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Project Title: Epidemiology of Almond Leaf Scorch Disease in the San Joaquin Valley of California: Factors Affecting Pathogen Distribution and Movement.

Project No.: 04-RG-01

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Introduction:

California almond production experienced record harvests for a fifth consecutive year in 2004 with yields exceeding 1 billion pounds. The almond industry leads the world in total production and supplies nearly all domestic consumption (www.almondboard.com/trade/content.cfm). The industry has averaged nearly 22% annual growth in the past 5 years with further increases expected. Expansion of the industry in California's SJV, however, may be impacted by the recent emergence of almond leaf scorch (ALS) disease. This disease poses a serious threat in many almond growing regions of California's Central Valley where ALS incidence has increased notably in the last 4-5 years. This disease, caused by the xylem-limited bacterium, *Xylella fastidiosa* (*Xf*), exhibit symptoms that appear as tan-colored, marginal scorching at the tips and margins of leaves with a characteristic chlorotic, band separating the scorched and green leaf tissues.

The bacterium has a diverse host range including both monocots and dicots and is transmitted by xylem feeding sharpshooters (Cicadellidae) and spittlebugs (Cercopidae). In California, there are at least 20 species of sharpshooter/spittlebugs capable of transmitting the pathogen; however, only four species are considered to be epidemiologically important in transmission of *Xf* to grapes. The primary vector(s) of ALS strains of *Xf*, however, have not been as well documented. Nevertheless, some leafhopper and spittlebug species have been implicated as possible vectors of *Xf*-ALS strains.

While *Xf* has long been present in the SJV, the incidence of ALS appears to have emerged, or reemerged as the case may be, as a significant disease threat to numerous locations throughout much of almond producing region. In particular, across the south-central portions of the SJV, in Kern, Tulare, and Fresno counties alone, *Xf* has been isolated from affected trees at 30 separate field locations with notable differences in varietal susceptibility. The overall goal of this project is to increase our understanding of the epidemiology of ALS in the central and southern SJV of CA with a focus on factors that influence the geographical distribution and movement of the pathogen. The primary focus will be to accurately identify the natural vectors of *Xf*-ALS strains, to characterize temporal and spatial patterns of disease incidence within selected orchards, and to determine the genetic structure of *Xf* strains associated with ALS. This ongoing research will provide fundamental, research-based information necessary to manage ALS in central valley orchards. An accurate knowledge of which vector species transmit *Xf*-ALS strains in the central and southern SJV, where they acquire the pathogen, when they move into orchards, and when they spread the pathogen to almonds is critical to understanding and managing the spread of this disease.

Objectives

1. Identify the primary vectors of *Xylella fastidiosa* causing almond leaf scorch disease and determine their (a) seasonal population dynamics and (b) patterns of movement into and within selected orchards from the surrounding agricultural landscape.
2. Comparatively characterize *Xylella fastidiosa* populations associated with (a) almond leaf scorch disease, (b) insect vectors immigrating into almond orchards, and (c) identify potential reservoir hosts in and around almond orchards.
3. Monitor the progress of almond leaf scorch disease incidence within selected orchards to evaluate the extent of tree-to-tree spread of *Xf* that may occur among trees and identify whether affected trees can serve as inoculum sources.

Results:

Objective 1.

Seasonal Dispersal. The seasonal population dynamics of potential *Xf* vectors dispersing into selected almond orchards has been monitored since January 2004. Throughout the season, migrating sharpshooter adults were monitored using combinations of yellow sticky traps, vertical mesh sticky traps, and universal black light traps systematically placed within and surrounding 5 pre-selected almond orchards; 3 in Fresno County and 2 in Kern County, CA, each with a recent history of ALS. In the survey locations, green sharpshooters (GSS), *Draeculacephala minerva* are the only known *Xf* vector species captured on dispersal traps among the 5 survey locations. No redheaded sharpshooters (RHSS), *Xyphon fulgida*, have been collected at any of the locations during the course of the experiments. Across all experimental locations, yellow card traps captured the fewest dispersing GSS (N=17) compared to both mesh (N=255) and blacklight traps (N=185) through May, 2005. Seasonal patterns of GSS capture generally reflect adult movement represneting two of the three known summer generations. Specifically, a modest peak in adult capture occurred in early May, 2004 with a larger peak following in late July and early August consistent with the species' multivoltine phenology. Again in 2005, peaks in GSS dispersal occurred in late May averaging 6.9 adult GSS per mesh trap across the 3 locations in Fresno County and 3.3 adults/trap averaged across 2 locations in Kern County.

