NEW PERSPECTIVES ON THE EPIDEMIOLOGY OF CITRUS STUBBORN DISEASE IN CALIFORNIA ORCHARDS

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CHAPTER I

INTRODUCTION

Citrus is a major crop in United States, and California ranks first in fresh fruit production (16). Historically, citrus stubborn disease (CSD) was a significant problem in the region (9), but its studies were done mainly during the 1960s and 1970s (2, 4, 15). Although CSD has been present in California since 1915 (6), its impact in the San Joaquin Valley has had greater visibility in recent years, since more citrus growers have reported the occurrence of symptoms including general stunting, short leaf internodes, leaf mottling, unseasonal blossoming and lopsided fruits, all of which are consistent with CSD (6).

Stubborn disease is caused by *Spiroplasma citri*, a phloem-inhabiting, cell wall-less bacterium in the class Mollicutes (8, 17). *S. citri* is transmitted in a propagative manner by several species of leafhoppers that are common inhabitants of California citrus groves and natural habitats (11, 14). *Circulifer tenellus* (Baker), the beet leafhopper, was reported as the major vector of the pathogen but other species of leafhoppers could also be important in disease epidemiology (11, 14). The general distribution of CSD-infected plants in commercial crops suggests a migration of the infected insects from weeds to the commercial crops. The ability to feed on different plant species and to migrate long distances make this insect a key element in disease epidemiology (7). *S. citri* can be transmitted by vectors to several weed and crop species, and the occurrence of new crop host, such as carrots, indicates that the host range of the vector may be increasing, and that the emergence of new vectors could be occurring (10). Bacterial survival during environmental and host changes is facilitated by gene evolution, which is driven by small local changes in nucleotide sequence, intragenomic reshuffling and acquisition of

DNA from other organisms (12). The very small genome of *S. citri* easily acquires or deletes genetic components, thereby becoming more fit (13). For example, continuous graft transmission of *S. citri* from periwinkle to periwinkle resulted in a chromosomal inversion and genomic deletions in *S. citri* BR3-3X that were associated with loss of transmissibility by the natural vector, *C. tenellus* (18, 19). High passage in artificial medium also altered *S. citri* transmissibility (18). These mechanisms, alone or in conjunction, could generate new genes that increase the ability of *S. citri* to adapt to changes in the host or the environment.

Stubborn epidemiology is influenced by factors related to the spiroplasma, its plant hosts, vectors, management practices and the environment. In California, disease spread was variable in different locations and incidence was higher in the interior valleys in comparison with the coastal region (5). Levels of transmission and symptom expression were correlated with temperature and were higher under warm conditions (1, 3). However, few epidemiological studies of stubborn diseases have been performed, limiting our understanding of this complex pathosystem and our ability to develop optimal measures of management and control.

In this research we investigated the severity and epidemiology of CSD in California. Understanding the reasons for the possible recent re-emergence of the disease, after a relatively quiescent period, will be critical in the development of management strategies that are effective, economical and safe for people and the environment. Specific objectives of this research were:

i) Optimize sampling protocols and tools for detection of *S. citri* in citrus plants and analyze the incidence of stubborn disease in California orchards

ii) Evaluate the genetic diversity among strains of *S. citri* from different locations, countries, hosts and time of isolation

iii) Assess the impact of *S. citri* on citrus development and production in one commercial citrus orchard

iv) Assess the relationship of citrus stubborn disease symptoms to *S. citri* genotype and spiroplasma titer in sweet orange plants

v) Confirm *S. citri* as the causal agent of carrot purple leaf disease and evaluate the specificity of *S. citri* strains to citrus and carrot plants

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CHAPTER II

REVIEW OF LITERATURE

1. Citrus

1.1. Uses and origin

Oranges are the third most consumed fresh fruit in the United States, after bananas and apples, with a per capita consumption of 12.3 pounds in 2000/01. As juice, this fruit becomes the most consumed fruit, with U.S. residents consuming an average of 5.19 gallons per year, the equivalent of approximately 74.1 pounds of fresh fruit. This usage is more than twice that for apple juice, the second most consumed juice. The consumption of oranges is not related to annual consumer income, but rather to the customs of the regions within the U.S. (47). Besides fresh fruit and juice, oranges and other citrus fruits also are used to produce marmalade, perfume, pectin and cattle feed (12).

The origin of oranges and all the other species of the genus *Citrus* is believed to be Asia. The first written record of this genus, in 310 B.C. concerned citron (*Citrus medica* L.). Later, reports of sour orange (*Citrus aurantium* L.), lemon (*Citrus limon* Burm. f.) and sweet orange (*Citrus sinensis* [L.] Osb.) appeared. Sweet orange was reported in Europe around the 15th century, in South America in 1549, Central America in 1568, southern Africa in 1654 and Australia in 1788 (12, 50). In the U.S. the first planting occurred around 1565 in Florida and plantings followed in South Carolina and Georgia in 1577, Arizona in 1683 and California in 1769 (50).

1.2. Citrus producers

World citrus production is concentrated in few countries and the estimated production for 2004/05 was 70 million tons. Brazil is the greatest producer (16.4 million tons) followed by China (12.9 million tons), the United States (11.4 million tons), Mexico (6.3 million tons) and Spain (5.7 million tons). Other countries, including Egypt, Italy, South Africa, Turkey, Morocco, Argentina, Greece, Australia, Cuba and Israel, are also significant producers (15).

Citrus production in the U.S. occurs primarily in four states: Florida, California, Texas and Arizona. Florida is the greatest producer, with 7.5 million tons, followed by California with 3.3 million tons; and Texas and Arizona together produced less than a half million tons in the period 2004/05 (15). Around 68% of all the American citrus produced is transformed into juice or other drinks and almost all of the oranges produced in Florida (96%) are processed. California fruit, on the other hand, is almost all sold as fresh fruit (59).

1.3. Botany

The earliest taxonomic system for citrus was proposed in 1875; later classifications were proposed based on morphology, DNA characteristics and origin (12). The present classification places citrus in the Rutaceae family, subfamily Aurantioideae, tribe Citreae and sub-tribe Citrinae. The most important genera are *Poncirus* (trifoliate orange), *Fortunella* (kumquat) and *Citrus* (oranges, mandarins, grapefruit and others) (54).

The most important commercial genera are characterized by evergreen, medium-sized trees that produce white flowers, single leaflet leaves (except for *Poncirus*, which has three), and a winged leaf petiole that is useful in identification. Seeds are produced by sexual fertilization and also by adventitious nucellar embryos, which are asexual and genetically identical to the mother plant, in contrast to zygotic embryos, which are products of cross or self pollination. Plants from different citrus genera are able to hybridize, a characteristic that provides a genetic resource to citrus breeders (54).

The fruit of citrus, a hesperidium berry, arises from the development of a fertilized ovary. Despite its name, this fruit is not a true hesperidium because it lacks the latter's characteristic peel surrounding the fruit like grapes and tomatoes (12). The outer layer of a citrus fruit, called the exocarp, or flavedo, is composed of tabular parenchyma cells. This layer contains oil glands, and the parenchyma cells contain chloroplasts that give young fruits their green color. Underneath the exocarp is a white spongy layer called the albedo or mesocarp and a third layer called the endocarp, which contains section walls, seeds and juice vesicles. The core of the fruit is a white structure called the columella, which is filled with vascular bundles that transport nutrients from the stem and the root into the fruit and leaves (12, 49).

1.4. Citrus cultivars

Commercial citrus trees usually consist of a rootstock, which supplies water and nutrients, and a scion or fruit-bearing portion. Four groups of citrus are commercially cultivated worldwide as scions or rootstock. Because of its adaptability to different climatic conditions and its wide range of cultivars, *Citrus sinensis* (sweet orange), which originated from the northeastern region of India and central China, is considered to be the most economically important group of citrus. This group can be divided into four sub-groups according to morphology and season of maturity (12, 54):

- Round orange: This sub-group, also known as the common orange, can be used for juice and fresh fruit and is the most-planted citrus type in the world. Round orange cultivars have different fructification cycles, but commercial orchards usually plant several cultivars including one of an early season maturity (such as 'Hamlin'), a mid season maturity (such as 'Pera' or 'Shamouti') and one of late season (such as 'Valencia' or 'Natal') which allow harvesting for a long period of time.
- Navel orange: Fruit of this group usually is seedless because of partial or complete ovule sterility. High levels of limonin, which causes bitterness in juice, limit the use of this group to fresh fruit. The most popular cultivars are Baianinha, Navelina, Navelate, Washington, Atwood, Fisher, Leng and Newhall.

- Blood orange: Also named pigmented orange, because of the red anthocyanin pigmentation, this group of citrus is mainly important in the United States, Italy, Spain, Morocco, Algeria and Tunisia.
- Acidless orange: Sweet oranges of this sub-group lacks the flavor and the aroma of typical citrus because of the lack of acidity in their fruits, which usually are consumed in the fresh market.

Mandarin, tangerine and soft orange are different names for *Citrus reticulata* Blanco, *Citrus unshiu* Marc. and *Citrus deliciosa* Ten., which are widely consumed as fresh fruit or in juice blends to improve color. Limes (*Citrus aurantifolia* L.), the third most important member of the genus *Citrus*, are limited to the tropics and warm conditions. They are divided into two main groups, Tahiti and Key. *Citrus limon* Burm. f. (lemons) have seedless to moderately seedy fruit and are commonly planted in warmer areas (12, 54).

Citrus paradise Macf., the fourth most commercially important group, probably originated from natural crosses between pummelo (*Citrus grandis* [L.] Osb.) and sweet orange. Its fruit, among the largest, is consumed primarily in North America, Europe and Japan (12).

1.5. Citrus cultivation and biotic disease as a limiting factor

Citrus is a common name applied to all commercial genera. Most citrus plants are perennial trees cultivated in subtropical areas between 35° and 15° latitude, where temperatures are at least 20 °F. The orchards must be fertilized by synthetic and/or organic fertilizers and irrigated when necessary. Pest management strategies are applied when economically justified (54).

Combating diseases caused by fungi, bacteria, viruses or nematodes is one of the most expensive elements of citrus production, representing around 5% of the total direct costs of production (48). The distribution and importance of each disease varies among countries (61).

In the United States the most important citrus diseases are different in the two main producer states, Florida and California. In the eastern states, tristeza, caused by the citrus tristeza virus (CTV), citrus canker (*Xanthomonas axonopodis* pv. *citri*), huang-

longbing (*Candidatus liberibacter asiaticus*), greasy spot (*Mycosphaerella citri*), scab (*Elsinoe falwcettii*), melanose (*Diaphorte citri*), Alternaria brown spot (*Alternaria alternata*), post bloom fruit drop (*Colletotrichum acutatum*), foot root (*Phytophthora nicotianae*) and brown root of fruit (*Phytophthora spp*.) are the most damaging diseases. In the western states, the viral and bacterial diseases seem to be more numerous and important than the fungal diseases (10).

Of the few diseases that occur in California citrus, tristeza is a major concern. Another damaging disease not yet found in Florida, but prevalent in California, is citrus stubborn, which is caused by a spiroplasma (10). Tristeza is a worldwide problem whose management usually involves vector control, cross-protection, and the use of disease free buds and root stock, among other practices (12). Stubborn, on the other hand, is much less characterized, particularly with respect to epidemiological features that might provide clues for the development of effective control strategies.

2. *Spiroplasma citri*

2.1. Cell and colony morphology

Initially, stubborn disease was attributed to a virus (46). The isolation and completion of Koch's postulates showing *Spiroplasma citri* to be the etiological agent were reported in the United States in 1972 (22) and in France in 1973 (52). Pleomorphic organisms were cultured from symptomatic citrus plants from California in medium containing cholesterol. Irregular bodies (0.5-2 μm across and 7 μm long) were observed by electron microscopy and, after subculture onto solid medium produce fried-egg colonies (22). *S. citri* reached turbidity in liquid medium and changed the pH of liquid medium from 7.8 to 5 in 2-3 days. Dependency on exogenous animal serum and cholesterol, an optimal growth temperature (32°C), resistance to penicillin and sensitivity to tetracycline were also reported (52).

Cells of *S. citri* are usually helical in the plant host and certain media, but non-helical isolates also have been reported in media and in insects (56). Helical cells of *S. citri* have between one and more than ten turns. Active and growing cultures usually have a high proportion of four-turn and two-turn helices. The terminal portions of two-turn

cells usually are blunted on one end and tapered on the other, but similar terminals on both ends occur (23). As the culture matures, the average numbers of turns increases, and aggregates, consisting of multiple spiroplasmas, form. Eventually, all the cells become spherical bodies (23, 55). In solid media with low agar concentrations helical spiroplasmas give rise to colonies having a central mass surrounded by satellites created by motile cells that migrate from the center. On the other hand, non-helical strains, independent of agar content, form colonies without satellites that resemble fried eggs (55).

Spiroplasmas lack a cell wall, but are surrounded by a cholesterol-rich membrane and are shaped by a cytoskeleton composed of fibrils arranged in parallel organization. Membrane and fibrils are coiled in a dynamic structure along spiroplasma cell (57). This morphological structure allows the organism to move by propagation of kink pairs through the length of the body (53).

Among all *S. citri* membrane proteins spiralin is the most abundant in *S. citri* and has a significant role in insect transmission (14). This protein has an unusual chemical makeup, lacking methionine, histidine, tryptophan and arginine (6). The gene that encodes spiralin usually has a very conserved sequence in the first 24 amino acids at the N-terminus. Spiralins of different strains of *S. citri* have the same number of amino acids but are usually polymorphic along the sequence (20).

2.2. Isolation

Isolation of *S. citri* begins with an initial surface sterilization of plant tissue or leafhopper followed by maceration in broth medium and filtration (7). Media optimal for *S. citri* is different from that used for other spiroplasma species and must contain inorganic salts, amino acids, organic acids, carbohydrates, cholestorerol and other minor nutrients (11, 38). To avoid possible spiroplasmastatic effects caused by plant tissue inhibitors, subculturing is recommended (39). After isolation the multiplication rate of *S. citri* is variable, but the doubling time usually is about 4 hours at 31 °C, and after 2 to 4 days of isolation the titer is around 10^9 colony forming units mL^{-1} (38, 55).

2.3. Phylogeny and taxonomy

S. citri is phylogenetically related to Gram positive bacteria from the family *Bacillaceae*, genera *Bacillus*, *Lactobacillus* and *Streptococcus* (63). The present classification of *S. citri* places this organism in the Domain Bacteria, Phylum *Firmicutes*, Class *Mollicutes*, Order *Entomoplasmatales*, Family *Spiroplasmataceae*, and Genus *Spiroplasma* (24).

Spiroplasma group members may be classified according to serological characteristics such as cross-serological growth inhibition and organism deformation. Initial classification placed *S. citri* in Serogroup I, along with the honey bee spiroplasma and *S. kunkelli*, causal agent of corn stunt (13). Present classification includes 34 group designations. *S. citri* is still considered as a member of Serogroup I but this group includes seven members (24, 62).

3. Symptoms

The citrus disease caused by *S. citri* in California was initially called “stubborn”, because of the difficulty in controlling it, while in Israel it was called “little leaf” because of its symptoms (9, 41). Eventually the name “stubborn” became universal.

Citrus trees with stubborn disease are characterized by unseasonal flushes of leaves, stems and flowers. Leaves usually are smaller than normal and mottled. In severe infections there are multiple buds and an excessive number of shoots and internodes, along with a general stunting of the tree. Fruit of infected trees usually are lopsided with a curved columella; the albedo may become blue (mainly in grapefruit and tangelos) and the flavor may be insipid, sour or bitter. Roots also may be stunted (9).

Symptom expression is affected by temperature (5, 46). Citrus inoculated by grafting and kept in greenhouses have a latent period of around two months under temperatures of 35 °C/27 °C (day/night). Plants grown at temperatures below 35 °C/27 °C (day/night) did not develop symptoms at all until they were transferred to warmer conditions. Infected trees grown at high temperatures had a higher percentage of symptomatic plants and decreased shoot length, indications that the disease has become more severe. In addition to the influence of temperature, the occurrence of mildly and severely symptomatic CSD trees under field conditions can also be associated with bacterial titer within the plant and/or strain virulence (8, 9).

Little is known about the mechanisms of *S. citri* pathogenicity and plant symptom expression (27). Mutagenesis using random insertions of the transposson Tn*4001* demonstrated that the fructose operon is somehow related to *S. citri* virulence and to the length of the latent period (27). The fructose operon comprises three genes (*fruR, fruA* and *fruK*) that normally transcribe two messenger RNAs. Mutation within the fructose operon resulted in lack of transcription, preventing fructose utilization by mutated spiroplasmas (27). The inability to utilize fructose as a source of energy by fructose-operon mutated *S. citri* strains reduced the aggressiveness of the pathogen, resulting in plants having symptoms milder than those induced by the wild type spiroplasma (26, 27).

Symptoms of stubborn are relatively nonspecific and may be confused with those of other biotic diseases such as citrus tristeza, exocortis and vein enation. The mottling in leaves is similar to that seen with abiotic conditions caused by iron, zinc and manganese deficiency (54). Among all citrus species, *C. sinensis, C. paradise, C. reticulata X C. paradise* and *C. reticulata* are the most susceptible in the field (9, 10).

In addition to citrus, *S. citri* also infects two other commercial crops, horseradish (*Armoracia rusticana* (Gaertn., Mey., Scherb.) and carrots (37). Possible migratory routes of the leafhopper vector *Circulifer tenellus* (19) probably introduced *S. citri* in to the northern states of United States, causing a disease in Illinois and Maryland horseradish called “brittle root”, which is characterized by stunting and chlorosis (16, 18). Recently, carrots grown in the state of Washington showing purple leaves, general stunting, secondary tap and bunchy roots and were shown to be infected by *S. citri* and/or a phytoplasma, becoming the third naturally infected commercial crop (36). Natural occurrence of *S. citri* in zinnia (*Zinnia elegans* Jacq.), aztec marigold (*Tagetes erecta* L.), viola (*Viola cornuta* L. ‘Alba’) and foxglove (*Digitalis purpurea* L.) and the weeds london rocket (*Sisybrium irio* L.) and wild turnip (*Brassica tournefortii* Gouan) were also reported (1, 29, 31).

Plants artificially inoculated with *S. citri* also could present symptoms similar to those of stubborn. Brassicaceas, Fabaceas, Asteraceaes, Caryophyllaceaes, Malvaceaes, Plumbaginaceaes, Ranunculaceaes, Rosaceaes, Violaceaes and Liliaceaes were families reported to show symptoms of interveinal chlorosis, apical rosette, proliferation of lateral buds, stunting and wilt 2 to 4 months after artificial inoculation (9).

4. Identification

Detection of *S. citri* can be done by several methods including Dienes' staining of phloem sieve tubes, fluorescent dye (4'6-diamidino-2-phenylindole), electron microscopy, polyacrylamide gel electrophoresis, DNA-DNA hybridization, genomic sequencing and host range (17). However, spiroplasma isolation in culture media and later observation using dark field microscopy (58) and polymerase chain reaction (PCR) are more commonly used today (67).

5. *Spiroplasma citri* dispersal

5.1. Leafhopper transmission

Insect transmission of *S. citri* occurs by leafhoppers in the Order Hemiptera: suborder Homoptera: family Cicacellidae: subfamily Deltocephalinae (17). *Scaphytopius nitridus* (DeLong) was the first reported insect vector, transmitting *S. citri* from citrus to periwinkle and citrus under greenhouse conditions (30, 44). Later, *Circulifer tenellus* (the beet leafhopper), collected from California orchards, was shown to harbor and to transmit *S. citri* to citrus and periwinkle (45) and to the weed London rocket (29). Under experimental conditions only, *Macrosteles fascifrons* transmited the spiroplasma from aster (*Callistephus chinensis*) to aster, and from horseradish to the Brassicaceous weed yellow rocket (*Barbarea vulgaris*), aster, and turnip (43).

Circulifer haematoceps (synonym: *Neoaliturus haematoceps*) is a key vector in the Mediterranean region including Turkey, Morocco, Syria and France (Corsica) (21). In southern Turkey five other leafhoppers were found to carry *S. citri* in their bodies, but only one of them, *Circulifer opacipennis* (Lethierry), was effective in transmission to *Catharanthus roseus* L. (32).

Among all species reported to transmit *S. citri* in the United States *C. tenellus* is the primary vector of *S. citri* (10).The mode of transmission is propagative, the mollicute entering the gut lumen, passing through the gut wall by traversing the epithelial cells, moving to the base of the intestinal membrane and into the hemocoel. *S. citri* cells are transported by the hemolymph, where they multiply, to the salivary glands, where they

enter salivary gland cells, multiply, and finally move into the salivary canal. The leafhopper then is able to infect plants (40).

During leafhopper acquisition, spiroplasmas orient and attach in the host cell membrane via their tapered ends (2). In the insect the prokaryote may damage membranes and basal lamina and cause disorganization of the endoplasmic reticulum (35), which increases leafhopper mortality (40). Spiroplasmas propagated by multiple plant grafts or multiple sub-cultures lost their ability to cross gut and salivary gland barriers and the ability to be transmitted by *C. tenellus* (60). After multiple subcultures or graftings, isolates also lacked three proteins (146, 144 and 92 kDa) suggested to be involved in transmission (19).

Some proteins may be important in *S. citri* adherence to cells of its vector during transmission (68). Spiralin is the most abundant protein of *S. citri* and mutants defective in the spiralin gene, when injected into insects, can multiply as well as wild type spiroplasmas but their transmission rates were lower than those of the wild type (14). This decrease in transmission efficiency suggests that spiralin acts as a binding factor in interactions with glycoproteins of the vector (34). Besides spiralin, P89 (designated SARP1) encoded on plasmid pBJS-O (28, 68), P58 (65), and P32 encoded on plasmid pSci6 (4, 33) are also related to *S. citri* transmission.

5.2. Grafting

Contaminated bud citrus propagating material may be another way of *S. citri* infection since this pathogen is easily inoculated by grafting with infected scions (51). Use of *S. citri* free buds may be an easy way to avoid the disease where it is not endemic (10).

6. Genetic information

S. citri has one of the largest genomes among Mollicutes, with a size around 1.8 Mbp. It is characterized by a high adenosine-thymidine content and utilization of UGA to encode tryptophan instead of being a stop codon as in other organisms. Beyond the

circular chromosome, *S. citri* also contains plasmids and virus genomes that contribute to genetic information (42, 65).

Genetic variations of spiroplasmas are attributed to DNA acquisition and loss, DNA replication and repair, homologous recombination and transposition (42). Different maintenance conditions, such as graft transmission or multiple passages in medium, can lead to genome modifications due to chromosomal and extrachromosomal inversions and deletions (25, 66).

