). Thus, assessment of impact was imprecise. The recent development of more sensitive molecular techniques to detect *S. citri* (Yokomi *et al.*, 2008) allowed us to identify mildly or non-symptomatic, but *S. citri*-positive, trees for inclusion in our study.

Based on a two year study at one location the majority of the symptoms related to tree development and fruit quality parameters assessed were associated primarily with severely symptomatic *S. citri*-positive trees, while trees having no, or mild, symptoms were relatively, or completely, indistinguishable from spiroplasma-free trees.

Differences in tree development and productivity among infected and non-infected trees in general were greater in 2007 than in 2006. The average air and soil temperatures in the region near the orchard were very similar in both years (data not shown) and the only significant climatic difference between the years was the occurrence of a 20-day freezing period in January 2007 when temperatures reached a low of -8.3°C. Overall, there were 40 days of hard freezes in the winter of 2006-2007 vs. 5 days in 2005-2006. Trees having smaller and defoliated canopies (a symptom of CSD) could have suffered more from the frost (possibly also related to the greater impact on fruit production observed in that year in comparison with 2006). In addition, the fact that a larger number of trees was sampled in 2007 could have decreased the data variability, thereby emphasizing the differences between the two years of evaluation.

CSD affects both tree height and canopy diameter. Sweet orange trees *S. citri*-positive were 13% shorter and had 6% smaller canopy widths than did *S. citri*-negative trees in the 2007 assessment, with severely symptomatic trees accounting for most of the statistical differences encountered. Previous reports from California (Calavan and Christiansen, 1966) indicated that plants graft-inoculated with *S. citri* were up to 55% shorter than *S. citri*-negative controls. The difference between our findings and previous reports are likely due to the fact that trees in our study were infected naturally by leafhoppers, and therefore received a much lower spiroplasma inoculum dose than do graft-inoculated trees. In Cyprus (Kyriakou *et al.*, 1996), the presence of *S. citri* in Navel sweet orange trees caused no significant impact on tree development. However, trees in that study ranged from mildly to severely symptomatic, so our finding that only severely affected trees are likely to be smaller than *S. citri*-negative trees suggests an explanation for the different results in our studies.

CSD affects fruit production and yield in several ways. Navel orange trees *S. citri*-positive produced 26 to 32% fewer fruit than did *S. citri*-free trees, and the loss was even greater (53 and 45% in 2006 and 2007, respectively) when only severely symptomatic trees were considered. Lower yield was influenced also by earlier fruit drop and the production of lighter and smaller fruit on *S. citri*-positive trees than on *S. citri*-negative trees.

Symptoms related to tree size and fruit yield are likely related to the fact that *S. citri*, a phloem resident, requires carbohydrates and sterols from its plant host (Andre *et al.*, 2005; Chang, 1998). While living in plants, spiroplasmas compete with their hosts for these energy sources, causing depletion of some sugars and hormones and accumulation of others. The resulting imbalance affects the normal metabolism of the citrus plant, causing stunting, leaf mottling, production of smaller and fewer fruit and off-season blooming.

In our work, as in an earlier study in Cyprus (Kyriakou *et al.*, 1996), the quantity and quality of juice from fruit of *S. citri*-positive trees were similar to those from *S. citri*–free trees. However, others (Calavan and Oldfield, 1979) reported insipid, sour or bitter flavor in fruit of *S. citri*-positive trees. In our study, a few *S. citri*-positive trees produced fruit with unusually high citric acid content, although these differences were not statistically significant. The inconsistency among these different studies could be due to the reported variability in chemical composition of fruit on *S. citri*-positive trees (Bové *et al.*, 1961). Additionally, we assessed fruit from at least 10 *S. citri*-positive trees whereas previous studies compared fruit from only two trees (Bové *et al.*, 1961).

Although it is logical to expect that the impacts of CSD on citrus tree development and production would be greater in severely affected trees than in mildly symptomatic trees, our study documented and quantified those differences for the first time. Others have suggested that symptom severity could be related to different bacterial strains and/or titer (Calavan, 1969; Calavan and Bové, 1989). We found *S. citri*-positive leaf petioles and fruit central axis in higher numbers, and from significantly more of the randomly selected sites within the tree canopy, in severely symptomatic, than in mildly symptomatic plants. The differences could be due to higher *S. citri* multiplication rates and/or a higher amount of initial inoculum in the former than in the latter. The differences we encountered in the time required for visual confirmation of bacterial growth after cultivation from samples from severely and mildly symptomatic trees also suggest that the titer of the bacterium is higher in trees having severe symptoms than in those having mild symptoms, especially since we found no statistical difference in the growth rates of spiroplas-

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ma isolates from these two tree groups.

Although many anecdotal observations have been reported, and previous work has documented some of the impacts of CSD on citrus production and quality in California, this is the first comprehensive work to characterize and quantify these impacts. Our study focused on a naturally-infected Navel orange orchard in the San Joaquin Valley. Trees having severe symptoms of CSD sustained a highly significant impact on fruit production due to lower yield and number of fruit, and lower fruit quality, whereas mildly symptomatic trees rarely sustained major impacts in comparison with the *S. citri*-negative controls. Thus, the management of infected trees should be evaluated according to the conditions in each grove. In orchards where the incidence of severely CSD symptomatic trees is high, the removal and replacement of such plants should be analyzed as one alternative to restore normal production of the orchard in the short-term. Citrus is not a preferred host of the main vector of *S. citri*, *C. tenellus* (Oldfield *et al.*, 1976), and CSD-infected plants are not likely to serve as a significant inoculum source to *S. citri*-negative citrus plants. However, *S. citri* asymptomatic or mildly CSD symptomatic trees could become severely symptomatic with time, and, in the long-term management of *S. citri* infected orchards these plants should be inspected periodically to monitor the progress of the disease. When disease severity increases rouging of *S. citri*-positive trees and tree replacement should be considered.

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