Among the mechanisms of DNA acquisition and loss by spiroplasmas, bacteriophages play an important role. The first virus reported to infect *S. citri* and introduce DNA by horizontal transference was SpV1. This circular, single stranded DNA virus was reported to integrate its full length fragment in the *S. citri* chromosome, and sometimes part of this fragment was deleted, resulting in incomplete inserts in the core DNA (3). The biological importance of inserted viral sequences is still unknown, but the physical map of the *S. citri* genome shows that this bacteriophage could be present in up to 17 copies in the genome, accounting for up to 8% of the entire genome content (3, 64).

Nucleotide substitutions, which may occur in unfavorable environmental conditions or be associated with incorrect DNA polymerization and homologous recombination, are other possible sources of genetic variation in spiroplasmas. These factors alone, or in combination, allow spiroplasma evolution under different situations.

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CHAPTER III

ASSESSMENT OF CITRUS STUBBORN DISEASE INCIDENCE IN CITRUS

Abstract

Citrus stubborn disease, caused by *Spiroplasma citri*, has occurred in California for over 90 years; however, detection methods for estimating disease incidence have not been optimized. Two 8 ha commercial citrus plots were sampled in July and August, 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 bulk sampling, all trees in 6 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.

Introduction

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 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-11 sweet orange (*Citrus sinensis* [L.] Osbeck) 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, 17).

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 the row were sampled. Each group of 4 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 were sampled. 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 (Fig. 1-C1). 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 (Fig. 1-C2). 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 is active on that site.

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. (17) 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* (16). Furthermore, they also showed results of melting curves from real time PCR assays with SYBR-green. 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. (16) 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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Figure caption

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; C. Every-tree blocking sampling (ETBS), six blocks of 64 trees each were sampled in orchard 1 (C1) and orchard 2 (C2); blocks of sampled trees indicated in gray.

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

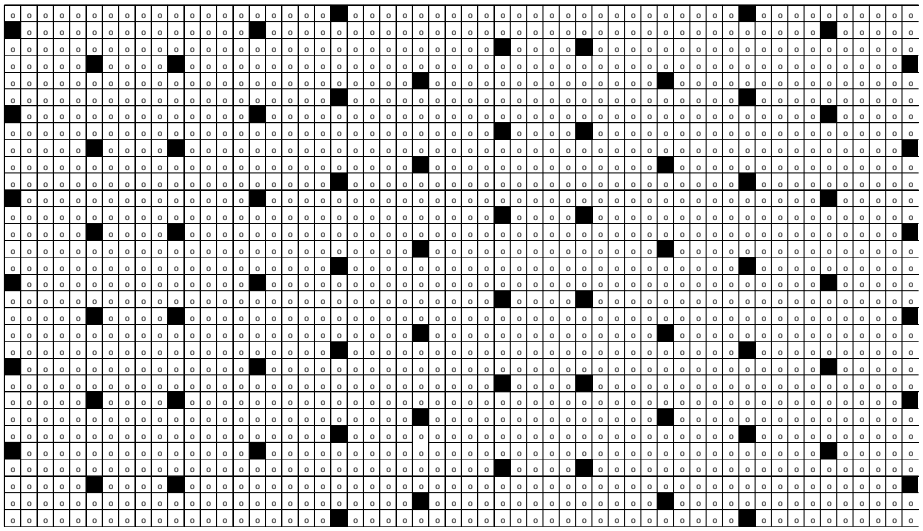
[3] ND= not done

TABLE 2. Incidence of citrus stubborn in two California sweet orange commercial orchards evaluated by stat, hierarchical and every-tree block sampling techniques.

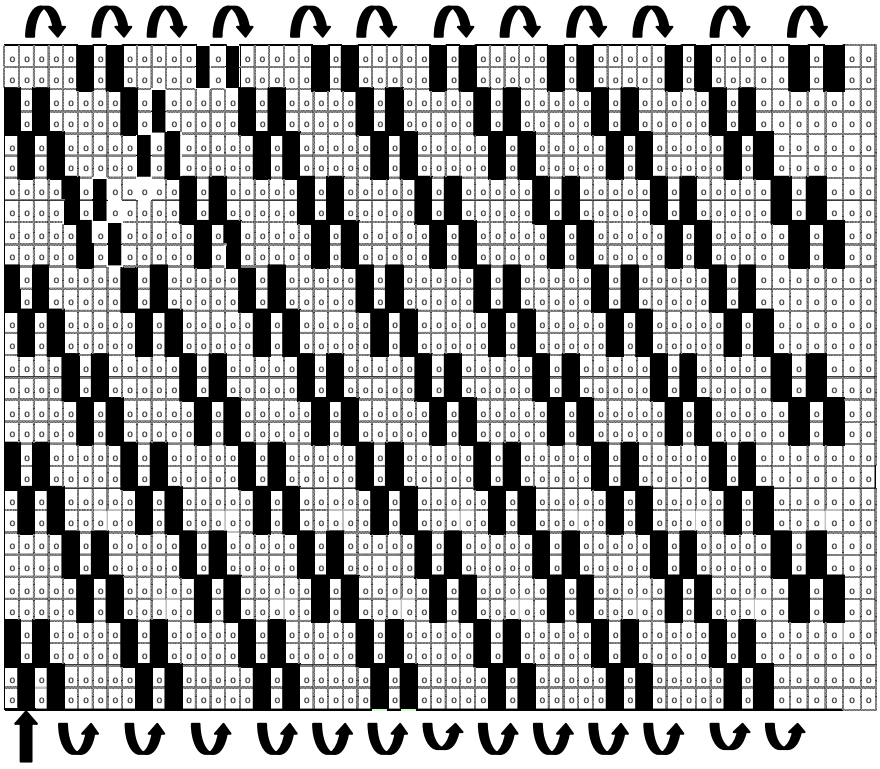
Sampling method	Stat[a]	Hierarchical*			Every-tree block		
Detection method	Culturing	Culturing	PCR	Total[b]	Culturing	PCR	Total[b]
				Orchard 1			
Total number of samples	74	105	105	105	382	382	382
Number of positive samples	34	75	77	89	191	223	225
Incidence (%)	45.9	71.4	73.3	84.8	50	58.4	58.9
				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

A.



B.



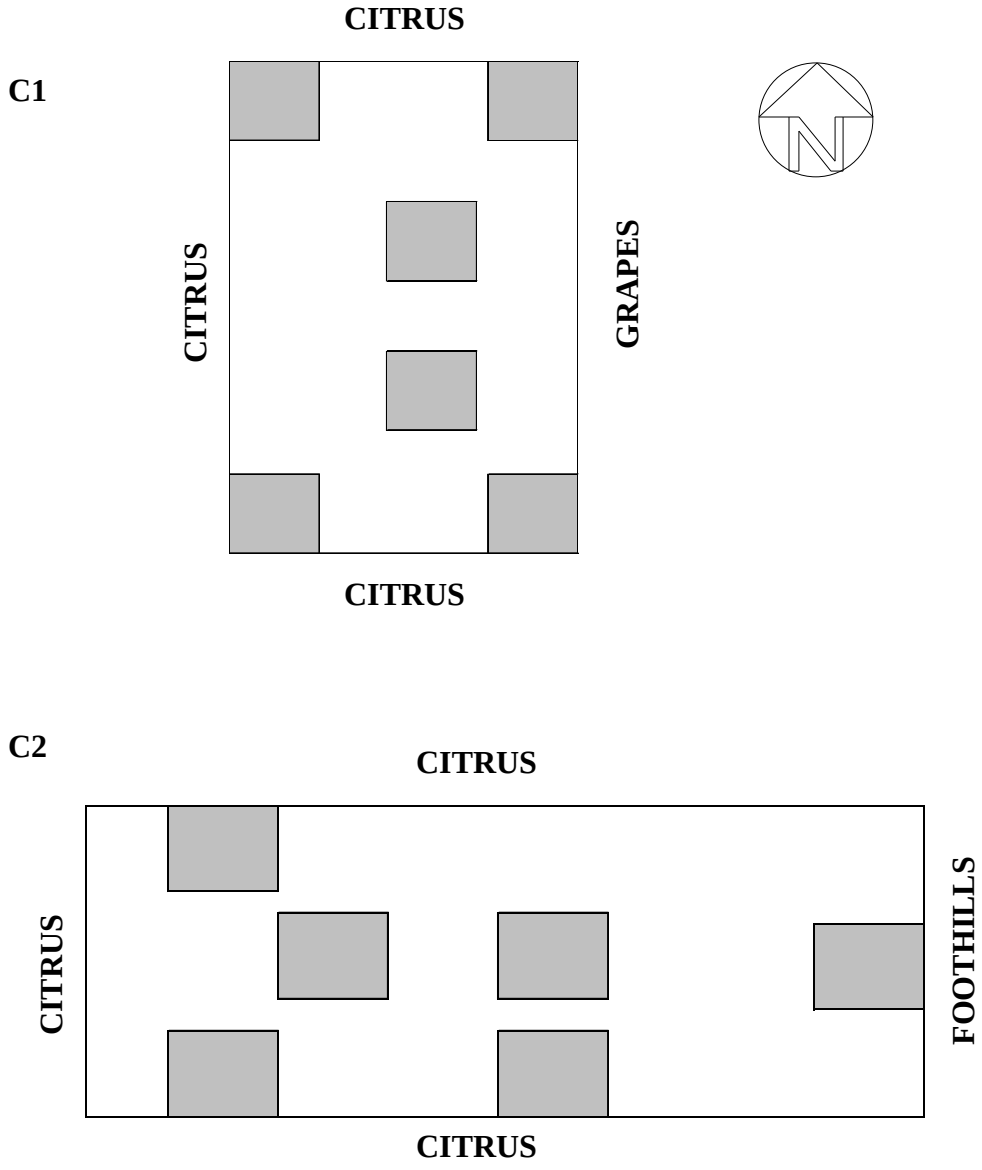

C1
CITRUS
CITRUS
GRAPES
CITRUS
C2
CITRUS
CITRUS
FOOTHILLS
CITRUS

Fig. 1.

CHAPTER IV

GENETIC DIVERSITY OF *SPIROPLASMA CITRI* STRAINS FROM DIFFERENT REGIONS, HOSTS, AND ISOLATION DATES

Abstract

Spiroplasma citri, a phloem-limited pathogen, causes citrus stubborn disease (CSD). Losses due to CSD in California orchards have grown over the past decade. To investigate the possibility of introduction or emergence of a new strain, a study of genetic diversity among *S. citri* strains from various locations was conducted using RAPD-PCR of 35 strains cultured from 1980 to 1993, and of 35 strains cultured from 2005 to 2006. Analysis using 20 primer pairs revealed considerable diversity among strains. However, no unique genetic signatures were associated with recently collected strains compared with those collected 15-28 years ago, and no geographically associated pattern was distinguishable. *S. citri* strains from carrot and daikon radish contain some unique DNA fragments, suggesting some host plant influence. Multiple strains from single trees also showed genetic diversity. Sequencing of five RAPD bands that differed among strains showed that diversity-related gene sequences include virus fragments, and fragments potentially encoding a membrane lipoprotein, a DNA modification enzyme and a mobilization element. No differences in colony morphology were observed among the strains. The lack of correlation between PCR patterns and isolation date or collection site is inconsistent with the hypothesis that recent infections are due to the introduction or emergence of novel pathogen strains.

Introduction

Oranges and other citrus fruits are among the most popular fruits in the United States, and California is the major citrus producer for the nation's fresh fruit market (30). California citrus orchards have been affected by citrus stubborn disease (CSD) since 1915 (4). Initially attributed to a viral agent (10), in 1972 the etiologic agent was confirmed as a wall-less bacterium, *Spiroplasma citri* (15, 32). Severely affected trees are stunted and have short leaf internodes, leaf mottling, unseasonal blossoms and lopsided fruits (4).

S. citri, a phloem-limited mollicute, is transmitted in a circulative-propagative manner by several species of leafhoppers (21, 28). The primary vector of *S. citri* in the U.S., *Circulifer tenellus* (Baker), is a polyphagous insect able to transmit the spiroplasma from several weed species commonly found in the foothills of the San Joaquin Valley, California (3). The general distribution of CSD infected plants in commercial crops suggests a migration of the infected insects from the weeds to the commercial crops. The ability to feed on different plant species and to migrate long distances make this insect a key element in disease epidemiology (12). Besides *C. tenellus*, *Scaphytopius nitridus* (DeLong) also was reported as vector of *S. citri* (16, 28) but its importance in CSD epidemiology remains unclear.

Although CSD has been present in the San Joaquin Valley for many years, its impact in the region apparently increased after a series of freezes in California citrus orchards during the 1990s, as more growers reported CSD symptoms. *S. citri* also was detected for the first time in carrots, first in Washington State (19) and shortly thereafter in California (this paper), possibly reflecting niche expansion and adaptation of the pathogen to a new plant host.

The very small genome of *S. citri* easily acquires or deletes genetic components, thereby enhancing its fitness (23). For example, continuous graft transmission of *S. citri* from periwinkle to periwinkle resulted in a chromosomal inversion and genomic deletions in *S. citri* BR3-3X that were associated with loss of transmissibility by the natural vector, *C. tenellus* (39, 41). High passage in artificial medium also altered *S. citri* transmissibility (39).

Randomly amplified polymorphic DNA (RAPD-PCR), using low stringency conditions (22) and random primers having short nucleotide sequences has been used

efficiently to discriminate genetic diversity among some plant pathogenic bacteria, including *S. citri* strains (24). Although the reproducibility of RAPD fingerprints can be influenced by the reagents, thermocycler (38) and intensity of amplicons used to score the fingerprint (36), under well-established parameters the results can be very reproducible within a laboratory.

Repetitive element PCR (rep-PCR), which amplifies DNA sequences between repetitive sequences on the bacterial chromosome, also is used to assess genetic diversity in plant pathogenic bacteria (22). The effectiveness of the common rep primers ERIC and BOX, however, apparently is limited in assessing genetic diversity of *S. citri* (26).

The *S. citri* genome has been shown to evolve over relatively short periods of time (23). The possibility that the apparent increase in CSD incidence in California orchards is due to the occurrence of a new *S. citri* strain, led us to compare the genetic diversity among *S. citri* strains recently cultivated from different plant hosts in California (2005 to 2006) with *S. citri* strains cultivated from various locations between 1980 and 1993. This work was previously published (25)

Materials and methods

Bacterial strains and isolation

S. citri strains were obtained from various locations and in different years (Tables 1 and 2). Thirty five strains, collected over the past 25 years from sweet orange (*Citrus sinensis* [L.] Osb.), grapefruit (*Citrus paradisi* Macf), horseradish (*Armoracia rusticana* P.G. Gaertn., B. Mey. & Scherb*)*, peach (*Prunus persica* (L.) Batsch), broccoli *(Brassica oleracea L.)* and the beet leafhopper (*C. tenellus)*, were available in J. Fletcher's collection. Thirty three additional strains were collected during this study by cultivation from *S. citri* infected sweet orange, carrot (*Daucus carota* L.), the weed lamb's quarter (*Chenopodium album* L.) and daikon radish (*Raphanus sativus* L.) plants from several different locations in the San Joaquin Valley, California. Sampling occurred during 2005 and 2006 (Table 2, Figure 1). Two California strains (C189 and S600, Table 2) maintained continuously *in planta* were obtained from the Citrus Clonal Protection Program (CCPP), University of California, Riverside. A single strain from sweet orange

was collected in Israel in 2006. Cultivation was performed using standard procedures in LD8 medium (2, 20), and strains were triply cloned and stored at -80ºC. One to 6 strains per orchard were cultured and, except for one experiment designed to assess within-tree diversity, each strain was obtained from a different plant. Strains were named, in general, according to site location (1 through 12) and plant (A through F) (Table 2). Strains of *S. melliferum*, *S. floricola*, *S. phoeniceum* and *S. kunkelii* also were included (Table 1).

DNA isolation

Spiroplasmas were grown in 25 mL LD8 broth (20) (30 ºC) to a titer of 10^8 cells/mL. Cells were harvested, pellets were re-suspended in CTAB buffer and DNA extraction was accomplished via standard procedures (9). The DNA pellets were dissolved in water and quantified in a spectrophotometer (Nanodrop, ND-1000, Wilmington, DE). The DNA solution was diluted to 4 ng/ µL and stored at -20ºC.

RAPD-PCR and rep-PCR

Twenty 10-oligonucleotide primers, chosen arbitrarily (OPA-09, OPA-18, OPB-01, OPB-16, OPC-03, OPC-13, OPH-08, OPN-11, OPQ-06, OPY-05, OPY-15, OPZ-15, OPAW-05, OPAX-02, OPBF-05, OPAF-07, OPA-13, OPA-14, OPA-15, OPB-20, Operon Technologies, Alameda, CA), were used in RAPD amplifications. PCR reaction mixtures (25 µL) contained 5.7 µL autoclaved distilled water, 2.5 µL GoTaq Flexi buffer (10X) (Promega, Madison, WI), 2.5 µL $MgCl_2$ (25 mM), 4 µL dNTP mix (1 mM, each) (Fisher Scientific, Fair Lawn, NJ), 20 ng template and 1.5 units GoTaq DNA polymerase (Promega, Madison, WI). PCR reactions without DNA template were used as negative controls. Initial denaturation was performed at 94 ºC (6 min), followed by forty cycles of 94 ºC (1 min), 35 ºC (2 min), 72 ºC (2 min). PTC-200 thermocycler (MJ Research, Inc, Ramsey, MN) was used for all experiments. Rep-PCR conditions were similar, except that the BOX 1AR primer was used (18), and the initial denaturation was 95 ºC (2 min), followed by thirty cycles of 94 ºC (3 sec), 92 ºC (30 sec), 50 ºC (1 min) and 65 ºC (8 min). PCR products were electrophoresed in 1.5 % TAE-agarose at 100 V/cm. Bands were compared to a 1 Kb Plus DNA Ladder (Invitrogen, Carlsband, CA). Gels were

stained with ethidium bromide and visualized using an AlphaImager and Alphaease FC™ software (Alpha Innotech Corporation, San Leandro, CA).

Patterns of RAPD and rep-PCR DNA fingerprints were assessed visually. The presence or absence of bands in each strain was transformed into binary data (presence =1, absence =0) using an Excel (Microsoft) processor and the data saved as a text file. Data reliability was assessed using SEQBOOT (PHYLIP) (11), followed by the MIX parsimony program (PHYLIP) (11). Consensus trees (Consense, PHYLIP) were generated using *S. floricola* as outgroup. The tree was visualized in the TREEVIEW program (29). Complementary binary data were analyzed using the SAS/PRINCOMP procedure, SAS software 9.1(34).

Within-tree *S. citri* genetic variability

To assess within-tree spiroplasma variability, three infected sweet orange trees from each of two orchards (orchards 4 and 5, Fig. 1) were selected randomly. On May 17, 2006 one fruit was harvested from each of four different locations within each tree (total of 4 fruits per tree), and spiroplasmas were cultured in LD8 broth from columella tissue (2, 20). Initial culture filtrates were diluted and sub-cultured on agar-solidified LD8 medium; seven to eight different individual colonies per tree were increased in liquid LD8 broth. DNA extraction and RAPD-PCR were performed as described above using primers OPA-09, OPY-05, OPC-13 and OPB-20, which had previously proved useful to differentiate strains.

Sequencing of differential bands

Five differential amplicons were extracted from agarose gels and purified with a Geneclean *Turbo* Kit (Qbiogene, Inc., Carlsbad, CA). DNA was cloned in *Escherichia coli* (strain Mach 1™ - T1^{R}) using the Topo TA Cloning Kit (Invitrogen, Carlsbad, CA). Plasmids from successfully transformed clones were extracted by a small-scale preparation alkaline lysis and cleaned with chloroform:phenol separation, and dissolved in water (33). The target inserts were confirmed by restriction digests with *Eco*RI according to product specifications (Promega, WI). Sequencing was performed using

standard methods in an automated 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Sequences were compared by nucleotide blast and submitted to NCBI data base (1).

S. citri colony morphology in LD8 medium

To assess whether genetic diversity was related to *S. citri* colony morphology, two historical (strains 19 and 35, Table 1) and nine recently collected *S. citri* strains (strains 40, 41, 47, 54, 56, 63, 68, 73 and 74, Table 2) were selected for further study.

Twenty-four hour old cultures of *S. citri* strains were diluted in PBS buffer and plated on LD8, agar (0.6 or 1.0% noble agar). Colony morphology was evaluated after 10 or 30 days of incubation at 30 ºC in the dark. Dienes' stained (8) plates were observed using stereo and light microscopy. Images were acquired in bright field using an Olympus BX51 microscope (Olympus Optical Co., Tokyo, Japan). From strains that yielded more than one type of colony, single cells were sub-cultured in LD8 medium to assess the stability of the colony morphology. This experiment was repeated two times.

Results

RAPD-PCR and rep-BOX

All 20 RAPD primers yielded differential amplification patterns among the five spiroplasma species evaluated, and 17 did so among *S. citri* strains. Patterns that revealed the greatest diversity among strains are presented (Fig. 2 and 3). The 159 differential RAPD amplicons ranged from 3.0 to 0.25 kbp in size. No consistent differences were observed between new strains of *S. citri* collected in this study and strains cultured from up to 27 years ago ("historic" strains) (Fig. 2). Most of the amplicon patterns observed from new strains of *S. citri* varied from site to site of geographical origin; however, a few correlated consistently with the site of collection (Fig. 2B, arrow). Some fragments from carrot (Fig. 3A, arrow) or daikon radish (Fig. 3B, star) strains were not amplified from citrus strains. Banding profiles produced by BOX-PCR were indistinguishable among the strains (data not shown).

Transformed binary data from the RAPDs was used to generate a phylogenetic tree (Fig. 4) in which *S. floricola* (S.f.), *S. phoeniceum* (S.p.), *S. kunkelii* (S.k.) and *S.*

melliferum (S.m.) formed four separate branches. *S. citri* strains fell into two major clusters, both containing mixtures of historic and new strains. One cluster included new strains from sweet orange orchards 2, 4, 5, and 8, a new strain from Israel (Ir), and carrot strains from two different California sites. The other cluster included new strains from sweet orange orchards 3, 6, 7, 10 and from the lamb's-quarter, collection site 9 (Fig. 4).

Spiroplasma strains from carrot were all highly similar to one another and most clustered together (Fig. 4). In contrast, sweet orange strains newly cultured from 8 different groves were widely distributed in the two main brackets of the dendrogram (Fig. 4). Although orchards 3, 4, 5 and SL were adjacent to one another along the Sierra Mountain foothills in Kern County, CA, high variability in RAPD patterns resulted in the placement of strains from these orchards into two separate clusters (Fig. 4). Moreover, significant genetic diversity among spiroplasmas from orchards 4 and 5 was observed; these strains clustered more loosely than did strains from orchards 3 and 6, which were highly similar to one another (Fig. 4).

Both historic and new strains of *S. citri* were distributed widely among branches of the phylogenetic tree, presenting little evidence of correlation between clustering and date of isolation (Fig. 4). Exceptions were strains R8A2, Maroc, MR3, M200HX and M200H, all of which are from the same geographical region; in fact, the latter three are laboratory derivatives of the first (35). Interestingly, strain BR3, the progenitor of BR3-42, BR3-80, BR3-P, BR3-T and BR3-G, clustered closely only with BR3-42, which was derived from BR3 by 42 successive sub-cultures (14). It was somewhat removed from BR3-80 and BR3-P, which were derived from it by 80 or 130 successive sub-cultures, respectively (39), and from BR3-T and BR3-G, which were derived from it by successive transmission by beet leafhoppers or periwinkle grafting, respectively (39). In addition, *S. citri* strains BR15, BR17 and BR18, from adjacent horseradish field sites in Illinois, also showed significant genetic diversity (Fig.4).

Principal component analysis (PCA) of *S. citri* historic and new strains showed that the first two principal components explained 30.3% of the variation encountered in the analysis. Primers OPA-15, OPA-18 and OPN11 appeared to have a great influence on the first two components. In agreement with the phylogenetic tree, there was no clustering between historic and new strains (Fig. 5A.). PCA of *S. citri* strains cultivated from

orchards 3-6 showed that in some orchards the genetic variability within a grove was very limited and in others the genetic variability within groves was greater (Fig. 5B.).

Within-tree genetic diversity

S. citri genetic diversity within single sweet orange trees in orchards 4 and 5 was evidenced by differential banding patterns obtained using primers OPA-09 and OPC-13. In orchard 4, only one of the three sweet orange trees evaluated yielded multiple genotypes (data not shown). In orchard 5, two of the three plants evaluated were infected by more than one type of spiroplasma in the same plant. One of the trees yielded two, and the other tree yielded three, different RAPD patterns (Fig. 6).

Sequencing of differential amplicons

Sequencing of five RAPD amplicons that were produced differentially among strains (diversity-related gene sequences) identified two plectroviral fragments and fragments potentially encoding parts of a membrane lipoprotein, a DNA modification enzyme, and a mobilization element (Table 3).

***S. citri* colony morphology**

Of the 11 *S. citri* strains plated onto solid medium, only strain 35 (ASP-1) presented a stable colony morphology in both experiments performed, independent of the agar concentration used; its colonies were of the “fried egg” type (colony type B, Fig. 7). All other strains produced some colonies with fried egg morphology and others having a dense center surrounded by satellite colonies (colony type A, Fig. 7). When colonies of either type were picked, sub-cultured in LD8 broth medium and re-plated, they again yielded colonies of both types (Fig. 7).

Discussion

Genetic diversity in bacteria can be assessed by examining specific restriction sites, repetitive elements, genome sequences or the amplicons produced by random primers (RAPD) (22). RAPD-PCR, when optimized for a particular application, provides

effective discrimination among species and strains (31). RAPD primers used in the present study were suitable for differentiating *S. citri* from other members of spiroplasma serogroup I (*S. melliferum*, *S. kunkelii*, and *S. phoeniceum)* and serogroup III (*S. floricola*) and also for discriminating genetic differences among *S. citri* strains.

Significant genetic variability of *S. citri* apparently has existed in California, Maryland and Illinois for a long period of time. Strains obtained in 1993 (GO3 and GO5) from two grapefruit trees in the same Coachella Valley, CA, orchard and located only two trees apart, yielded so many differential amplicons that these strains were separated into the two main branches of the phylogenetic tree. The same phenomenon was observed with some *S. citri* strains cultivated from 1981-1984 from horseradish plants with symptoms of brittle root disease from adjacent fields in Illinois or Maryland (7, 13). Yokomi et al. (42) have shown the presence of two genetically different populations of *S. citri* in field trees from central California and from historical strains collected from southern California in the 1960s.

It is challenging to evaluate the significance of the differences among strains and clusters in the RAPD-PCR generated phylogenetic tree of Fig. 4. One measure of significance can be generated by examining two cases in which several different *S. citri* lines, derived from a common parent strain under laboratory conditions, generated different RAPD patterns in the present work. Such comparisons are useful because, since we know the derivation histories and some of the genomic information about these strains, we can apply that information to inform our interpretation of the RAPD results in this study. In one example, strain BR3, which was cultivated originally from *S. citri*-infected Illinois horseradish, generated derivative genotypes after successive sub-culturing *in vitro* or after extended cycles of either leafhopper transmission or plant graft transmission (14). Previous work revealed differences in protein profiles of the non-transmissible BR3-derivative lines BR3-P and BR3-G, obtained by *in vitro* and graft transmission, in comparison with BR3, the parent line, and with BR3-T, a still-transmissible leafhopper-transmitted derivative (14). In addition, BR3-G had sustained a chromosomal inversion and two deletions compared to its progenitor (41), mutations that likely were involved in its loss of transmissibility. In the lineage proposed by our RAPD analysis (Fig. 4), BR3 and BR3-T are each more closely aligned with other *S. citri* strains

than to each other. In a second example, RAPD pattern differences were observed among strains M200H, M200HX and MR3, all of which are subculture derivatives of strain R8A2. MR3 is able to resist infection by a spiroplasma virus isolated from *S. melliferum*, unlike its parent, which remains susceptible (35). Consideration of the phylogenetic tree clusters in tandem with previous knowledge of the same strains provides a sense of the scale of the differences identified in this study.

Despite the great variability in RAPD profiles encountered among the 70 *S. citri* strains evaluated in this study, no particular pattern differentiates *S. citri* strains collected between 2005-2006 from strains collected between 1980 to 1993. Comparison among strains recently collected from California orchards showed that genetic patterns of *S. citri* within an orchard could be similar or very different. Since the majority of the orchards sampled were located near the foothills in the east side of the Central Valley, the different degrees of genetic diversity may reflect greater or lesser exposure of the trees to insect vectors, and, consequently, more or fewer inoculation events. Genetic homogeneity in some groves could also be consequence of dispersion by nursery propagation. One RAPD primer (OPA-13) differentiated between carrot and citrus strains of *S. citri*. This result is different from an earlier report that showed no significant genetic differences among citrus and carrot strains but since this first study used the 16S rDNA region that is a more taxonomy marker than RAPD these results are not comparable (19).

The *S. citri* genome is one of the largest among Mollicutes, and is characterized by high adenosine-thymidine content. In addition to its circular chromosome, *S. citri* also harbors plasmids and virus genomes, which likely serve as sources of genetic information (23, 40). Genetic variations of spiroplasmas are attributed to DNA acquisition and loss, DNA replication and repair, homologous recombination and transposition (23). Amplicons sequenced in the present work showed that, in addition to the fact that bacteriophages occupy a considerable proportion of the *S. citri* genome, genetic differences among strains may result from several different mechanisms within the genome. Such inserts included a hypothetical gene for a DNA methyltransferase, an enzyme associated with DNA protection and repair, a transmembrane lipoprotein which the C-terminal is also virtually identical to SpV1-R8A2 B phage ORF product and a mobilization-like protein.

S. citri cells usually are motile and helical when cultured on LD8 medium, and move linearly in medium solidified with a low agar concentration, forming colonies with indistinct edges due to the formation of satellite colonies. Strains having low motility in agar medium yield colonies with well-defined edges, sometimes called “fried-egg” colonies. These colony types have been found in natural populations from CSD citrus trees in the Middle East (37), and also in motility-impaired mutants generated by Tn*4001* transposition (17). In our study, except for *S. citri* ASP-1, previously reported as a non-helical, non-motile, fried-egg colony-producing strain, none of the spiroplasma strains maintained a single, stable colony morphology during successive subculturing. Colony morphology apparently is unreliable as a means of differentiating strains. Whether such variation is related to the phenomenon of phase variation, in which a variety of molecular mechanisms in mollicutes give rise to variable phenotypes (27), is not known.

S. citri genetic diversity within single trees in citrus orchards 4 and 5 showed that, under conditions of commercial citrus orchards, populations of *S. citri* may be heterogeneous. The explanation for such diversity could be multiple natural infections of single trees by heterogeneous strains, a series of *in planta* genetic alterations, or grafting of young rootstocks with mixtures of field-infected and nursery-contaminated bud-wood.

The lack of major differences among historic and new strains leads us to conclude that genetic changes in *S. citri* genome were not a significant factor in any re-emergence of CSD in California orchards. The epidemiology of CSD is complex. The pathogen infects several plant host species including commercial crops and weeds, and has several different leafhopper vectors, all of which could influence the impact of CSD in California.

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Figure captions

Fig. 1. Location of citrus orchards and other sites in the San Joaquin and Antelope Valleys, CA where *Spiroplasma citri* was collected for this study. Each grey square represents 1.61 km^2 in which a commercial orchard of twenty-five or more citrus trees were planted.

Fig. 2. Random amplified polymorphism DNA (RAPD) fingerprints generated using primer OPA-15 and a template of historic **(A)** or new *Spiroplasma citri* strains **(B)** from California and Israel. Numbers below bars indicate strain; lanes C: the control reaction without template DNA, lanes M: DNA ladder 1 kb plus; size fragments are listed on the right. Above the bar: **A**, Historic strains: 1 to 10, from California; 11 to 24, from Illinois horseradish; 25 to 27, from Maryland horseradish; 28 to 33, from Morocco; 34 and 35, from Iran (In) and Israel (Ir); 36 to 39, different species of spiroplasma (DS), and 40, new strain from site 11. **B**, New strains: site 1, daikon radish, sites 2 to 8 and 10, citrus orchards, site 9, lamb's quarter, sites 11 and 12, carrot. Asterisk: strains cultivated from grafted plants from the Citrus Clonal Protection Program, Ir, new strain from Israel. Arrows indicate *Spiroplasma citri* amplicon specific to some citrus sites and to lamb's-quarter.

Fig. 3. Random amplified polymorphism DNA fingerprints after amplification with primers OPA-13 **A**, and OPZ -15 **B**; template DNA from new *Spiroplasma citri* strains from California and Israel. Numbers below the bar show strain identification; lanes C: the control reaction without template DNA; lanes M: DNA ladder 1 kb plus, size fragments on right. Above the bar: site 1- daikon radish, sites 2 to 8 and 10 - citrus orchards, site 9 – lamb's quarter, sites 11 and 12 carrot, * strains cultivated from grafted plants obtained from Citrus Clonal Protection Program, Ir- new strain from Israel. Arrows indicate a specific amplicon in carrots (40, 41 and 69-73) (**A**) and the star indicates a specific amplicon in daikon radish (57) (**B**).

Fig. 4. A phylogenetic tree constructed by mix parsimony (PHYLIP) based on 159 characters of the 70 different strains of *Spiroplasma citri* and of *S. melliferum* (S.m), *S. floricola* (S.f.), *S. phoeniceum* (S.p.) and *S. kunkelii* (S.k). Numbers at the nodes indicate

the levels of bootstrap support (percentage) based on 1000 repetitions for the branches immediately to the left.

Fig. 5. Arrangement of *Spiroplasma citri* strains based on principal component analysis using as input the differential characters determined by random amplified polymorphism DNA reactions. **A**, Analysis including historic *S. citri* strains (black squares) and new strains (open squares), **B**, *S. citri* isolates from orchards (sites) 3 to 6. Majority of strains from orchards 3 and 6 formed a tight cluster at principal coordinate 1 (3) and principal coordinate 2 (0).

Fig. 6. Random amplified polymorphism DNA fingerprints, after amplification with primer OPA - 09. DNA extracted from seven to eight *Spiroplasma citri* strains, selected by single-cell cloning from multiple fruits from three different sweet orange trees in orchard 5, served as template. Above the bar: tree identification; below the bar: strain identification; tree1, strains A to H, tree 2, strains I to P, tree 3, strains Q to X and the control reaction without template DNA (-). Lanes Y: DNA ladder 1 kb plus, size fragments listed on right. Arrows indicate differential patterns: 1, presence of 3.0 and 1.65 Kbp amplicons; 2, absence of 3.0 and 1.65 Kbp amplicons and 3, presence of 3.0 Kbp and absence of 1.65 Kbp amplicons.

Fig. 7. Mixed colony types of *Spiroplasma citri* obtained from strain 41, cultivated from infected carrot plants on agar-solidified (0.6%) LD8 medium. A, colony with satellites and B, fried egg colony.

TABLE 1. Strains of *Spiroplasma citri* and other *Spiroplasma* species analyzed in this study.

Nº.	Strains/Species	Geographical origin	Host	Source/ (reference)	Isolation date
1	Ex-Cal	California	Sweet orange	B. Kirkpatrick	1984
2	O1	California	Sweet orange	B. Kirkpatrick	1984
3	O-62	California	Peach	B. Kirkpatrick	1984
4	O-202	California	Peach	B. Kirkpatrick	1984
5	O-15	California	Broccoli	B. Kirkpatrick	1984
6	B105	California	Sweet orange	R. Whitcomb	1983
7	B106	California	Sweet orange	R. Whitcomb	1983
8	CB1	California	Leafhopper	R. Whitcomb (5)	1983
9	GO-3	Coachella Valley, California	Grapefruit	G. Oldfield (isolated by J. Fletcher)	1993
10	GO-5	Coachella Valley, California	Grapefruit	G. Oldfield (isolated by J. Fletcher)	1993
11	BR1	Illinois	Horseradish	J. Fletcher (13)	1980
12	BR6	Illinois	Horseradish	J. Fletcher (13)	1980
13	BR11	Illinois	Horseradish	J. Fletcher	1984
14	BR 12	Illinois	Horseradish	J. Fletcher	1984
15	BR 14	Illinois	Horseradish	J. Fletcher	1984
16	BR 15	Illinois	Horseradish	J. Fletcher	1984
17	BR 17	Illinois	Horseradish	J. Fletcher	1984
18	BR 18	Illinois	Horseradish	J. Fletcher	1984
19	BR3	Illinois	Horseradish	J. Fletcher (13)	1980
20	BR3G	Derived from BR3	Horseradish	J. Fletcher (38)	NA[a]
21	BR3T	Derived from BR3	Horseradish	J. Fletcher (38)	NA
22	BR3-42	Derived from BR3	Horseradish	J. Fletcher (38)	NA
23	BR3-80	Derived from BR3	Horseradish	J. Fletcher (38)	NA
24	BR3P	Derived from BR3	Horseradish	J. Fletcher (38)	NA
25	MDHR2	Charles County, Maryland	Horseradish	R. Davis (7)	1981
26	MDHR4	Charles County, Maryland	Horseradish	R. Davis (7)	1981
27	MDHR5	Charles County, Maryland	Horseradish	R. Davis (7)	1981
28	M200H	Derived from R8A2	Sweet orange	R. Davis (34)	NA
29	M200HX	Derived from R8A2	Sweet orange	R. Davis (34)	NA
30	MR3	Derived from R8A2	Sweet orange	R. Davis (34)	NA
31	R8A2	Morocco	Sweet orange	R. Davis (31)	1985
32	Beni-Mellal	Morocco	Sweet orange	S. Purcell	1984
33	Maroc	Morocco	Sweet orange	R. Whitcomb	1983
34	Iran	Iran	Sweet orange	R. Whitcomb	1983
35	ASP-1	Israel	Sweet orange	R. Davis (36)	1981
36	*Spiroplasma melliferum* / TS-2	Maryland	Honey bee	R. Davis (5)	1991
37	*Spiroplasma phoeniceum*	Middle East	Periwinkle	R. Davis	1982
38	*Spiroplasma floricola* / 23-6	Maryland	Tulip tree	R. Davis (6)	1978
39	*Spiroplasma kunkelii* / CR2	Alajuela, Costa Rica	Corn	J. Fletcher	1987

[a]NA. Does not apply.

TABLE 2. *Spiroplasma citri* strains cultivated in 2005 and 2006 and analyzed in this study.

#	Site*	Strains		Origin	Host	Source / Reference	Isolation
		This paper designation	Original designation				
40	11	C5	C5	Kern County, California	Carrot	This study	2005
41	11	C17	C17	Kern County, California	Carrot	This study	2005
42	2	2A	Ca 1	Tulare County, California	Sweet orange	This study	2005
43	3	3A	Ca 7	Tulare County, California	Sweet orange	This study	2005
44	3	3B	Ca 12	Tula re County, California	Sweet orange	This study	2005
45	3	3C	Ca 16	Tulare County, California	Sweet orange	This study	2005
46	3	3D	Ca 20	Tulare County, California	Sweet orange	This study	2005
47	3	3E	Ca 23	Tulare County, California	Sweet orange	This study	2005
48	3	3F	Ca 28	Tulare County, California	Sweet orange	This study	2005
49	6	6A	Ca 35	Kern County, California	Sweet orange	This study	2005
50	6	6B	Ca 40	Kern County, California	Sweet orange	This study	2005
51	6	6C	Ca 42	Kern County, California	Sweet orange	This study	2005
52	6	6D	Ca 46	Kern County, California	Sweet orange	This study	2005
53	6	6E	Ca 51	Kern County, California	Sweet orange	This study	2005
54	6	6F	Ca 53	Kern County, California	Sweet orange	This study	2005
55	4	4A	Ca 62	Kern County, California	Sweet orange	This study	2005
56	NA[b]	ISR	Israel	Bet-Dagan, Israel	Sweet orange	This study	2006
57	1	Dk	Ca 264	Fresno County, California	Daikon radish	This study	2005
58	4	4B	Ca 204	Kern County, California	Sweet orange	This study	2006
59	4	4C	Ca 198	Kern County, California	Sweet orange	This study	2006
60	4	4D	Ca 207	Kern County, California	Sweet orange	This study	2006
61	5	5A	Ca 160	Kern County, California	Sweet orange	This study	2005
62	5	5B	Ca 242	Kern County, California	Sweet orange	This study	2006
63	5	5C	Ca 256	Kern County, California	Sweet orange	This study	2006
64	NA	C189	C189	CCPP[c]	Sweet orange	CCPP (15)	2006
65	NA	600B	600B	CCPP	Sweet orange	CCPP	2006
66	9	W	Ca 263	Fresno County, California	Lamb's-quarter	This study	2006
67	7	7A	Ca 144	Kern County, California	Sweet orange	This study	2005
68	8	8A	Ca 170	Fresno County, California	Sweet orange	This study	2006
69	12	C1	C1	Kern County, California	Carrot	This study	2006
70	12	C3	C3	Kern County, California	Carrot	This study	2006
71	12	C6	C6	Kern County, California	Carrot	This study	2006
72	12	C7	C7	Kern County, California	Carrot	This study	2006
73	12	C8	C8	Kern County, California	Carrot	This study	2006
74	10	SL	SL	Kern County, California	Sweet orange	This study	2006

[a]See map (Fig. 1).
[b]NA. Does not apply.
[c]CCPP, Citrus Clonal Protection Program, University of California, Riverside.

TABLE 3. Description and putative functions of sequences of strain-differential amplicons of *Spiroplasma citri* obtained by random amplified polymorphism DNA-polymerase chain reaction

Strain sequenced	Amplicon size (bp)	Accession number	Function/product
35[a]	520	EU005544	Plectrovirus Spv1-c74
68[b]	176	EU005545	Plectrovirus Spv1-R8A2b
43[c]	227	EU005546	Hypothetical lipoprotein transmembrane
40[d]	1186	EU005547	MOB-like protein
63[e]	688	EU005548	Hypothetical DNA methyltransferase protein

[a]Amplicon unique to historic strains 19, 22, 24, 34, and 35; the new Israeli strain 56; and a new strain from site 8 in California, 68, using primer OPA-09.
[b]Amplicon present in new strains 40-42, 57-63, 66, and 68; and historic strain 35, using primer OPA-13.
[c]Amplicon present only in trees from sites 3, 6, 7 and 9 (new strains 43-54 and 65-67) and in historic strains 3, 7, 10, 11, 15, 17, 18, 25 and 27, using primer OPA-13.
[d]Amplicon present only in carrot strains 40, 41 and 69-73, daikon radish strain 57, and historic strains 5, 8, 11-14, 16-19, 21-24 and 26, using primer OPA-13.
[e]Amplicon present in some, but not all, trees of site 6, using primer OPC-13.

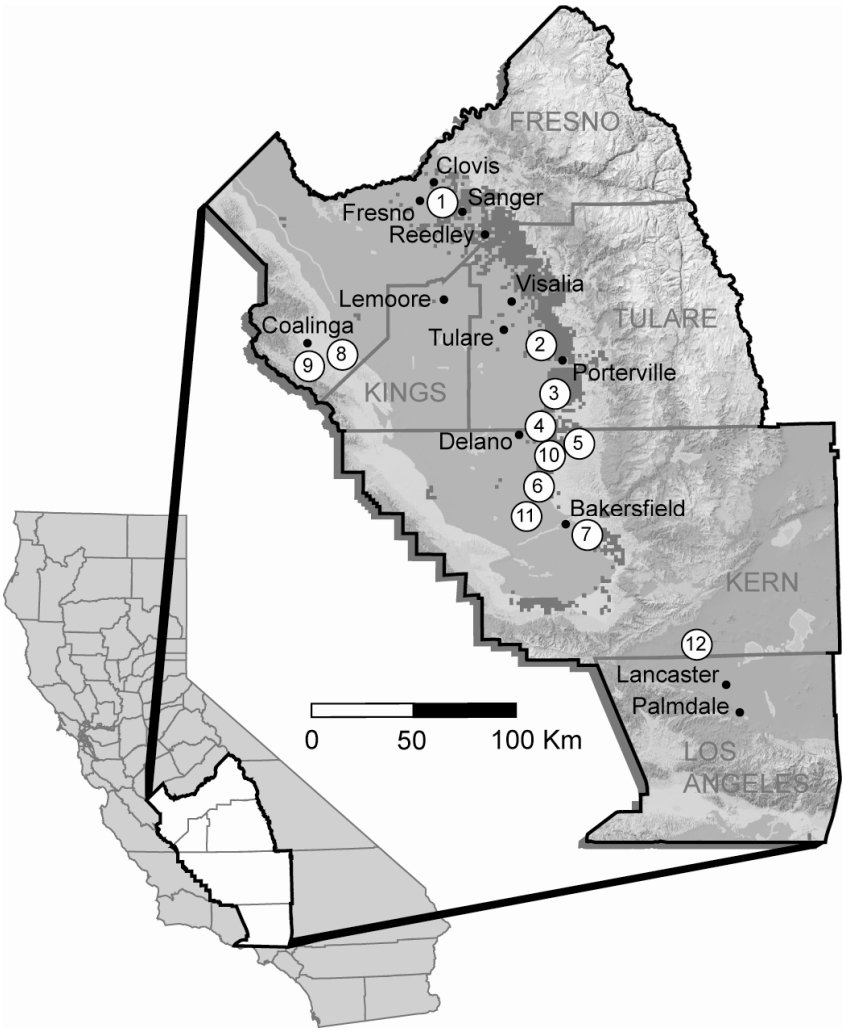
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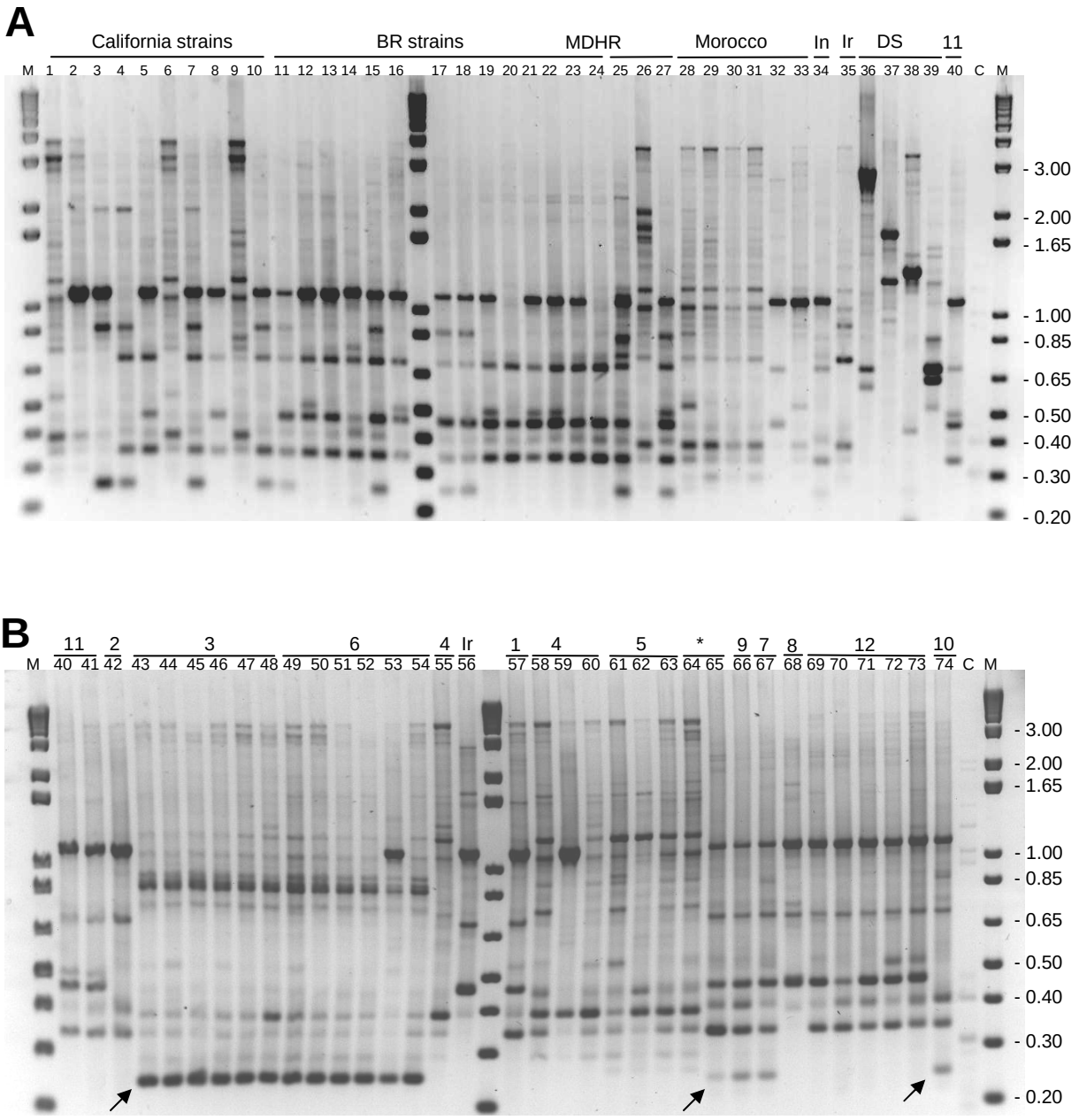
Fig. 1.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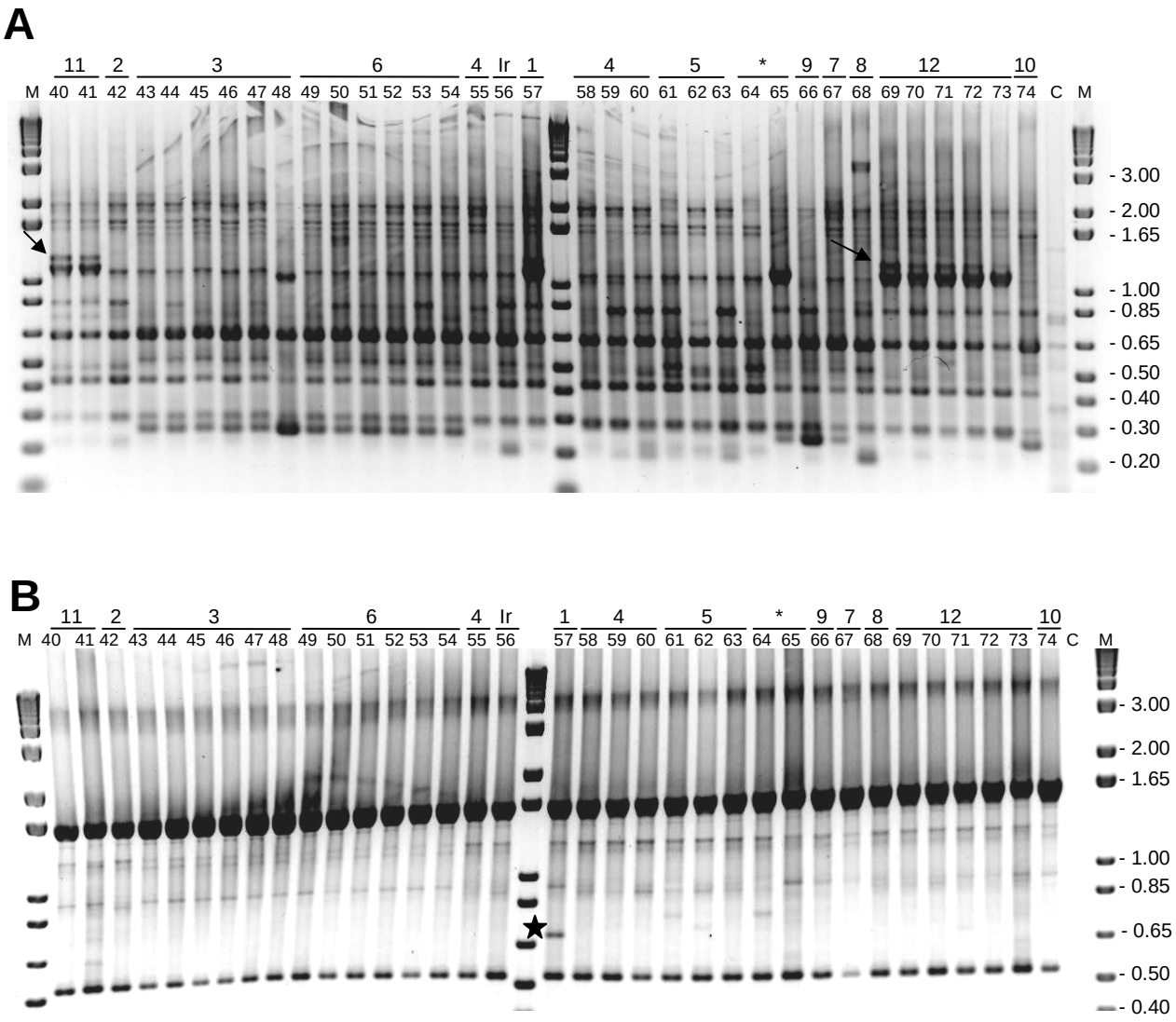
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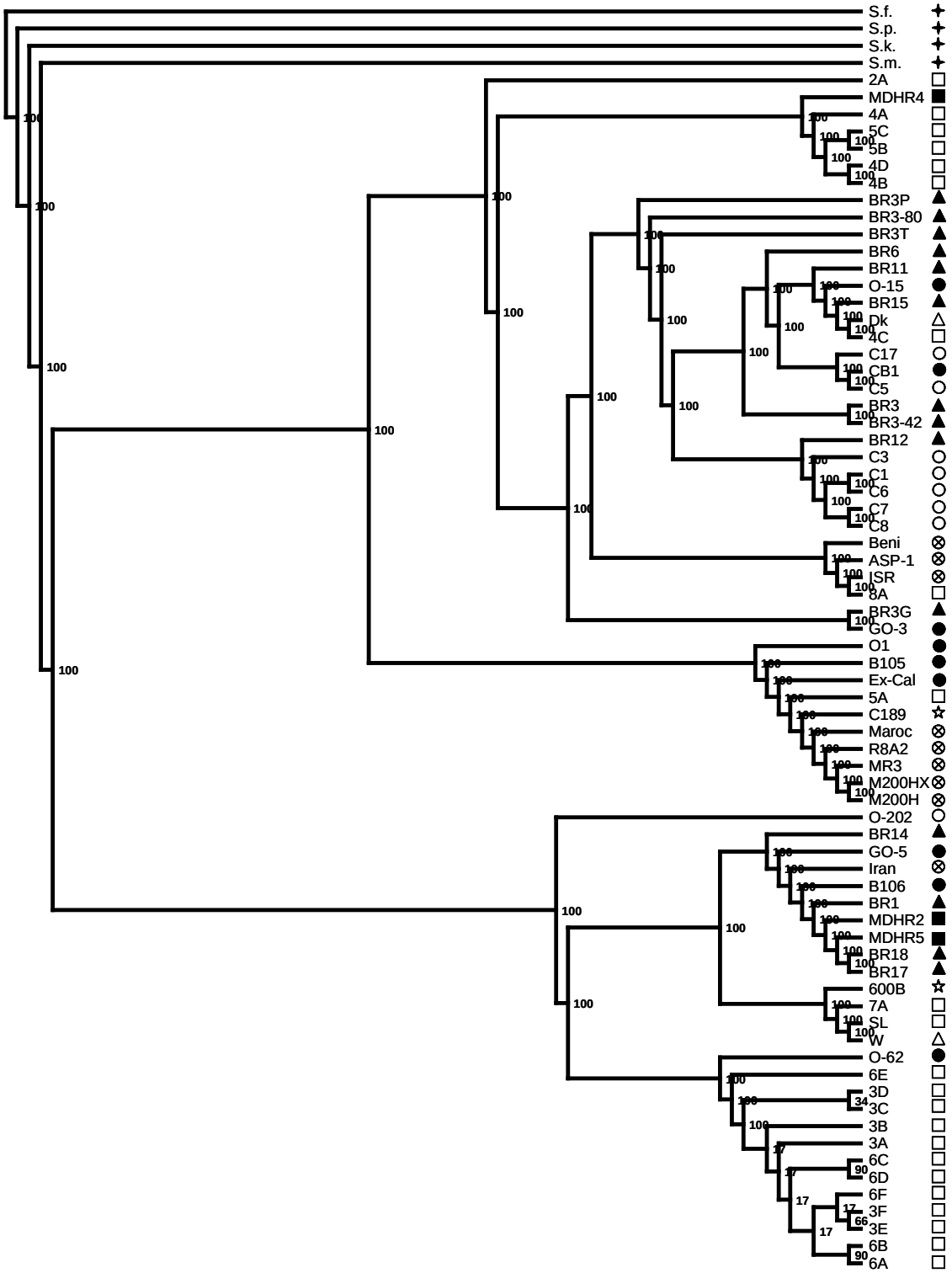
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Fig. 4.

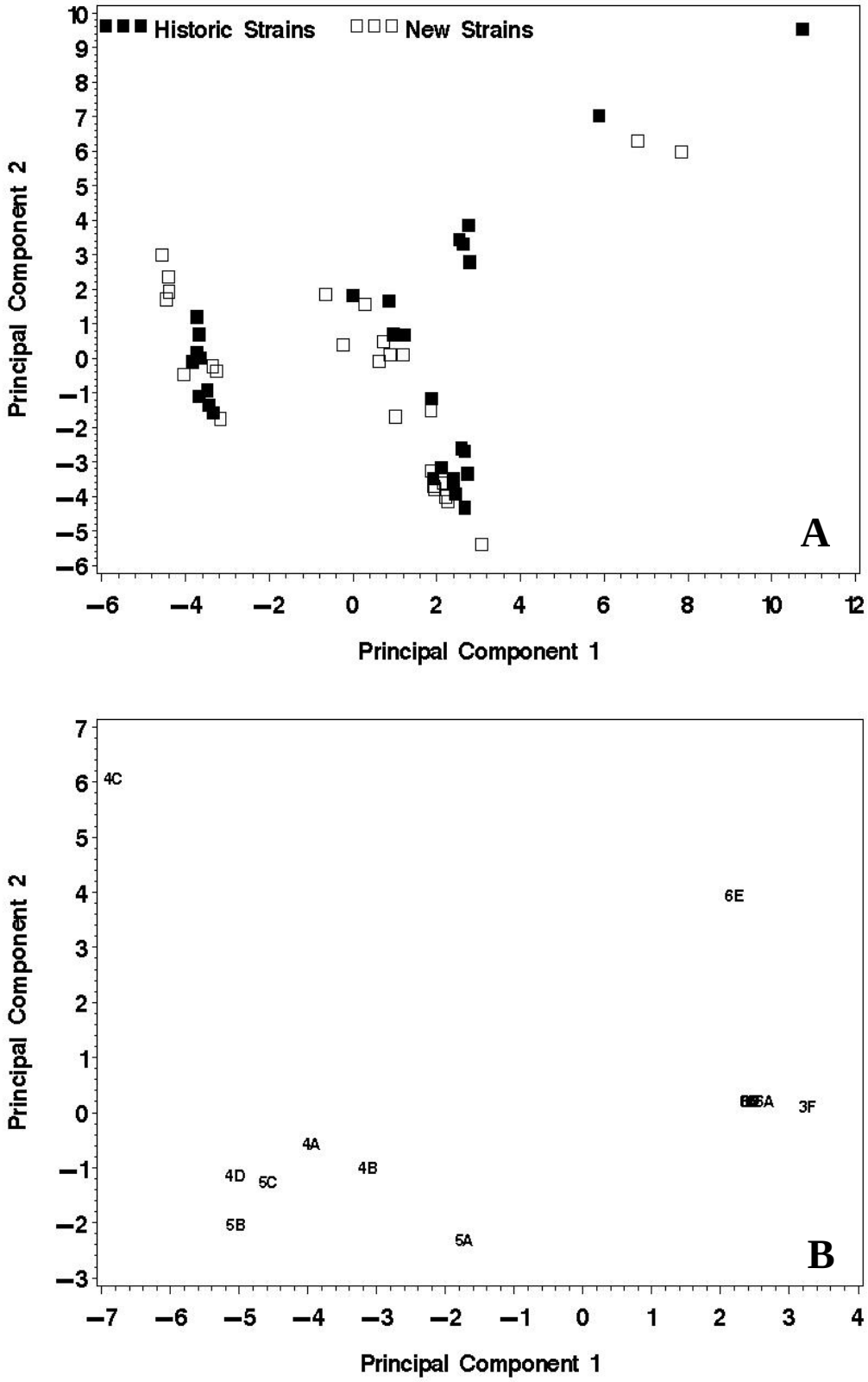
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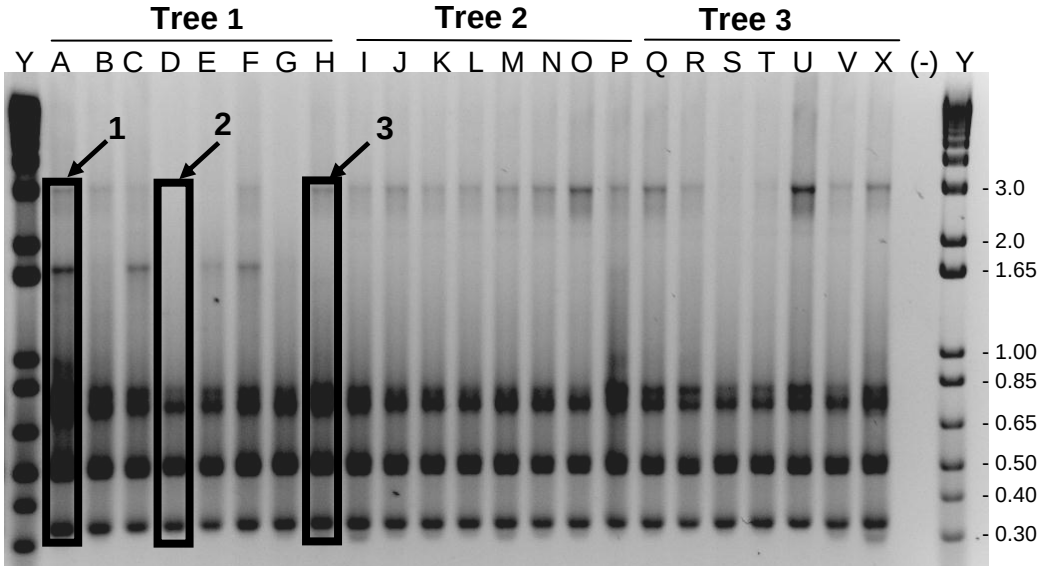
Fig. 5.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0.30

Fig. 6.

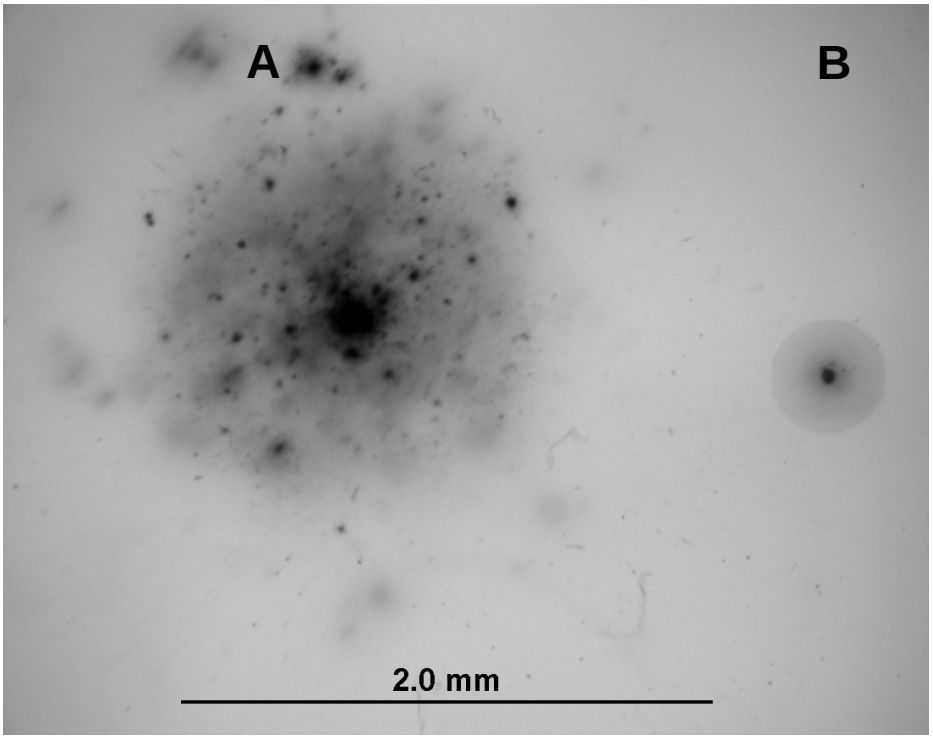

Fig. 7.

CHAPTER V

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

Abstract

The impact of citrus stubborn disease (CSD), caused by *Spiroplasma citri*, on citrus cultivated under commercial conditions is not fully understood or quantified. The objective of this work was to measure the impact of *S. citri* infection on citrus and assess the distribution of the bacterium in trees having different degrees of symptom severity. Infected and healthy navel orange trees were selected in a commercial grove in California. Measurements included tree canopy height and width, trunk diameter, fruit number, and weight and number of fruits dropped prematurely. Thirty fruits per tree were measured, weighed and evaluated for color, size and presence of sunburn. Juice was extracted and weighed, and total soluble solids and titratable acidity were measured. Distribution of the bacterium in plants with mild or severe symptoms was assessed by q-PCR and spiroplasma culture. Fruits from infected trees were lighter, smaller, and more likely to be mis-shapen than those from healthy trees. Significant yield reduction occurred only in severely symptomatic trees in which *S. citri* was broadly distributed within the tree canopy, as assessed by cultivation and q-PCR. All the other variables were statistically indistinguishable between infected and healthy trees, regardless of symptom severity. The significant reductions in fruit yield and quality associated with *S. citri* infection validate the concern among citrus growers in California's Central Valley that CSD is a significant constraint to production and marketability.

Introduction

Citrus fruits (oranges, grapefruits, lemons and limes) are among the most popular fruits in the United States, following only bananas and apples in fresh fruit consumption (24). Citrus fresh fruit production in the United States is concentrated in California, where dry summers, hot days, and cool nights enable uniform fruit ripening (28). Citrus stubborn disease (CSD), caused by *Spiroplasma citri*, has impacted yields and fruit quality in this state for many years. The disease was attributed initially to a viral agent (11), but in 1972 the etiologic agent was confirmed as a wall-less bacterium by two different research groups working independently (13, 25).

S. citri is a phloem-limited mollicute transmitted by several species of leafhoppers in a propagative manner or by grafting of infected plant material (15, 21). 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* infected trees near orchard edges suggests migration of infected insects from weeds to the commercial crops during the summer when the environment becomes unfavorable to annual weeds (7).

Although CSD has been present in California since 1915 (9), its impact in the San Joaquin Valley has had greater visibility in the past 5 years, since more citrus growers have reported the occurrence of symptoms consistent with CSD including general stunting, short leaf internodes, leaf mottling, unseasonal blossoming and lopsided fruits (9). Samples from such trees, subjected to bacterial culturing and PCR (19, 29, 30), 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 cv. Valencia Frost sweet oranges under commercial conditions in California (6). Yield of infected trees ranged from 44 to 74% lower than that of healthy trees, and fruits from diseased trees weighed 6 to 17% less than those from healthy trees, depending on the root-stock used (6). In Cyprus, natural infections of *S. citri* in cv. Frost Washington Navel trees decreased citrus production by 28%, and fruits produced by such trees were 20 to 38% lighter than those produced by *S. citri*-free trees. Fruits from infected plants also were 8 to 15% smaller in diameter than those from *S. citri*-free trees, but no effects were observed on the amount or quality of the juice (14). 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 infected plants were, respectively, 92% and 7 % lower than from healthy trees (8).

The impact of *S. citri* infection on yield and tree height correlated with the severity of CSD symptoms on inoculated plants (6), but similar studies have not been done under field conditions. 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 (7) and/or strain virulence (6). The objective of this study was to estimate the impact of *S. citri* on Navel sweet orange production in a commercial orchard in California and to assess the possible correlation of pathogen distribution in trees with mild and severe CSD symptoms. Some results were previously reported (16, 17).

Materials and methods

Orchard location

The study plot, a commercial orchard adjacent to the foothills of the San Joaquin Valley in northeastern Kern Co., CA, contained approximately 1,800 sweet orange (*Citrus sinensis* [L.] Osb.) trees, cultivar Thompson Improved Navel, grafted onto Carrizo citrange (*Citrus sinensis* Osb. x *Poncirus trifoliata* L. Raf.) rootstock. Trees were approximately 20 years old.

Sampling

Evaluations were done in 2006 and 2007. On an initial screening four fruits from each of 380 trees were harvested in May of 2006 to confirm the presence of *S. citri*. Fruit receptacles were processed using standard procedures (5, 14, 18) for spiroplasma cultivation in LD8 medium and fruit columellas were subjected to polymerase chain reaction (PCR) using spiralin and P-58 based primers (30). 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 negative and half positive for *S. citri* by both culturing and PCR. Infected trees were classified as mildly or severely symptomatic. Trees designated “mildly symptomatic” were generally asymptomatic but some of them

had 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*-infected 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 (12), all 32 trees were visually inspected for the presence of bark cracks with oozing sap, symptoms typical of infection by *Phytopthora spp.*; root samples were incubated in modified Seinhorst mist apparatus (mist chamber) (2) 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) (20).

Fruit yield and quality evaluations

Field and laboratory evaluations were performed in October of 2006 and 2007. Tree height and width, and trunk scion and root-stock diameters were measured. Fruits dropped within the tree canopy circumference were counted. Fruit yield was estimated with the aid of a 0.6 x 0.6 m plastic frame constructed of PVC pipe arranged to form an open square. 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 fruits within the frame area, extending inward to the trunk, were counted. After these field evaluations, 30 fruits from each tree were harvested randomly and transported to the ARS-USDA, Parlier, CA for laboratory analyses. Fruits were weighed, and the length and width of each fruit were measured with a digital caliper. The presence/absence of sunburn was recorded. Fruit 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, Los Angeles, CA) 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) (27). Results were evaluated using PC SAS version 9.1 (26). 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 severely symptomatic trees was assessed by harvesting ten fruits at random from each of ten CSD affected trees (5 mildly and 5 severely symptomatic) in August 2007. Fruit receptacles were processed for spiroplasma cultivation in LD8 broth (5, 14, 18). Cultures were evaluated daily for turbidity and spiroplasma growth was confirmed by dark-field microscopy using an Olympus BH-2 microscope (Olympus Optical Co., Tokyo, Japan) (1200 x). Non-turbid samples were evaluated weekly by dark-field microscopy for 60 days. Relative spiroplasma titer in fruit 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* strains 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 hours.

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. Fruits and leaves from the same ten 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 columella or leaf 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 columellas and leaf petioles (29, 30). The presence or absence of *S. citri* in fruit columella and leaf petiole samples was analyzed by a chi-square test using SAS software

9.1 and PROC FREQ (26). Data were sorted first by disease status (mild vs. severe) and canopy aspect and then by status alone. The suitability of fruit columella and leaf petiole as sources of DNA template was compared by a chi-square analysis.

Results

Fruit yield and quality evaluations.

No evidence of parasitic nematodes or *Phytophtora 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, in the citrus orchard evaluated, CSD-infected trees produced fewer fruits and fruits were of lower quality (smaller and misshapen) than those from spiroplasma-free trees.

Infected trees sustained yields 25 and 32% lower than those of spiroplasma-free trees in years 2006 and 2007, respectively (Table 1). Yields of severely symptomatic trees were negatively impacted in comparison with those of their healthy counterparts (52 and 45% lower in 2006 and 2007, respectively, $p<0.01$). Yield from CSD mildly symptomatic trees were not statistically different from its healthy 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 of mildly symptomatic trees exceeded that of healthy 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 analysis. However, in 2007 trees harboring *S. citri* were approximately 13% shorter and 6% smaller in canopy width than were the healthy trees. Severely symptomatic trees sustained greater differences in tree size than did mildly symptomatic trees ($p=0.00$) (Table 1).

Fruits from infected trees were lighter in weight and smaller than those from healthy trees. Significant fruit sunburn, which results from the lack of leaf shading due to shorter leaves internodes and/or defoliation on infected plants, which dries the juice vesicles, was observed only in 2007 (Table 2). Infected trees had approximately 8% more misshapen fruits than did healthy trees. This difference was even greater (15.4% more)

when the comparison was restricted to healthy vs. severely symptomatic trees (Table 2). Of the three different variables measured by the digital colorimeter (light, chroma, and hue angle) only chroma was different, and it was lower in infected trees than healthy trees during the 2007 evaluation. These data, which reflect delayed or incomplete ripening, indicate that fruits from infected trees were, in general, greener than those from healthy trees. This difference was much greater in severely symptomatic trees ($p=0.04$) than in mildly symptomatic ones (Table 2). No significant differences between healthy and infected trees were observed in the juice weight and quality evaluations (Table 3).

Distribution of *S. citri* in mildly and severely affected citrus trees.

Fruit receptacles of severely symptomatic trees yielded positive *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* strains obtained from mildly and severely symptomatic trees multiplied at identical 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 presence of *S. citri* was related to neither canopy aspect (east vs. west) nor tier (lower, medium and upper part of tree canopy), regardless of whether the DNA template source was fruit columella or leaf petiole (Table 4). Tree disease status (mildly or severely symptomatic), on the other hand, was significantly correlated to the real-time PCR reaction (Table 5). 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 columella (Table 5). Leaf petioles generated fewer positives than did fruit columellas, but petiole samples from severely symptomatic trees also provided a greater number of positive real-time PCR results than did samples from mildly symptomatic trees (Table 5).

Discussion

Although CSD has been present in California orchards since 1915, until recently disease diagnosis was based exclusively on symptoms, spiroplasma culturing and serological tests (30). Symptoms of CSD can be confused with nutritional deficiencies, other plant diseases, or environmental impacts (4, 23). Thus, assessment of impact was imprecise. The recent development of more sensitive molecular techniques facilitates sensitive detection of *S. citri* (30) and allowed us to identify mildly or non-symptomatic, but infected, trees for inclusion in our study.

Our two year evaluations showed that the majority of the symptoms related to tree development and fruit quality parameters assessed were associated primarily with severely symptomatic *S. citri*-infected trees, while trees having no, or mild, symptoms were relatively, or completely, indistinguishable from spiroplasma-free tree in these respects.

CSD affects both tree height and canopy diameter. Sweet orange trees infected by *S. citri* were 13% shorter and had 6% smaller canopy width than healthy trees in the 2007 assessment, with severely symptomatic trees accounting for most of the statistical differences encountered. Previous reports from California (8) showed that plants graft-inoculated with *S. citri* were up to 55% shorter than healthy controls. The differences between our findings and previous reports are likely due to the fact that trees in our study were inoculated naturally by leafhoppers, and therefore received a much lower spiroplasma inoculum dose than graft-inoculated trees. In Cyprus (14), 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 healthy trees suggests an explanation for the difference in our studies.

CSD affects fruit production and yield in several ways. Navel orange trees infected with *S. citri* produced 26 to 32% fewer fruits 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 fruits on infected trees than those on healthy 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 (1, 10). 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 fruits and off-season blooming.

In our work, as in an earlier study in Cyprus (14), the quantity and quality of juice from fruits of *S. citri*-infected trees were equivalent to those from *S. citri*–free trees. However, others (9) reported insipid, sour or bitter flavor in fruits of *S. citri*-infected trees. In our study, a few infected trees produced fruits 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 fruits on infected trees (3). Additionally, we assessed fruits from at least 10 infected trees whereas previous studies compared fruits from only two trees (3).

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 (6, 7). We found *S. citri*-positive tissues 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 the former than in the latter, especially since we found no statistical difference in the growth rates of spiroplasma strains from these two tree groups.

Although many anecdotal reports exist, 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. We chose to focus on the most important commercial citrus cultivar in California, Navel sweet orange, and we used

a naturally-infected 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 fruits with lower quality whereas mildly symptomatic trees rarely had any major impact in comparison with the healthy controls. In our understanding, 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 plot in the short-term. Citrus is not a suitable host of the main vector of *S. citri*, *C. tenellus* (22), and CSD-infected plants are not likely to serve as inoculum source to healthy 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. If disease severity increases trees should be replaced.

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Figure captions

Fig. 1. Percentage of *Spiroplasma citri* cultures obtained from receptacles of sweet orange fruits from mildly or severely symptomatic citrus trees affected by citrus stubborn disease. 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 hours after initial sub-culture in LD8 broth. Strains 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.

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

2006 Evaluation									
Variables	**Infected vs Healthy[a]**			**Mild vs Healthy[b]**			**Severe vs Healthy[c]**		
	Infected	Healthy	P value	Mild	Healthy	P value	Severe	Healthy	P value
Height (m)	2.4	2.5	0.06	2.7	2.8	0.14	2.0	2.3	0.22
Width (m)	2.6	2.7	0.41	2.7	2.9	0.34	2.4	2.6	0.84
RS (cm)	19.4	19.7	0.71	21.6	21.1	0.67	17.3	18.3	0.36
Scion (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
Yield [e]	5.9	7.9	0.02	7.1	6.0	0.37	4.7	9.9	0.00
2007 Evaluation									
Variables	**Infected vs Healthy[a]**			**Mild vs Healthy[b]**			**Severe vs Healthy[c]**		
	Infected	Healthy	P value	Mild	Healthy	P value	Severe	Healthy	P value
Height (m)	1.9	2.2	0.02	2.3	2.3	0.99	1.6	2.2	0.00
Width (m)	3.2	3.4	0.04	3.5	3.4	0.91	2.9	3.3	0.00
RS (cm)	19.6	20.1	0.65	21.3	20.8	0.72	17.9	19.3	0.32
Scion (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	0.00	20.0	7.5	0.05	33.7	7.2	0.00
Yield [e]	7.8	11.5	0.00	9.2	11.1	0.15	6.5	11.9	0.00

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

[a] Includes 10 pairs (2006) or 16 pairs (2007) of healthy and infected trees (mild and severe pooled together)

[b] Includes 5 (2006) or 8 (2007) mildly symptomatic trees and their corresponding healthy counterparts

[c] Includes 5 (2006) or 8 (2007) severely symptomatic trees and their corresponding healthy counterparts

[d] Number of fruits dropped within the boundaries of the tree canopy

[e] Average number of fruits from 4 sides of trees within the area of a 0.6 x 0.6 m PVC frame

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

2006 Evaluation

Variables		Infected vs Healthy[a]			Mild vs Healthy[b]			Severe vs Healthy[c]		
		Infected	Healthy	P value	Mild	Healthy	P value	Severe	Healthy	P value
Weight (Kg)		3.5	4.2	0.01	3.7	5.0	0.00	3.2	3.5	0.48
Length (L) (mm)		47.0	54.8	0.00	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	0.00
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	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 Evaluation

Variables		Infected vs Healthy[a]			Mild vs Healthy[b]			Severe vs Healthy[c]		
		Infected	Healthy	P value	Mild	Healthy	P value	Severe	Healthy	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	0.00	70.4	72.0	0.43	60.3	70.1	0.00
Width (W) (mm)		64.6	68.3	0.03	69.2	69.1	0.94	60.0	67.6	0.00
Ratio (L/W)		1.0	1.0	0.02	1.0	1.0	0.07	1.0	1.0	0.11
Normal fruit (%)		91.7	99.2	0.00	98.7	98.3	0.91	84.6	100.0	0.00
Sunburn (%)		6.0	1.9	0.00	2.5	1.2	0.40	9.6	2.5	0.00
	L	65.4	68.2	0.10	66.8	68.3	0.52	64.0	68.1	0.09
Peel color	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 fruits (2006 evaluation) or 960 fruits (2007 evaluation) harvested from pairs of healthy and infected trees (mild and severe pooled together)

[b] Includes 300 fruits (2006 evaluation) or 480 fruits (2007 evaluation) harvested from mildly symptomatic trees and their corresponding healthy pairs

[c] Includes 300 fruits (2006 evaluation) or 480 fruits (2007 evaluation) harvested from severely symptomatic trees and their corresponding healthy pairs

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

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

2006 Evaluation									
Variables	**Infected vs Healthy[a]**			**Mild vs Healthy[b]**			**Severe vs Healthy[c]**		
	Infected	Healthy	P value	Mild	Healthy	P value	Severe	Healthy	P value
Weight (Kg)	1.0	1.2	0.11	1.0	1.4	0.01	1.0	1.0	0.81
Brix (%)	10.5	10.4	0.26	10.0	10.3	0.63	10.9	11.5	0.27
TA (%)	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 Evaluation									
Variables	**Infected vs Healthy[a]**			**Mild vs Healthy[b]**			**Severe vs Healthy[c]**		
	Infected	Healthy	P value	Mild	Healthy	P value	Severe	Healthy	P value
Weight (Kg)	1.9	2.0	0.24	2.1	2.1	0.81	1.6	2.0	0.07
Brix (%)	12.9	13.5	0.37	13.1	13.3	0.75	12.8	13.6	0.34
TA (%)	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

Brix, measurement of dissolved sugar-to-water mass ratio; TA, titration acidity assay using citric acid equivalents

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

[a] Includes juice extracted from 600 fruits (2006 evaluation) or 960 fruits (2007 evaluation) harvested from pairs of healthy and infected trees (mild and severe pooled together)

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

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

Table 4. Distribution of *Spiroplasma citri* in different parts of Navel sweet orange trees based on q-PCR of fruit columella and leaf petiole.

Status	Fruit and petiole tier				Fruit and petiole aspect		
	Lower	Medium	Upper	P value	East	West	P value
Positive samples from columella (%)[a]	33.3	45.0	43.3	0.37	41.1	40.0	0.88
Positive samples from columella of mildly affected trees (%)[c]	20.0	26.7	26.7	0.78	28.9	20.0	0.50
Positive samples from columella of severely affected trees (%)[c]	46.7	63.3	60.0	0.39	53.3	60.0	0.33
Positive samples from petiole (%)[b]	13.3	8.3	16.7	0.39	14.4	11.1	0.52
Positive samples from petiole mildly affected trees (%)[c]	6.7	0.0	6.7	0.35	6.7	2.2	0.31
Positive samples from petiole severely affected trees (%)[c]	20.0	16.7	26.7	0.63	22.2	20.0	0.80

[a]Includes fruit columella from 5 mildly and 5 severely symptomatic trees (60 samples from each fruit tier and 90 samples from each aspect)

[b]Includes petioles from 5 mild and 5 severely symptomatic trees (60 samples from each petiole tier and 90 samples to each aspect)

[c]30 samples from each fruit columella/petiole tier and 45 samples from each fruit columella/ petiole aspect

Table 5. Presence of *Spiroplasma citri* in different canopy tiers and aspects of Navel sweet orange trees based on q-PCR of fruit columellas and leaf petioles from mildly and severely citrus stubborn symptomatic trees.

Status	Fruit columella			Petiole		
	Mild symptoms	Severe symptoms	P value	Mild symptoms	Severe symptoms	P value
Number of positive samples (%)[a]	24.4	56.7	0	4.4	21.1	0
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	0.00	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	0.00	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

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

[b]Includes fruit columellas or petioles from 5 mildly or 5 severely symptomatic trees (45 samples from each tree status)

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

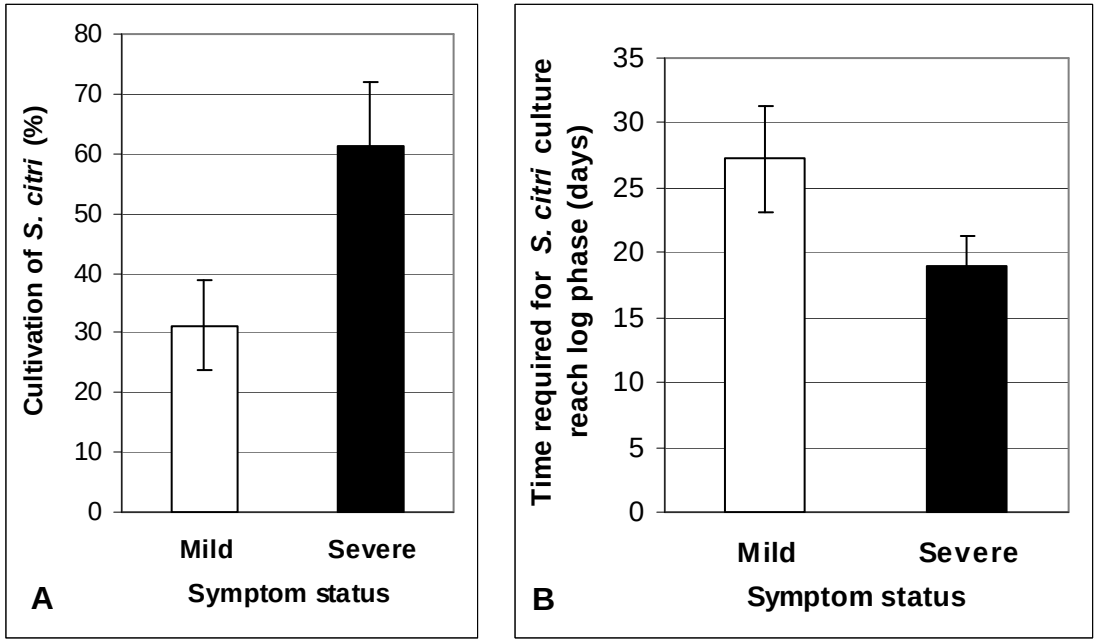
Cultivation of S. citri (%)
80
70
60
50
40
30
20
10
0
Mild
Severe
Symptom status
A
Time required for S. citri culture reach log phase (days)
35
30
25
20
15
10
5
0
Mild
Severe
Symptom status
B

Fig. 1.

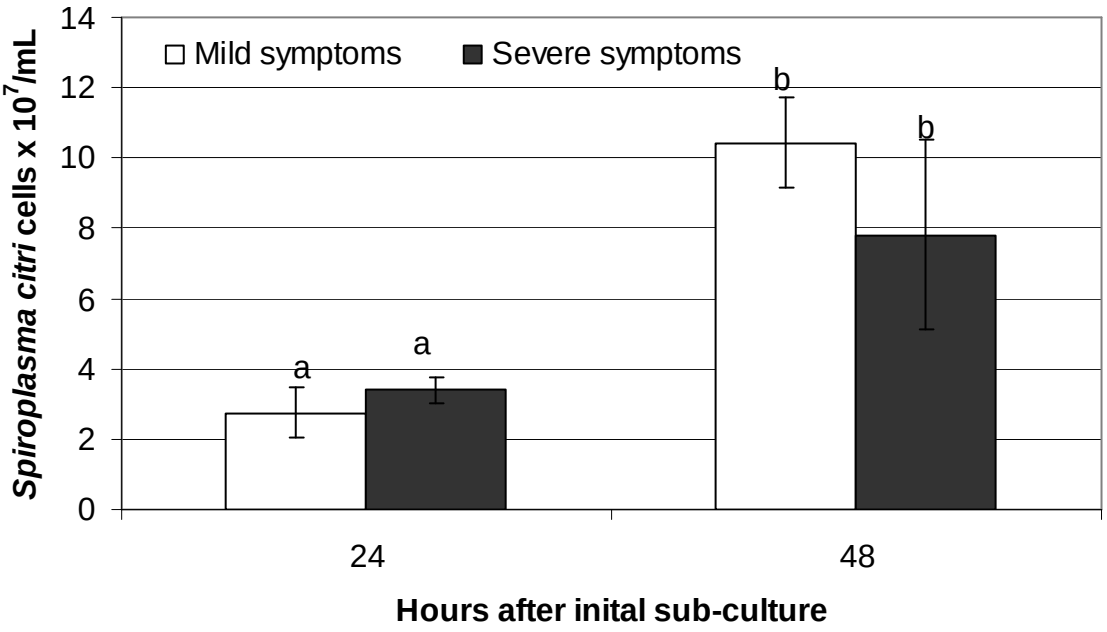
14
12
10
8
6
4
2
0
Mild symptoms
Severe symptoms
Spiroplasma citri cells x 10^7/mL
a
a
b
b
24
48
Hours after inital sub-culture

Fig . 2.

.

CHAPTER VI

SYMPTOM SEVERITY OF SWEET ORANGE TREES WITH CITRUS STUBBORN DISEASE IS ASSOCIATED WITH *SPIROPLASMA CITRI* TITER

Abstract

The impact of citrus stubborn disease (CSD), caused by *Spiroplasma citri*, on citrus production is associated with the symptom severity on infected citrus trees. To assess whether symptom severity was associated with spiroplasma virulence and/or titer in the plant, 58 *S. citri* strains were cultivated from severely and mildly infected trees and, using DNA template from these strains, RAPD and SSR fingerprinting differentiated four *S. citri* populations. Each of the four types was present in both mildly and severely symptomatic trees, suggesting that pathogen strain differences do not account for differences in disease severity. PCR reactions performed using primers specific for the genes of the pathogenicity-related fructose operon yielded amplicons of expected size in strains from both severely and mildly symptomatic trees. Quantitative PCR (q-PCR), using as template DNA extracted from fruit columellas of severely or mildly symptomatic trees, demonstrated that spiroplasma titer is over 6000 times higher in severely symptomatic than mildly symptomatic trees. The genotypic similarities among *S. citri* strains obtained from severely and mildly symptomatic trees, and the consistent evidence of higher bacterial titer in severely symptomatic trees compared to mildly infected ones, suggests that the latter, but not the former, is, at least in part, responsible for the higher severity in some of the *S. citri* affected trees in the orchard evaluated.

Introduction

Citrus stubborn disease (CSD) has been reported in California for over 50 years and *Spiroplasma citri* was confirmed as its causal agent in 1972 and 1973 by two separate research groups (13, 31). *S. citri*, a phloem-limited mollicute, is transmitted in a propagative manner by several species of leafhoppers or by grafting of infected plant tissues (18, 27). In turnip *(Brassica rapa* L.), leaf inoculation of the pathogen by the U.S. predominant leafhopper vector, *Circulifer tenellus* Baker, showed that the pathogen moves first to the roots and then to young leaves (11).

During infection and colonization *S. citri* utilizes carbohydrates and sterols from its plant host (1, 8), and competes with the host for these energy sources; this causes the depletion of some sugars and hormones and the accumulation of others. The resulting imbalance affects the metabolism of the plant causing stunting and leaf mottling (1). In addition, *S. citri*-infected citrus plants produce smaller and fewer fruits, and have off-season blooming, multiple axillary buds and shortened internodes (7).

CSD symptom expression is influenced by temperature, and during warm weather (30 to 35 ºC) leaf mottling and stunting were obvious 5 to 8 weeks after spiroplasma inoculation (3, 29). Changes in temperature also can affect symptom expression, turning symptomatic plants asymptomatic and vice-versa (3, 29). The impact of *S. citri* on citrus production seems to be related to symptom severity, since severely symptomatic citrus trees had lower yield and produced fewer and smaller fruits than did mildly symptomatic trees (21). The reason for variation in symptom severity under field conditions is not fully understood, but could be associated with bacterial titer within the plant and/or variations in strain virulence (6, 7).

The very small genome of *S. citri* easily deletes or acquires genetic components, thus enhancing the microbe's fitness (20). Continuous graft transmission of *S. citri* from periwinkle to periwinkle resulted in a chromosomal inversion and genomic deletions in *S. citri* BR3-3X that were associated with loss of transmissibility by the natural vector, *C. tenellus* (37, 39). High passage in artificial medium also altered *S. citri* transmissibility (37).

Randomly amplified polymorphic DNA (RAPD-PCR), using low stringency conditions (19) and random primers having short nucleotide sequences, has been used to efficiently discriminate genetic diversity among some plant pathogenic bacteria, including *S. citri* strains (22). Although the reproducibility of RAPD fingerprints can be influenced by the template and $MgCl_2$ concentration (14), the thermocycler used in the laboratory (26, 35) and the intensity of amplicons used to score the fingerprint (33), RAPD fingerprints can be very reproducible under well-established laboratory conditions (25).

Short sequence repeats (SSRs) are single or multi-nucleotide sequences, repeated along the genomes of prokaryotic and eukaryotic cells, arising from slipped-strand mispairing, inadequate mismatch repair and/or mutagenesis (36). The function of SSRs is not well established but is assumed to be related to protein encoding sequences or cell walls (36). SSRs are frequently used as molecular markers and are useful in assessing the genetic structure of populations of plant pathogenic bacteria (9).

The *S. citri* genome has evolved over a relatively short period of time (20). Genomic changes could lead to the occurrence of *S. citri* strains having enhanced aggressiveness, enabling more efficient use of carbohydrates and/or sterols and causing greater nutritional imbalance in the host, thus increasing symptom severity in citrus. Alternatively, an earlier inoculation or higher initial inoculum titer could lead to a greater distribution and multiplication of the spiroplasma in the trees, thereby increasing disease severity.

Variations in CSD symptom severity within a single orchard led us to compare the genetic diversity among *S. citri* strains from severely symptomatic trees with those from mildly symptomatic trees using RAPD and SSR markers and primers designed with homology to the pathogenicity-related fructose operon (15, 16). To elucidate whether the difference in CSD symptom severity in infected trees was related to *S. citri* strain or spiroplasma titer, quantification of the pathogen in severely and mildly CSD symptomatic trees was performed by quantitative PCR (q-PCR).

Materials and methods

Bacterial strains and isolation

S. citri strains were obtained from a plot within a commercial orchard in northeastern Kern Co., CA, that contained approximately 1,800 sweet orange (*Citrus sinensis* [L.] Osb.) trees, cultivar Thompson Improved Navel, grafted onto Carrizo citrange (*Citrus sinensis* Osb. x *Poncirus trifoliata* L. Raf.) rootstock. Trees were approximately 20 years old. Ten fruits were harvested at random from each of fifteen CSD affected trees (7 mildly and 8 severely symptomatic) in August, 2007. Fruit receptacles were processed for spiroplasma cultivation in LD8 broth (4, 17, 24). Cultures were evaluated daily for turbidity and spiroplasma growth was confirmed by dark-field microscopy using an Olympus BH-2 microscope (Olympus Optical Co., Tokyo, Japan) (1200 x). Also included were strains of *S. citri* cultivated from horseradish (*Armoracia rusticana* P.G. Gaertn., B. Mey. & Scherb) BR3-3X (12), carrot (*Daucus carota* L.) C17 (25) and citrus (34) and *Spiroplasma phoeniceum* and *S. kunkelii*.

DNA isolation

Spiroplasmas were grown in LD8 broth (17) (30 ºC) to a titer of 10^8 cells/mL. Cells were harvested, pellets were re-suspended in CTAB buffer and DNA extraction was accomplished via standard procedures (10). The DNA pellets were dissolved in water and quantified in a spectrophotometer (Nanodrop®, ND-1000, Wilmington, DE). The DNA solution was diluted to 4 ng/ µL, stored at -20 ºC and used in RAPD, SSR and PCR evaluations.

RAPD-PCR

Eleven 10-oligonucleotide primers, previously reported to discriminate *S. citri* strains (OPA-09, OPA-13, OPA-15, OPA-18, OPN-11, OPC-03, OPC-13, OPH-08, OPB-20, OPQ-06, and OPAW-05; Operon Technologies, Alameda, CA), were used in RAPD amplifications (25). PCR reaction mixtures and conditions were as previously reported (25). PCR reactions without DNA template were used as negative controls. A PTC-200 thermocycler (MJ Research, Inc, Ramsey, MN) was used for all experiments and reactions were performed twice. PCR products were electrophoresed in 1.5 % TAE-agarose (32) at 100 V/cm. Gels were stained with ethidium bromide and visualized using an AlphaImager

and Alphaease FC^{TM} software (Alpha Innotech Corporation, San Leandro, CA). Bands were compared to a 1 kb Plus DNA Ladder (Invitrogen®, Carlsband, CA).

SSR

Thirty seven contiguous chromosomal sequence blocks (contigs AM285302 to AM285339) from the *S. citri* strain GII-3 genome were retrieved from National Center for Biotechnology Information (NCBI). Sequences of four plectroviruses from *S. citri* SpV1-R8A2, SpV1-C74, SVTS2, and SVGII-3 (accession numbers NC_001365, NC_003793, NC_001270, and AJ96242, respectively) were also obtained from NCBI. Sequences were evaluated by the program Tandem Repeat Finder (2). Six contigs containing SSRs having at least 5 copies and a cutoff of 80 % of sequence match within the repeats were used for primer design. Primers were selected 40 to 268 nucleotides upstream or 14 to 195 nucleotides downstream of the SSR to avoid excessive thymine terminals (Table 1). PCR reaction mixtures were the same as described for RAPDs. PCR conditions consisted of an initial denaturation at 95 ºC (3 min), followed by thirty cycles of 95 ºC (15 sec), 50 ºC (30 sec), 72 ºC (1 min) and a final cycle of 72 ºC (5 min). PCR reactions without DNA template were used as negative controls. PCR products were electrophoresed in 3.0 % TAE-agarose at 50 V/cm. Gel staining and visualization were the same used in RAPD reactions. Four amplicons obtained from SSR reactions were sequenced using standard methods in an automated 3730 DNA analyzer (Applied Biosystems, Foster City, CA), and sequences were compared by clustal analysis with sequences retrieved from NCBI.

Fingerprint patterns of RAPD and SSR analyses were assessed visually. The presence or absence of bands in each strain was transformed into binary data (presence =1, absence =0) and were analyzed by principal component analysis using the SAS/PRINCOMP, SAS software 9.1.

Fructose operon

Five primer pairs were designed from the sequences of the three genes (*fruR*, *fruA* and *fruK*) and the translation initiation factor (*infB*) of the fructose operon, NCBI accession number AF202665, using Primer 3 software (30) (Fig.1, Table 2). PCR mixtures were the

same as used in the RAPD and SSR analyses and PCR conditions were the same as used in the SSR evaluation. PCR reactions without DNA template were used as negative controls. PCR products were electrophoresed in 1.5 % TAE-agarose at 100 V/cm. Gel staining and visualization were the same used in RAPD reactions.

Q-PCR

Triply cloned strain *S. citri* 160, which was obtained in 2006 (25) from the same orchard sampled in this study, was sub-cultured in LD8 broth. Cells were diluted 10-fold in 10% PBS-sucrose and plated onto 0.8% LD8 agar. Plates were incubated at 30ºC and the number of colonies (CFU) was assessed 11 days after sub-culture.

The same *S. citri* suspension utilized in the serial dilution was used for DNA extraction. Using a protocol adapted from Oliveira et al., 2002 (28), one mL of *S. citri* culture (3.40 x 10^8 cells) was harvested at 10,000 *g* for 10 min, the supernatant was discarded and the pellet mixed with 0.6 grams of finely minced citrus fruit columella (*S. citri*-free). One mL of 2.5X CTAB buffer was added and the mixture was homogenized in a MiniBeadBeater-96 (Bio-Spec Product, Bartlesville, OK) for 3 min. DNA extraction was accomplished via standard procedures (10), and DNA pellets were dissolved in 50 µL of water and quantified using a spectrophotometer (Nanodrop®, ND-1000, Wilmington, DE) and analyzed in 1.5 % TAE agarose gels. The DNA solution was diluted to 100 ng/ µL and stored at -20 ºC.

Six citrus trees, three with mild and three with severe symptoms of CSD, were used in the evaluation. Fruits were harvested from specific locations within the tree: two canopy aspects (one facing east and the other facing west) and three canopy tiers (top, middle and base), for a total of 18 samples per tree. DNA was extracted separately from each fruit columella, processed as described above, and adjusted to 100 ng/ µL. DNA was then stored at -20 ºC

Primers used in q-PCR were designed with homology to the single copy gene of the membrane-located spiralin protein gene, SP1 219f 5’ AAGCAGTGCAAGGAGTTGTAAAAA3’ and SP1 298r 5’ TGATGTACCTTTGTTGTCTTGATAAACA 3’ (R. Yokomi, personal communication).

A real-time PCR assay was developed using the DNA-binding fluorophore SYBR Green I, using PCR mixtures previously reported (40). Reactions were performed on a iQ5 Real-Time PCR System (BioRad, Hercules, CA) and the amplification consisted of an initial denaturation at 95° C for 3 min, followed by 37 cycles at 95 °C for 20 s and 55° C for 45 s. Control samples in each run included distilled water, DNA extracts from fruit columellas of *S. citri*-free citrus plants, and DNA extracted from *S. citri* cultures. To confirm the size and the specificity of the real-time amplicons, a melting curve was generated at 55 to 95° C at 0.5° C/10s. Real-time PCR products were separated in 3% agarose and bands were visualized by staining with ethidium bromide.

Sample titer was estimated by interpolation of the cycle thresholds (Ct) obtained from the field samples on a standard curve developed with the Ct and the quantity of DNA ($\log_{10}$ of the initial quantity of DNA template). Q-PCR reactions were performed twice and the Cts obtained in both evaluations were averaged.

Statistical analyses was performed using PC SAS Version 9.1 (SAS Institute, Cary, NC). Analysis of variance was used to compare factor levels. The number of spiroplasma cells was transformed with a natural logarithm function to address homogeneity of variance. The three factors of interest were symptom status (mildly or severely symptomatic), aspect (east or west) and canopy tier (top, middle or base); were arranged in a split plot arrangement with status as the main unit factor and aspect and tier as split unit factors. The simple effect of each factor was assessed with a SLICE option in an LSMEANS statement. A 0.05 level of significance was used for all comparisons.

Results

RAPD and SSR variation

All 11 RAPD primers yielded differential amplification patterns among the three spiroplasma species evaluated, and three (OPA-09, OPN-11 and OPQ-06) differentiated *S. citri* strains cultivated from mildly or severely symptomatic trees. Patterns that revealed the greatest diversity among strains are presented in Fig. 2. The five differential RAPD amplicons ranged from 1.65 to 0.85 kbp in size. Two main genetic patterns, consistent with the three primers used, were identified. No consistent differences were observed between

S. citri strains cultured from severely symptomatic trees and those cultivated from mildly symptomatic trees (Fig. 2) and some trees contained both genotypes. Control reactions without DNA yielded amplicons in some reactions, but these were attributed to natural contamination of *Taq* DNA polymerase since their size differs from those of the template DNA; hence they were not considered a problem in the data analysis (14).

From the 42 contigs evaluated 28 had SSRs identified by Tandem Repeats Finder software (2). The number of repeats per contig ranged from 1 to 33 but very few repeats were 100 % identical (data not shown). The scarcity of perfect repeats within the contigs led us to design additional primers homologous to sequences having percentage matches as low as 83% (Table 1). Six SSR primers were designed from five different contigs with an expected amplicon size ranging from 155 to 810 bp, with a minimum of three nucleotide repeats, and at least minimum of 8.7 copies (Table 1).

Five of the six SSR primers used yielded amplicons. Primers SSR 03 f/r did not yield amplicons that were independent of changes in PCR annealing temperature (data not shown). From the five primers used in PCR only SSR 02 yielded polymorphic amplicons among *S. citri* strains (Fig. 3). No correlation of amplicon size and strain origin (mildly or severely symptomatic trees) was observed (Fig. 3). Sequencing of amplicons obtained by PCR reactions with primers SSR 02, 20 A and 20B, showed that the numbers of copies of SSRs were different from those of the original sequences retrieved from NCBI. Amplicons obtained with SSR 02 had 10 or 13 TAA repeats whereas the original sequence from *S. citri* strain G II-3 had 15 repeats. Amplicons obtained from primers 20 A and 20B had 3.7 and 2.3 repeats, while the reference copy numbers from NCBI were 8.7 and 23.3, respectively.

Five clusters of *S. citri* strains were obtained by principal component analysis of five RAPD and two SSR differential amplicons. All clusters except that for coordinates 2 and -0.3 included *S. citri* strains from both mild and severely symptomatic trees. The four clusters that contained strains from both symptom types were so tightly structured that the black squares representing strains from severely symptomatic trees overlapped with the blank ones that represent strains from mildly symptomatic trees (Fig. 4).

Fructose operon

The five primers homologous to the three genes of the fructose operon (*fruR*, *fruA* and *fruK*) and the translation initiation factor (*infB*) yielded amplicons of the expected size with no amplification from the control reactions lacking DNA template (Fig. 5). No difference in amplicon size, which would suggest occurrence of insertion and/or deletion events, was observed (Fig. 5).

Quantification of *S. citri* by q-PCR

Primers homologous to the single-copy spiralin gene were highly specific with no amplication from controls. No formation of primer dimers occurred, as shown by the single peak in the melting curve of all reactions, which yielded single bands on 3% agarose gels (data not shown). Quantified amounts of *S. citri* DNA corresponding to $4.3x10^0$ to $4.3x10^6$ CFU of *S. citri* were used in the establishment of the standard curve (Fig. 6). Increasing the amount of DNA template in q-PCR reactions yielded lower Ct values (Fig. 6). Q-PCR was able to reliably amplify reactions with initial number of copies of DNA template ranging from 4,300 to 4,300,000, yielding Cts of 32.3 and 20.47, respectively. The corresponding linear regression was y=-4.0333x+44.367 with a regression coefficient of 0.98.

S. citri titers in severely and mildly CSD symptomatic trees were calculated by extrapolating the mean Ct value for the test samples into the standard curve previously developed (Fig. 6). The average *S. citri* titer on severely symptomatic trees (7.1×10^3 cells/mg) was statistically different from that on mildly symptomatic trees (1.15 cells/mg). No statistical differences were related to tree canopy tier (base, middle or top) (data not shown) and the only tree aspect (east or west) statistically different was in the comparison of severely symptomatic CSD trees in the upper tier (Table 3).

Discussion

The occurrence of varying severity of CSD symptoms in affected citrus trees has been reported since 1969, when the disease was still attributed to a virus-like organism (5). The factors underlying these different symptom levels could involve more aggressive pathogen strains (5, 7) and/or higher spiroplasma titer in severely symptomatic plants (6).

The association of disease severity with yield and fruit quality (21) led us to further study the determinants of CSD severity in one sweet orange orchard in California.

Genetic diversity in bacteria can be assessed by examining specific restriction sites, repetitive elements, genome sequences or the amplicons produced by random primers (RAPD) (19) and/or short sequence repeats (SSR) (36). RAPD and SSR primers used in the present study were suitable for differentiating *S. citri* from other members of spiroplasma serogroup I (*S. kunkelii*, and *S. phoeniceum)* and also among *S. citri* strains.

RAPDs and SSRs identified four main *S. citri* populations in the 15 sweet orange trees sampled. Both mildly and severely symptomatic trees contained all four *S. citri* populations, and some trees had mixed populations of the two, but no relationship between tree status (CSD severely or mildly symptomatic) and *S. citri* genotype was identified. Significant genetic variability of *S. citri* apparently has existed in California for a long period of time (25). Previous work showed that strains obtained from neighboring grapefruit trees in the Coachella Valley, CA, yielded as many differential amplicons as did trees located in orchards that were located apart. The presence of more than one genotype per tree was also previously reported (25).

The *S. citri* genome is one of the largest among Mollicutes and is characterized by a low guanine-cytosine content. In addition to its circular chromosome, *S. citri* also harbors plasmids and virus genomes, which likely serve as sources of genetic information (20, 38). Little is known about the mechanisms related to *S. citri* pathogenicity and plant symptom expression (16). Mutagenesis studies using random insertions of the transposson Tn*4001* demonstrated the relationship of the fructose operon to *S. citri* pathogenicity and delay of symptom expression (16). The fructose operon comprises three genes (*fruR, fruA* and *fruK*) that normally transcribe two messenger RNAs. Mutations within the fructose operon resulted in lack of transcription and prevented fructose utilization by mutated spiroplasmas (16). The inability to utilize fructose reduced the aggressiveness of *S. citri*, resulting in plants having milder symptoms than those inoculated with the wild-type spiroplasma (15, 16). The 58 *S. citri* strains utilized in this study yielded amplicons of the expected size from fructose-operon genes. This finding suggests that insertions/ deletions in this operon were unlikely in the spiroplasma population studied. We therefore reject the hypothesis

that *S. citri* strains having a disrupted fructose operon occurred in the *S. citri* population studied.

The use of q-PCR to quantify bacterial populations in citrus vascular tissues has been described (28). In our study, the *S. citri* titer in severely symptomatic trees was over 6000 times higher than that in mildly symptomatic trees, independent of tree tier or aspect. Recent studies using *S. citri* cultivation from fruit receptacles showed that cultures from mildly symptomatic trees required a longer period of time to achieve log phase than those cultivated from severely symptomatic trees, suggesting a higher titer of the bacterium in severely symptomatic trees (23). In the same study, q-PCR with primers homologous to the multi-copy P58-gene also showed that *S. citri* was present at more locations (was more widely distributed) in severely symptomatic trees than in mildly symptomatic trees, also suggesting a broader spiroplasma distribution in severely symptomatic trees (23). Our findings confirm higher *S. citri* titers in severely symptomatic trees and quantify *S. citri* populations in trees having these two levels of CSD severity.

In the sweet orange orchard evaluated in the present study, the severity of CSD symptomatic trees was associated only with bacterial titer. Different genetic approaches identified two main genotypes of *S. citri* in this orchard, and both were present in mild and severe CSD symptomatic trees. The findings elucidate an historic question about the probable cause of differences in CSD symptom expression levels. The reasons for the higher spiroplasma population and broader distribution on severely symptomatic trees remains unclear, but could be due to earlier infection and/or a higher number of infection sites.

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Figure captions

Fig. 1. Physical map of the fructose operon and orientation of the primers used to assess the amplicon size of *Spiroplasma citri* strains from mildly and severely symptomatic citrus trees affected by citrus stubborn disease (adapted from (16)).

Fig. 2. RAPD fingerprint after amplification with primer OPN-13. DNA extracted from 58 *Spiroplasma citri* strains, selected by single-cell cloning from fruits harvested from trees having mild or severe symptoms of citrus stubborn disease. Lanes 1 to 19; *S. citri* strains from mildly symptomatic trees. Lanes 20 to 58; *S. citri* strains from severely symptomatic trees. Lane 59, No DNA template. 60 to 62; *S. citri* strains BR3-3X, C17 and ASP-1 respectively. Lanes 63 and 64. *S. phoenicium* and *S kunkelii* respectively. L. DNA ladder 1 kb plus, size fragments listed on right.

Fig. 3. PCR fingerprints after amplification with primers designed from regions bordering short sequence repeats of *Spiroplasma citri* strains obtained from trees having mild or severe symptoms of citrus stubborn disease. L. DNA ladder 1 kb plus, size fragments on right. **A.** Lanes 1, 3 and 5. Amplicons obtained with DNA of *S. citri* strains from mildly symptomatic trees using primers SSR 06, 20 A and 20B, respectively. Lanes 2, 4 and 6. Amplicons obtained with DNA of *S. citri* strains cultivated from severely symptomatic trees using primers SSR 06, 20 A and 20B, respectively. Lanes 7 to 9. Control reactions with no template using primers SSR 06, 20 A and 20B, respectively. **B.** Differential amplicon sizes yielded by PCR reactions with primer SSR 02. Lanes 1 and 2. Amplicons obtained with DNA of *S. citri* strains from mildly symptomatic trees. Lanes 3 and 4. Amplicons obtained with DNA of *S. citri* strains from severely symptomatic trees. Lane 5. Control reaction with no template.

Fig. 4. Principal component analysis of *Spiroplasma citri* strains cultivated from trees with mild or severe symptoms of citrus stubborn disease symptoms, using, as input the differential characters obtained by random amplified polymorphic DNA and short sequence repeat analyses. All clusters except that on coordinates (2, -0.3) included *S. citri* strains from mildly and severely symptomatic trees. The four clusters that contained strains from both mildly and

severely symptomatic trees were so tightly structured that the black square representing strains from severely symptomatic trees overlapped the blank ones that represent strains from mildly symptomatic trees.

Fig. 5. PCR fingerprint after amplification using primers designed from genes of the fructose operon. L. DNA ladder 1 kb plus, size fragments on right. Lanes 1, 3, 5, 7 and 9. PCR amplicon obtained with DNA template of *Spiroplasma citri* strain 25F3 from a mildly symptomatic tree with primers InfB (f/r), fruR (f/r), fruA1 (f/r), fruA2 (f/r) and fruK (f/r) respectively. Lanes 2, 4, 5, 6 and 10. PCR amplicon obtained with DNA template of *S. citri* strain 8G from a severely symptomatic tree with primers InfB (f/r), fruR (f/r), fruA1 (f/r), fruA2 (f/r) and fruK (f/r), respectively. Lanes 11 to 15. Reaction controls for primers InfB (f/r), fruR (f/r), fruA1 (f/r), fruA2 (f/r) and fruK (f/r), respectively, using water as template.

Fig. 6. Standard curve of *Spiroplasma citri* concentrations following real time PCR amplifications; cycle threshold (Ct) is plotted against the log *S. citri* cells in 10-fold dilutions of spiroplasma template DNA mixed with citrus fruit columella tissue.

Table 1. Primers used to assess the number of short sequence repeats (SSR) of strains of *Spiroplasma citri* cultivated from sweet orange trees with mild or severe symptoms of citrus stubborn disease.

Primers				Contig	Period	Copy	Matches
Identification	Sequences	Location			size	number	(%)
SSR 02 F	TCATGATATGCGATATGTTCAGA	124021	124043	AM285302	3	15	90
SSR 02 R	CCATATATTGTAAAAGGCAATGACA	124151	124176				
SSR 03 F	GCTCTCCACAGTCAAACGGTA	36421	36441	AM285303	21	14.5	100
SSR 03 R	CCCCTGCTTTTTAATTGTTCTC	37210	37231				
SSR 06 F	GGTGCTAAATTACAAAAGAACAATTAGACC	16024	16053	AM285306	3	11	93
SSR 06 R	AGCCAATTTATTATTTATAATTGTAATAACATC	16228	16260				
SSR 12 F	TAAACTTGTTAATAGTATTTTTCAGTGTGC	6231	6260	AM285312	22	13.4	83
SSR 12 R	CAAATTCCTAACATAATTAATCACACTCC	6626	6654				
SSR 20A F	CGCTTAATTTCTCGTAAAATAGTACTACGATG	5721	5759	AM285320	3	8.7	100
SSR 20A R	GGTATATAAATGTTATGTATAGTCATTTGAGTTTTATG	5881	5908				
SSR 20B F	TACTATCATTGGTTTTTTAATTTGAGGTGA	16131	16160	AM285320	6	23.3	100
SSR 20B R	GCATTTACAGGATTCCATGATTAATAAG	16342	16369				

Table 2. Primers used to assess the presence of insertions or deletions in the fructose operon genes of strains of *Spiroplasma citri* cultivated from sweet orange trees with mild or severe symptoms of citrus stubborn disease.

Primers	Sequences	Regions	Amplicon size (bp)
InfB Fwd	CAGCTGACGATGGGGTAATG	64-83	943
InfB Rev	TCCTTCTGCTGCTGCAACTA	988-1007	
fruR Fwd	TTGCAATTATCACCAACAACA	1480-1500	602
fruR Rev	AATTTCAACTTCCGAACGAGA	2062-2082	
fruA-1 Fwd	CTACGCCATCTCAAGGAGGA	2431-2450	693
fruA-1 Rev	ATCATGCCGCAACATCACTA	3104-3124	
fruA-2 Fwd	CAGGCTGAGCAAATACATGG	3280-3299	793
fruA-2 Rev	TGCTACACCAATTGAAGCAC	4054-4073	
fruK Fwd	GGTGATTGGTGGAAAAGGAA	4353-4372	676
fruK Rev	CAGCAACCATTGAATCACCA	5013-5029	

Fwd. Forward, Rev. reverse

Table 3. Number of *Spiroplasma* cells detected in citrus trees presenting mild or severe symptoms of citrus stubborn disease in two different aspects (east or west) and three different tiers (base, middle or top).

Status	East*			West*		
	Base**	Middle**	Top**	Base**	Middle**	Top**
Mildly symptomatic trees	0.9Ba	1.1Ba	2.3Ba	2.6Ba	0.0Ba	0.0Ba
Severely symptomatic trees	2573.3Aa	3960.2Aa	2971.2Ab	17033.7Aa	12407.5Aa	3982.6Aa

*Includes fruit columella from 3 mildly and 3 severely symptomatic trees, 54 samples from each aspect (east or west)

**Includes fruit columella from 3 mildly and 3 severely symptomatic trees, 36 samples from each tier (base, middle or top)

Different capital letters within columns represent a statistical difference ($p<0.05$)

Different lower case letters within rows represent a statistical difference ($p<0.05$) within same tiers of different aspects

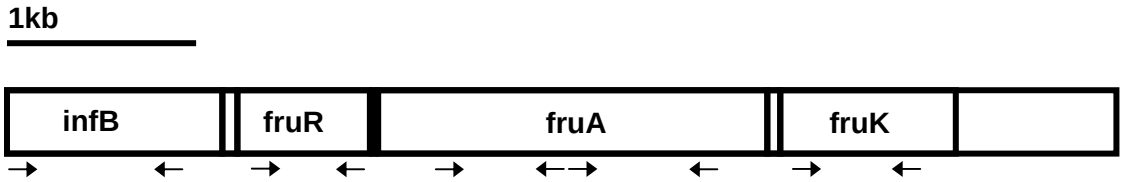

Fig.1.

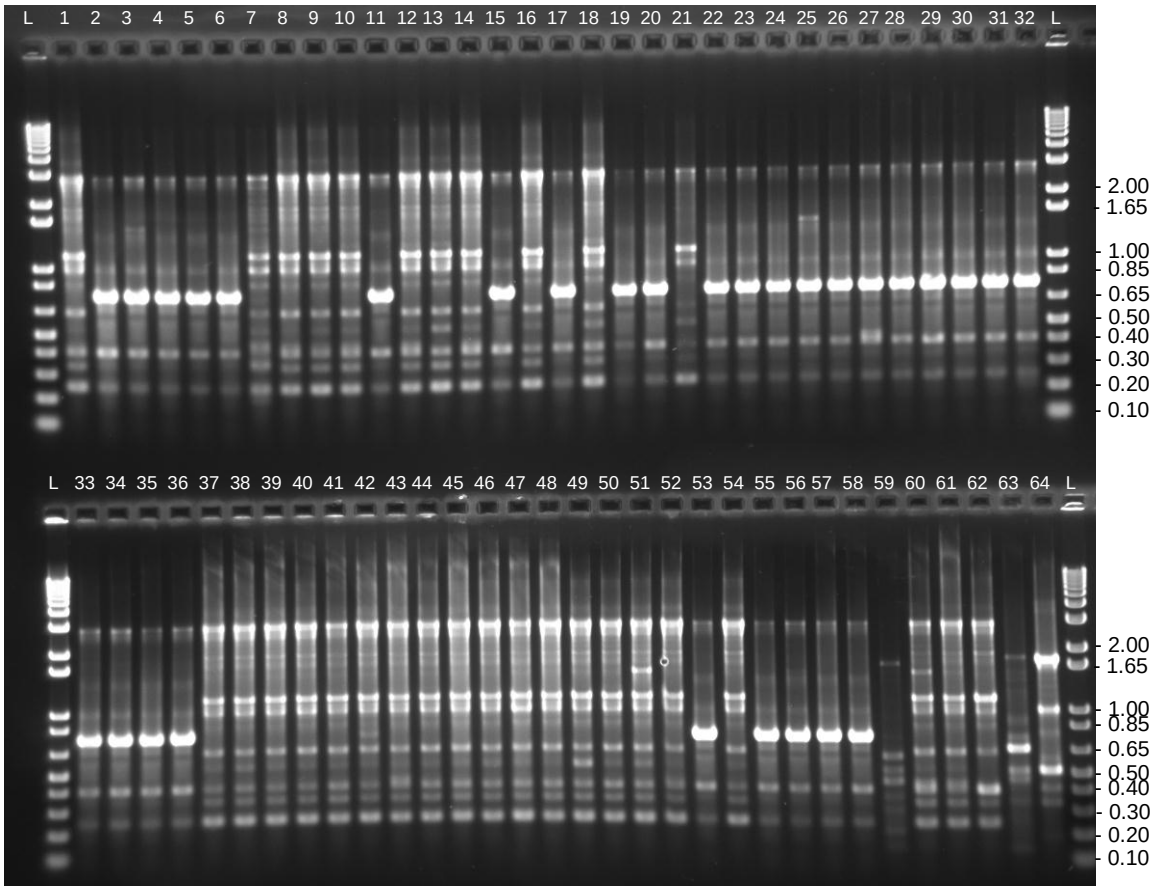
L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 L
2.00
1.65
1.00
0.85
0.65
0.50
0.40
0.30
0.20
0.10
L 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 L
2.00
1.65
1.00
0.85
0.65
0.50
0.40
0.30
0.20
0.10

Fig. 2.

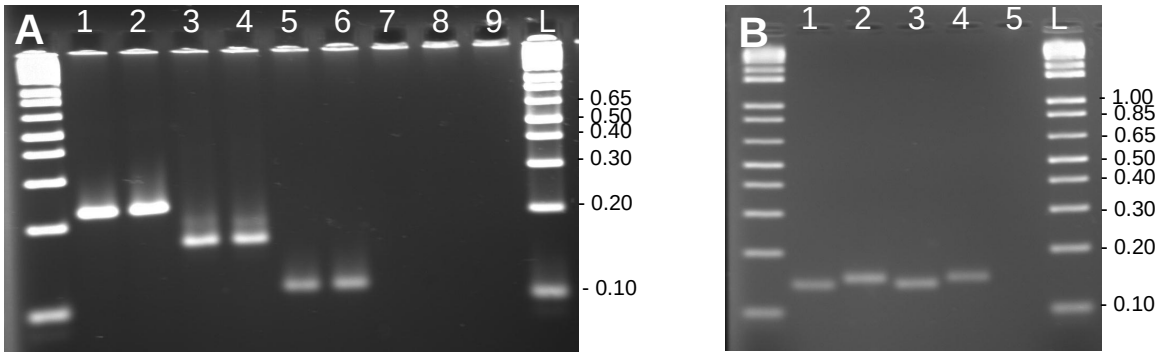
A
1 2 3 4 5 6 7 8 9 L
0.65
0.50
0.40
0.30
0.20
0.10
B
1 2 3 4 5 L
1.00
0.85
0.65
0.50
0.40
0.30
0.20
0.10

Fig. 3.

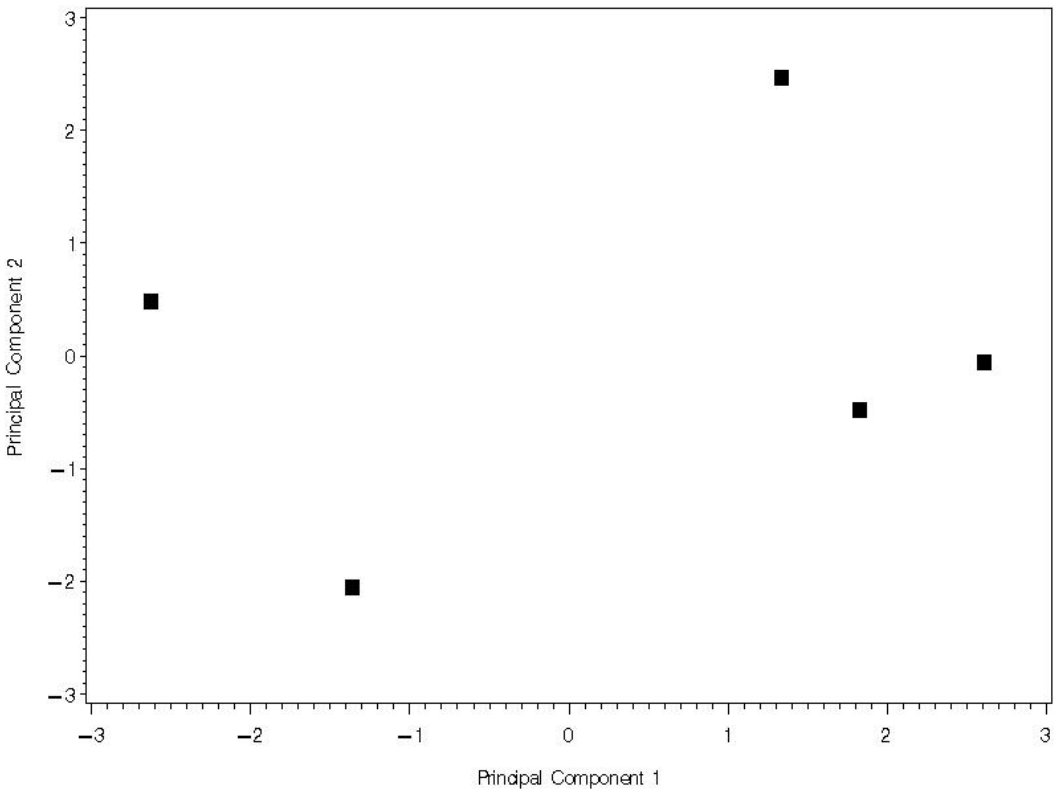
Principal Component 2
Principal Component 1
3
2
1
0
−1
−2
−3
−3
−2
−1
0
1
2
3

Fig. 4.

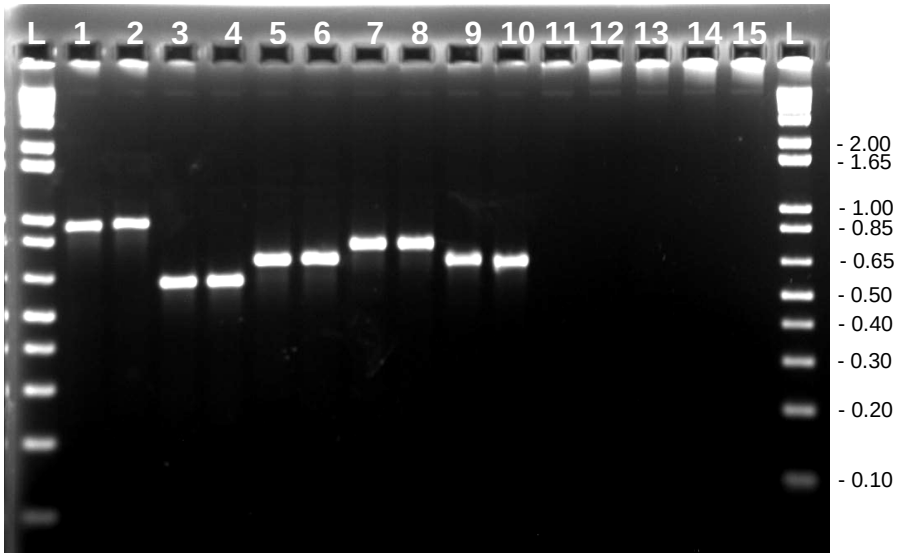
L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 L
- 2.00
- 1.65
- 1.00
- 0.85
- 0.65
- 0.50
- 0.40
- 0.30
- 0.20
- 0.10

Fig. 5.

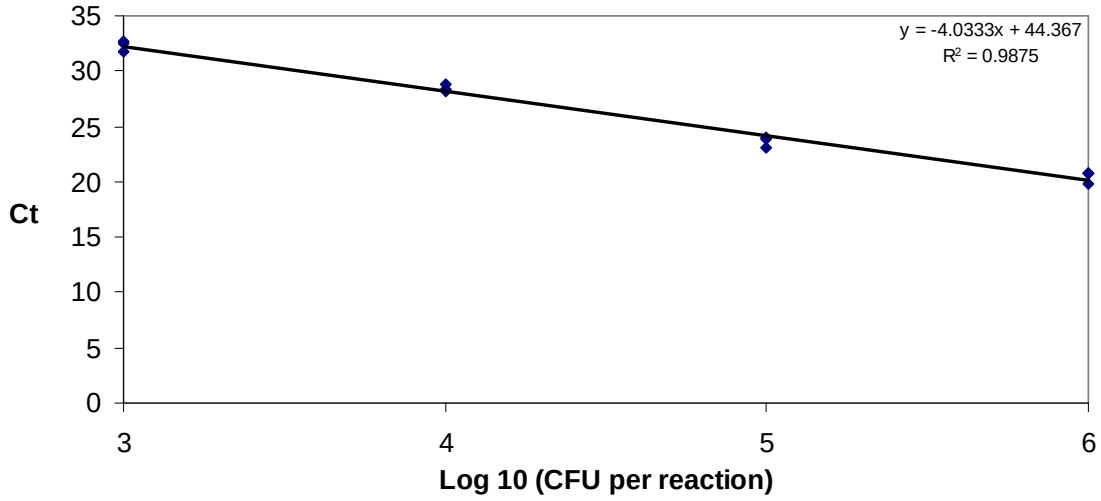
y = -4.0333x + 44.367
R2 = 0.9875
Ct
35
30
25
20
15
10
5
0
3
4
5
6
Log 10 (CFU per reaction)

Fig. 6.

CHAPTER VII

TRANSMISSION OF DIFFERENT STRAINS OF *SPIROPLASMA CITRI* TO CARROT AND CITRUS BY *CIRCULIFER TENELLUS* BAKER (HEMIPTERA:CICADELLIDAE)

Abstract

Carrot purple leaf disease (CPLD) was reported and associated with the presence of *Spiroplasma citri* in 2006 in the state of Washington, USA. The objectives of this work were to confirm *S. citri* as the causal agent of CPLD by fulfilling Koch's postulates, to determine whether carrot strains of *S. citri* can be transmitted by the beet leafhopper, *Circulifer tenellus* Baker, and to determine whether both carrot and citrus-derived spiroplasma strains are pathogenic to both of these plant species. Adults of the *S. citri* leafhopper vector, *Circulifer tenellus*, were exposed for 24 hours to feeding sachets containing spiroplasmas isolated from infected carrots and, after a 30 day latent period, insects were transferred to healthy carrot seedlings (5 leafhoppers/plant). Plants exposed to insects fed on buffer alone served as negative controls, while periwinkle plants exposed to insects fed on *S. citri* were used as positive controls. Confirmation of plant infection was based on the development of the expected symptoms in each host, spiroplasma re-isolation and PCR confirmation of bacterial identity. Purple leaves in carrots and small, chlorotic leaves in periwinkle became evident 10-45 days after plant exposure to insects fed on *S. citri*-buffer. No symptoms were present, and *S. citri* was not detected by PCR or culturing, in plants exposed to buffer-fed insects. Only symptomatic

plants of either species yielded cultures of spiroplasma and amplicons of expected size by PCR. *S. citri* was transmitted to 15% of the carrot plants and 50% of the periwinkle plants exposed to infected leafhoppers. Insects exposed to feeding sachets containing *S. citri* strains from carrot or citrus strains acquired and transmitted the pathogen to both their host of origin and to the other plant host (carrot or citrus), showing no strain-host-specificity. Our findings confirm the conclusion of Lee et al (14) that carrot is a host of *S. citri* and, because of its close association with the primary *S. citri* main leafhopper vector, *C. tenellus*, the occurrence of this disease in carrots are likely due to migration of infected leafhoppers to these crops.

Introduction

Spiroplasma citri, a phloem-inhabiting wall-less bacterium, causes stubborn disease (CSD) in citrus (*Citrus sinensis* [L.] Osb.) (11, 25, 26), brittle root in horseradish (*Armoracia rusticana* P.G. Gaertn., B. Mey. & Scherb*)*, (7, 10) and an un-named disease in periwinkle *Catharanthus roseus* (L.) G. Don in the United States (12). *S. citri* is naturally transmitted in a propagative manner by several species of leafhoppers (17, 23). The primary vector of *S. citri* in the U.S., the beet leafhopper, *C. tenellus*, is a polyphagous insect able to transmit the spiroplasma from weed species, such as London Rocket, commonly found in the foothills of California, to citrus plants (4) and is also implicated in the transmission of the pathogen to horseradish plants in Illinois (9). The location of stubborn diseased citrus trees, predominantly near the edges of orchards, and the presence of the insects in orchards only during summer (5) suggest a seasonal migration of infected insects from the weeds to the commercial crops during the dry season (4).

C. tenellus occurs commonly in arid areas of California, which coincide with areas of citrus production (24). The first report demonstrating the relationship between *C. tenellus* and *S. citri* showed that insects collected in California, exposed to citrus plants having CSD and then transferred to healthy citrus plants (24), transmitted the pathogen. High leafhopper mortality during the acquisition access period was observed, suggesting that citrus was not an optimal host for the insect. However, bacterial transmission by *C.*

tenellus was obtained when citrus plants and leafhoppers were caged with sugar beet plants, which are excellent hosts for the insect (22, 24). *C. tenellus* can acquire *S. citri* in less than one hour but the most effective rate of transmission was obtained when insects were exposed to infected plants for 48 hours (16). A minimum latent period of 24 days is required, during which the spiroplasma moves from the gut lumen, crossing the gut wall and reaching the salivary glands, from which they enter the salivary canal (17). Inoculation access periods of 48 h are optimal (16). During infection, the prokaryote damages insect membranes and basal lamina and causes disorganization of the endoplasmic reticulum (13), effects likely to increase leafhopper mortality (13, 17).

S. citri's very small genome easily acquires or deletes genetic components, thereby enhancing its fitness (19). Continuous graft transmission of *S. citri* from periwinkle to periwinkle resulted in a chromosomal inversion and genomic deletions in *S. citri* BR3-3X that were associated with loss of transmissibility by the natural vector, *C. tenellus* (28, 29). High passage of the spiroplasma in artificial medium also eliminated *S. citri* transmissibility (28).

The recent association of *S. citri* with purple leaf symptoms on carrot (*Daucus carota* L. subsp. Sativus (Hoffm.) Arcang) plants in Washington (14) suggested that carrot could be a third commercial crop (in addition to citrus and horseradish) affected by *S. citri*. However, Koch's postulates were not fulfilled in that study. Although populations of *C. tenellus* were smaller than those of other leafhopper species in Washington carrot-growing areas it was the only species found to harbor *S. citri* (14). Strains of *S. citri* from carrot in California differed slightly from those from citrus; random amplified polymorphic DNA (RAPD) analysis identified a mob-like protein present in *S. citri* carrot strains that was missing in citrus strains (21). Because of its genetic flexibility (19), *S. citri* is able to adapt its genetic content to different host and environmental conditions in short periods of time (19). The objectives of this study were to fulfill Koch's postulates to confirm the causal role of *S. citri* in CPLD, using *C. tenellus* as the insect vector, and to evaluate the plant host specificity of carrot and citrus-derived strains of *S. citri*.

Materials and Methods

Koch's postulates

Samples of carrot with or without symptoms of CPLD were collected from commercial fields in Kern County, CA, in 2006 and 2007. Carrot leaves and roots were processed for *S. citri* cultivation in LD8 broth (3, 15) and the presence of spiroplasmas was assessed daily by broth turbidity, and weekly by dark field microscopy at 1250x (27). Strains were triply cloned (21) and stored in aliquots at –80 ºC until used in experiments. DNA was extracted from triply cloned cultures (8) and used as template in polymerase chain reaction (PCR) using primers 132/710, homologous to the spiralin gene of *S. citri* at positions 132-151 TGCAACTGTAGCAACAGCAA and 710-729 TGCTTTTGGTGGTGCTAATG, (NCBI accession number U13998.1). *S. citri* strains R8A2, cultivated from citrus (26), and BR3-3X, cultivated from horseradish (10), were used as positive controls and water served as a negative control. PCR reaction mixtures (25 µL) contained 7.5 µL sterile distilled water, 4 µL GoTaq® Flexi buffer (10X) (Promega, Madison, WI), 4 µL $MgCl_2$ (25mM), 4 µL dNTP mix (1 mM, each) (Fisher® Scientific, Fair Lawn, NJ), 20 ng template and 1.5 units GoTaq®DNA polymerase (Promega, Madison, WI). Initial denaturation was performed at 95 ºC (3 min), followed by forty cycles of 95 ºC (15 seg), 55 ºC (30 seg), 72 ºC (1 min) with a final extension of 5 min. A PTC-100 thermocycler (MJ Research, Inc, Ramsey, MN) was used for all experiments. Reaction amplicons were confirmed by electrophoresis in 1.5 % agarose and PCR products were sequenced using standard methods in an automated 3730 DNA analyzer (Applied Biosystems, Foster City, CA). RAPD reactions, using primers and conditions previously reported (21), were performed to confirm that the strains isolated from carrot were *S. citri*.

The *C. tenellus* colony used in these experiments was initiated with insects collected in California (supplied by Gregory Walker, UC Riverside). One week old adults acquired the spiroplasma by feeding in sachets made of two layers of Parafilm® membrane stretched over a 50 mL plastic cup (28). Two different spiroplasma strains from carrots (C5 and C17) were used. Cultures grown in LD8 broth to a titer of 10^8 cells/mL were centrifuged at 10,000 *g* for 10 min and the pellet was then re-suspended in 500 µL of D10 buffer (1). The solution was gently vortexed and placed between the sachet membranes. Forty insects were transferred to each 50 mL plastic cup and the

acquisition access period (AAP) was 24 h under constant light at 30ºC. Control insects acquired *S. citri*-free D-10 buffer. After the AAP, aliquots of the feeding solution were checked by dark field microscopy (27) to assure the viability of spiroplasma cells used and insects were transferred to sugar beet plants for a latent period (LP) of 30 days.

After the LP, five insects each were transferred to tubular plastic cages 15 cm in height and 4 cm in diameter, covered with nylon mesh, and placed on carrot seedlings at the expanded leaf stage (Trinity, Sakata®) which were grown in plastic pots 25 cm in height and 6.4 cm in diameter. Periwinkle seedlings (3-4 expanded leaves) were used as positive controls. After an inoculation access period (IAP) of 48 h, insects were removed and the plants kept in a greenhouse for symptom expression. Sixty days after the IAP leaf samples from carrots and periwinkles were harvested and used for spiroplasma cultivation and DNA extraction. PCR was conducted using primers specific for the surface protein spiralin (2). Experiments were replicated 8 times, and the number of test plants per experiment was determined by the number of *S. citri*-exposed insects available.

Specificity of *S. citri* strains from carrot and citrus to their respective hosts

One strain isolated from carrot (C 17) and another from sweet orange (Ca 256) (21) were used. Feeding sachets were constructed using C17 or Ca 256 at 10^8 cells/mL in D-10 buffer, or with *S. citri* –free D-10 buffer as a control. The AAP and LP were the same as in the previous experiment. Host plants were used at the 3 to 4 expanded leaf-stage (carrot) (Trinity, Sakata®) or with 3-6 expanded leaves (sweet orange seedlings cv. Madam Vinous). After a 30-day LP, insects exposed to *S. citri*, or to D-10 buffer (healthy controls), were transferred to both citrus and carrot plants. The number of insects per plant and cage conditions was as reported above. After the IAP insects were removed from plants. Carrot seedlings were transferred to the greenhouse and citrus seedlings to a growth chamber (Model PGW36, Conviron, Ltd., Winnipeg, Canada) maintained at 35 ºC, 14 h light and 27 ºC, 10 h dark. Symptom expression was assessed weekly. Sixty days after inoculation the presence of *S. citri* in citrus and carrot leaves was confirmed by spiroplasma cultivation and PCR using the spiralin gene primers. Experiments were performed from November 2007 to August 2008 and replicated 8 times. A variable

number of repetitions were done per experiment, depending on plant and infected insect availability, but using a minimum of 40 plants per treatment.

Insect survival

The suitability of citrus as a host for *C. tenellus* is not fully understood (4). The high mortality rate observed for *C. tenellus* caged on citrus after the 48 h IAP (data not shown) during some experiments prompted us to further investigate the influence of the host on the *C. tenellus* survival. Five one-week old adult *C. tenellus* were caged on seedlings of citrus cv. Madam Vinous, or on carrots, using clip-cages (5.5x 5.2 x 1.8 cm), with one leaf per cage and one cage per plant. Leafhopper mortality was assessed daily for 10 days. As control treatments, insects were caged similarly on cotyledons of bean plants (*Phaseolus vulgaris* L.), a non host of *C. tenellus*, or on young leaves of sugar beet plants (*Beta vulgaris* L.), a suitable host (22). Experiments were replicated two times with 10 plants per treatment each time. Contingency tables at each time point were created to assess differences in mortality across hosts (PROC FREQ, SAS institute 1999). Pairwise comparison of hosts were determined and presented by letters and a 0.05 level of significance was used.

Results

Fulfillment of Koch's postulates

Spiroplasmas were visible by microscopic examination from symptomatic, but not asymptomatic, carrot plants from California, in LD8 broth 5 to 15 days after cultivation. Asymptomatic plants were culture-negative. DNA from such spiroplasma cultures, used as template in PCR reactions with spiralin primers, yielded the expected 597 bp amplicons, as did the control reactions containing DNA from *S. citri* control strains BR3-3X and R8A2 (Fig 1). The sequence of the PCR product from *S. citri* strains was 100% similar to that from the citrus control and RAPD fingerprints clearly demonstrated that the relationship of carrot strains to *S. citri* was closer than that to other spiroplasma species (21).

Purple leaves in carrots and small, chlorotic leaves in periwinkle became evident 15 to 45 days after plant exposure to *S. citri* infected insects. No symptoms were present, and *S. citri* was not detected by PCR or culturing, in plants exposed to buffer-fed insects. Only symptomatic plants of the two species yielded cultures of spiroplasma and amplicons of expected size by PCR. *S. citri* was transmitted to 15% of the carrot plants exposed to infected leafhoppers, a rate almost three times lower than that to periwinkle (50 %).

Transmission of *S. citri* strains cultivated from carrot and citrus to their hosts by *C. tenellus*

Symptom expression on carrot and citrus occurred in the same time frame as in experiments reported above. Cultivation and PCR, performed 60 days after the exposure of plants to *S. citri*-exposed leafhoppers, were positive only from symptomatic plants. Plants exposed to *C. tenellus* fed on *S. citri*-free buffer did not develop symptoms in any of the experiments performed (Table 2)

S. citri strains Ca 256 and C17, cultivated from citrus and carrots, respectively, were transmitted by *C. tenellus* to their host of origin and also to the challenge host (citrus or carrot) (Table 2). The percentage of infected plants was similar, regardless of the strain. The average of infected citrus plants was 17% on those exposed to the citrus strain, Ca 256, and 12% on those exposed to the carrot strain, C17. Approximately 6% of carrot plants exposed to the same strains became infected, averaging the results from 8 experiments.

Insect survival

Mortality of *C. tenellus* was higher on citrus and carrot seedlings than on sugar beet in all evaluations performed. Twenty four hours after transfer of *C. tenellus* from sugar beet plants to test plants, insect mortality rate was 10 and 11% on citrus and carrot seedlings, respectively (Fig. 2). During the ten day evaluation period the mortality rate was greater on citrus, carrots and beans than on sugar beet (Fig 2). On the last day of evaluation the percentage of surviving insects was 0% on citrus, 1% on beans, and 4% on

carrots, however; 95% survived on sugar beets. After 10 days of evaluation *C. tenellus* nymphs were present on sugar beet seedlings, indicating the suitability of this species, but none of the others tested, as a reproduction host (data not shown).

Discussion

The fulfillment of Koch's postulates is necessary to confirm the etiology of a plant disease. Spiroplasmas were cultivated from California carrot plants showing purple leaf discoloration and general stunting of shoots and leaves, confirming the findings of Lee et al. (14) from symptomatic carrot plants in the state of Washington. The average time required for spiroplasma cultures from carrot to achieve log phase, causing turbidity in the broth, was 5 to 15 days, similar to that for *S. citri* cultures obtained from citrus fruits in earlier studies (20).

Spiroplasmas from carrot, pelleted and re-suspended in D10 buffer, were acquired and transmitted to both carrot and periwinkle plants by *C. tenellus*. Symptoms on carrot plants exposed to *S. citri*-exposed leafhoppers included purple discoloration of leaves and stunting, while periwinkle plants exposed similarly, as plant controls, developed interveinal chlorosis, plant stunting and reduction of flower bud size, symptoms identical to those caused by *S. citri* strains from citrus (6). The rate of the spiroplasma transmission to periwinkle plants was three times higher than that to carrot plants, a result consistent with reports that carrots are not a preferred host for *C. tenellus* (22). Evidence for a potential role of the beet leafhopper in spiroplasma transmission to carrot was previously reported when insects harboring *S. citri* were collected in carrot fields in the state of Washington (14).

The high similarity of the spiralin sequence amplified from carrot-spiroplasma strains to that from a reference *S. citri* strain from citrus (R8A2) indicated that the spiroplasma causing CPLD is *S. citri*. The similarity of RAPD DNA fingerprints of spiroplasmas from carrot and from other plant hosts, and the fingerprint differences between the carrot strain in *Spiroplasma melliferum*, *S. floricola*, *S. phoeniceum* and *S. kunkelii*, confirmed *S. citri* as the causal agent of CPLD.

S. citri's very small genome easily acquires and/or deletes genetic components, thereby enhancing the microbe's fitness (19). Loss of transmissibility by the natural

vector, *C. tenellus*, in *S. citri* strains continuously graft transmitted from periwinkle to periwinkle or repeately sub-cultured in artificial medium (28, 29) are examples of such genetic plasticity. Our RAPD-PCR results demonstrated significant genetic diversity in current California *S. citri* strains and confirmed that strains from carrot can be distinguished from strains from citrus (21). Despite the modest molecular differences between *S. citri* strains from carrots and citrus, both are pathogenic to carrot and citrus. The transmission rates of the two strains to citrus and carrot were similar, suggesting little, if any, transmission specificity. We also found no correlation between transmissibility and RAPD fingerprints (21).

The high mortality of *S. citri*-free *C. tenellus*, when confined on carrot plants, confirmed a previous report of the unsuitably of this plant as a host to the beet leafhopper (22). The importance of citrus plants as hosts of *C. tenellus* is not fully understood (4), but our findings suggested that citrus, like carrot, is not as suitable as sugar beet for insect survival. Although carrot is not a preferred host plant, the concentration of CPLD symptomatic carrot plants at the edges of the production field (a pattern seen often with orchards affected by citrus stubborn disease) suggests that insects move from outside the field into the field (4, 5). Carrot production in the San Joaquin Valley occurs in two seasons: December to July and July to February (18). The latter season coincides with the summer, when annual weeds (major hosts of *C. tenellus*) dry up, forcing leafhopper migration into commercial crops (4). Young carrot seedlings in the fields at this time could be an attractive refuge for *C. tenellus*. The overall significance of CPLD in California is not known, but the prevalence of both *C. tenellus*, a natural inhabitant of the desert areas, and *S. citri* in the San Joaquin Valley suggest that closer assessments of disease impact and management are warranted.

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Figure captions

Fig. 1. Confirmation of carrot-derived spiroplasma strains C5 and C17 as *Spiroplasma citri* by polymerase chain reaction using spiralin primers 132/710, yielding an amplicon of 597 bp. Template DNA extracted from cultures isolated from carrots (strains C5 and C17), *S. citri* positive controls isolated form citrus R8A2 from citrus (A) and BR3-3X from horseradish (28) (B) and water (negative control). M: DNA ladder 1 kb plus, size fragments on right.

Fig. 2. Mortality of *Circulifer tenellus* 1 to 6 days after insect transfer from sugar beet plants to clip-cages without plants or clip-cages containing one leaf of carrot, citrus, bean or sugar beet. Different letters within days of treatments are statistically different ($p<0.05$).

Table 1 . Transmission* of *Spiroplasma citri* by *Circulifer tenellus* (BLH) to carrot and periwinkle plants 60 days after a 24 hour insect acquisition access period on D10 buffer containing *S. citri* strains C5 or C17 or D10 buffer (*S. citri*-free).

Experiments	Number of infected plants/ Number of plants exposed to inoculative BLH		Number of infected plants/ Number of plants exposed to non-inoculative BLH	
	Carrot	**Periwinkle**	**Carrot**	**Periwinkle**
	Strain C17		**D10 buffer**	
1	(0/6)	(1/3)	(0/3)	(0/2)
2	(0/1)	(3/4)	(0/2)	(0/1)
3	(1/1)	NE	(0/2)	NE
4	(0/5)	(0/3)	(0/2)	(0/3)
5	(2/7)	(1/5)	(0/2)	(0/2)
6	(0/6)	(0/3)	(0/3)	(0/5)
Sub-total	(3/26) 11.5%	(5/18) 27.8%	(0/14) 0%	(0/13) 0%
	Strain C5		**D10 buffer**	
7	(0/6)	(3/5)	(0/6)	(0/3)
8	(4/17)	(8/9)	(0/5)	(0/2)
Sub-total	(4/23) 17.4%	(11/14) 78.6%	(0/11) 0%	(0/5) 0%
Total	(7/49) 14.3%	(16/32) 50%	(0/11) 0%	(0/18) 0%

NE, Not evaluated; each plant was exposed to five insects.

* Confirmation of the presence of *Spiroplasma citri* done by symptom expression, bacterium culturing and polymerase chain reaction with primers SPN 132/710.

Table 2. Transmission* of *Spiroplasma citri* by *Circulifer tenellus* to healthy citrus and carrot plants 60 days after a 24 hour insect acquisition access period on D10 buffer containing citrus strain 256 or carrot strain C17 or on *S. citri*-free D10 buffer

Experiments		Strain 256-3X - Citrus strain						Strain C17-3X - Carrot strain						Healthy plants	
		Citrus			Carrot			Citrus			Carrots			Citrus	Carrots
		Positive	Total	(%)	Positive	Total	(%)	Positive	Total	(%)	Positive	Total	(%)		
1	11/30/07	1	5	20	0	0	0	1	13	7.7	0	0	0	5	0
2	01/05/08	1	2	50	0	2	0	1	4	25.0	1	5	20.0	2	3
3	03/02/08	5	9	55.6	1	9	11.1	1	8	12.5	0	8	0.0	6	3
4	04/06/08	0	3	0	1	6	16.7	2	9	22.2	1	10	10.0	5	6
5	05/06/08	0	11	0	0	3	0	0	4	0	0	4	0	6	6
6	05/21/08	0	4	0	0	11	0	0	0	0	0	0	0	4	9
7	06/10/08	0	4	0	0	8	0	0	0	0	0	5	0	4	5
8	06/14/08	0	3	0	0	3	0	0	3	0	1	10	10	5	5
	Total	7	41	17.1	2	42	4.8	5	41	12.2	3	42	7.1	37	37

* Confirmation of the presence of *Spiroplasma citri* done by symptom expression, bacterium culturing and polymerase chain reaction with primers SPN 132/710.

(%) Percentage of infected plants, each plant was exposed to five insects.

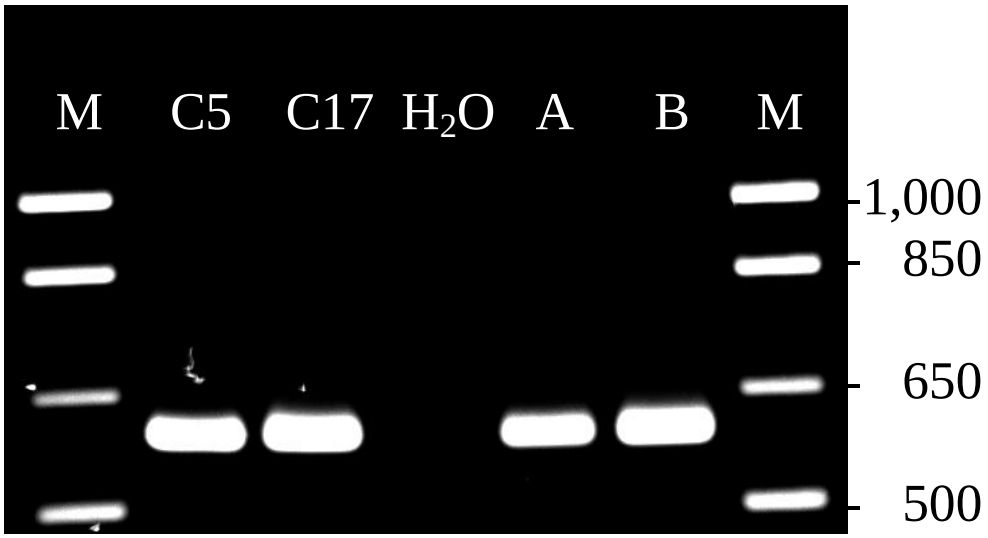

Fig. 1.

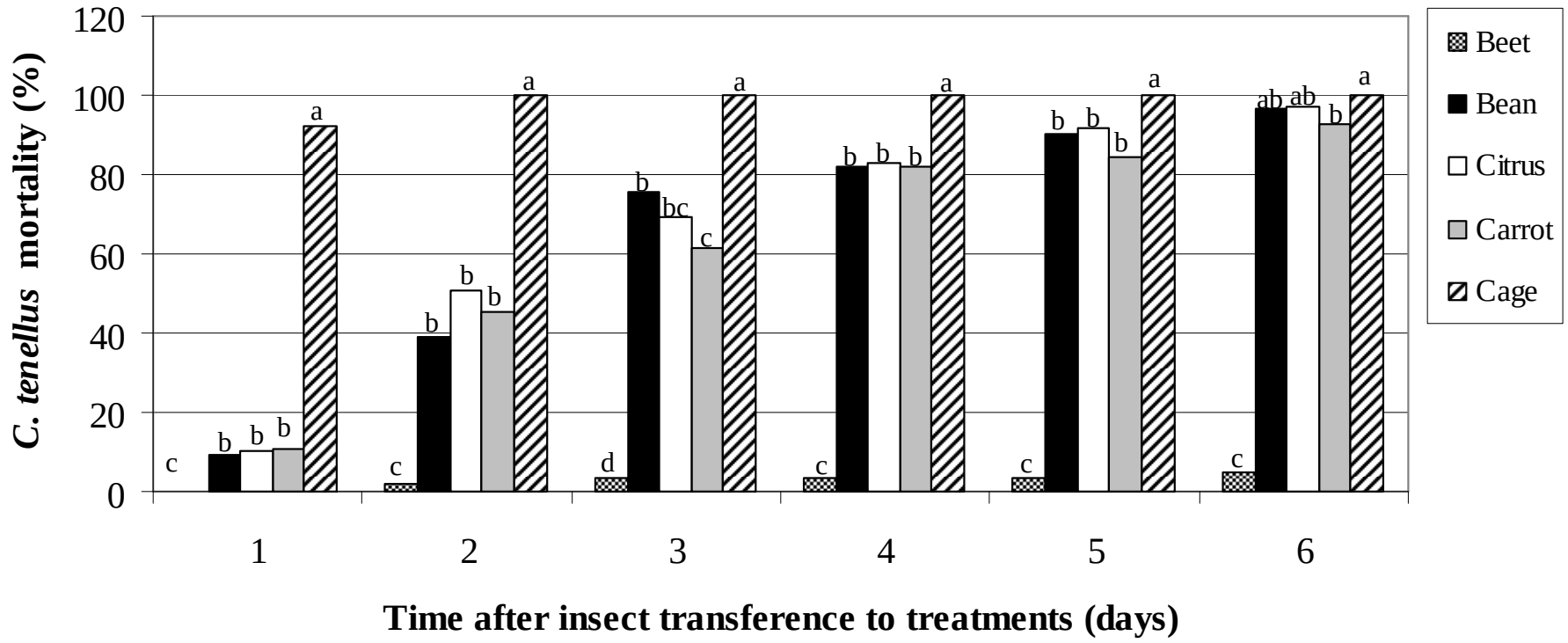
C. tenellus mortality (%)
120
100
80
60
40
20
0
1
2
3
4
5
6
Time after insect transference to treatments (days)
Beet
Bean
Citrus
Carrot
Cage
c
b b b
a
c
b
b b
a
d
b
bc
c
a
c
b b b
a
c
b b b
a
c
ab ab
b
a

Fig. 2.

VITA

Alexandre Furtado Silveira Mello

Candidate for the Degree of

Doctor of Philosophy

Dissertation: NEW PERSPECTIVES ON THE EPIDEMIOLOGY OF CITRUS STUBBORN DISEASE IN CALIFORNIA ORCHARDS

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Title of Study: NEW PERSPECTIVES ON THE EPIDEMIOLOGY OF CITRUS STUBBORN DISEASE IN CALIFORNIA ORCHARDS

Pages in Study: 131 Candidate for the Degree of Doctor of Philosophy

Major Field: Plant Pathology

Scope and Method of Study:

California is the major citrus fresh fruit producer in the US and citrus stubborn disease (CSD), caused by *Spiroplasma citri*, has been present in that state for a long period of time. CSD impact and epidemiology are not fully understood or quantified limiting the possibilities for science based management strategies. In this study, we evaluated symptom severity, disease incidence, pathogen diversity, and pathogen titer in symptomatic trees, thereby providing information useful to the improvement of disease management.

Findings and Conclusions:

Incidence of CSD in two commercial citrus orchards varied from low to high. Citrus fruit receptacles were more suitable than other tissues for spiroplasma cultivation. Molecular comparison of *S. citri* strains from different citrus orchards, carrot fields and weeds, collected from 1980 to 2007, showed that *S. citri* populations have a high degree of genetic variability, but strain identities were not correlated with the date or location of collection.

A two year field evaluation in one commercial sweet orange orchard showed that CSD severely symptomatic trees had a smaller tree canopy and yielded less fruit than mildly symptomatic trees. Fruits produced on severely symptomatic trees were also smaller, and more of them had sunburn than fruits from mildly symptomatic or healthy trees. Using a variety of molecular markers, no genetic differences were detected between *S. citri* strains from severely symptomatic trees and mildly symptomatic trees. However, bacterial titer, quantified by q-PCR, was higher in severely symptomatic than in mildly symptomatic trees.

The natural vector of *S. citri*, *Circulifer tenellus*, was able to acquire and transmit *S. citri* strains from feeding sachets to carrot plants, confirming the spiroplasma as the causal agent of carrot purple leaf disease. No strain-host specificity was identified.

ADVISER'S APPROVAL: Jacqueline Fletcher