Population Distribution. At monthly intervals beginning in April 2004 through May 2005, potential *Xf* vector species were collected from orchard trees, vegetation on the orchard floor, and within adjoining forage and pasture crops using sweep sampling. A total of 4,782 adult GSS have been collected from monthly sweep samples, the majority of which (N=3,522) were swept from adjoining pastureland and alfalfa forage crops. Less than 1% of the total GSS collected (N=39) were obtained from sweep samples of vegetation on the orchard floor(s). Peak periods of GSS collection on the orchard floor occurred between January – March, in both years of the study and averaged 0.2 adult GSS / standard 50-sweep sample. At no time throughout the course of the study were adult GSS swept from a subsample of almond foliage on select ALS-affected and healthy trees. The watercress leafhopper (*Acinopterus angulatus*) was the second most abundant leafhopper (N=122) swept from pasture and forage crops adjacent to ALS-affected orchards in both Fresno and Kern Counties. Here again, no adult RHSS were collected at any location through the course of the study.

Xf Detection. Genomic DNA of *Xf* has been amplified from adult GSS and *A. angulatus* collected from permanent forage grass pasture and alfalfa forage crops. Following a 12 h lyophilization and a CTAB minipreparation DNA extract procedure, insect head capsules are removed and homogenized in sterile phosphate-buffered saline and the presence of *Xf* genotypes determined using PCR formats. Among a total of 3,522 adult GSS collected in permanent pasture adjacent to an ALS-affected orchard in Fresno County, 610 have been assayed for the presence of *Xf*. Averaging over all sample dates and locations, the seasonal incidence of infection in GSS was estimated at 10.0%. Additionally, among a total of 122 *A*

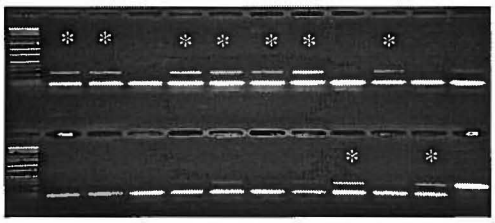

Figure 1. Detection of *Xf* (*) in adult GSS collected June, 2003 Fresno County from irrigated forage crops using primer pairs HL/5 & HL/6 producing an amplicon size of 221 bp.

angulatus collected, 48 have been assayed for the presence of *Xf*. An estimate of 6.3% (N=3) incidence of infection was determined although no current information is available concerning the vector status of this insect species. Further, we have conducted preliminary sensitivity assays for the detection of *Xf* genotypes in composite samples of adult GSS. By completing this objective we will better understand the *Xf* population(s) affecting almond in California, have the ability to better identify the important reservoir hosts which may serve as inoculum sources, and describe the vector(s) responsible for movement of the bacterium.

Objective 2. The genetic diversity of *Xf* population(s) associated with ALS in orchards in Fresno and Kern Counties was determined from collections made in both 2003 and 2004. Formal results of these data are included in the attached document (Chen et al. 2005. Two *Xylella fastidiosa* genotypes associated with almond leaf scorch disease on the same location in California. Phytopathology 95:708-714.). In brief, genomic DNA was isolated from the various *Xf* isolates collected from affected-almond and the population diversity assessed using combinations of RAPD and RFLP analyses, and single nucleotide polymorphisms (SNPs) from genome loci of taxonomic significance. There are clearly at least two genomic populations which co-exist simultaneously in affected orchards. At least two genotypically distinct types of *Xf* strains, G-type (grape) and A-type (almond), coexist simultaneously in the same infected almond orchard as well as in single infected leaves. Detailed microscopic observation of early passage cultures (presumably mixed or not pure) from the same leaf petiole revealed the presence of at least two colony types of *Xf*. The best observation for mixed colony types was in plates streaked from broth culture, probably resulting from better bacterial cell separation. PCR results from triple-cloned pure cultures separated from mixed colonies further confirmed the association of colony types with 16S rDNA SNP types and RFLP types of the RST31-RST33 locus. ALS epidemiology resulting from mixed strain populations has not been demonstrated previously and deserves further attention.

In addition to the large amount of genome sequence data accumulated recently, phenotypic descriptions of *Xf* and their biological nature, molecular basis, and genetic mechanism(s) are equally important for understanding pathogen and the development of disease control strategies. In recent studies undertaken in 2005, the colony morphologies of *Xf* strains with two distinct genotypes isolated from ALS-affected trees in the SJV of California were examined. Although significant variations occurred during the 14 sub-culturing passages, smooth colony morphotype was mainly associated with G-genotype strains. Rough colony morphotype was associated with A-genotype strains but smooth morphotype was observed to be descended from rough morphotype, indicating the presence of colony phase variation in *Xf*. Both A-genotype and G-genotype strains produced colony imprints or surface etchings on PWG and PW agar media, indicating enzymatic depolymerization of polysaccharides. *In silico* analyses identified four possible genes related to the imprint phenotype. This colony morphotyping in conjunction with PCR genotyping will further be used to survey genotype distribution among *Xf* strains of ALS collected in the central and southern San Joaquin Valley of California.

Objective 3. Incidence of ALS was mapped in 5 selected almond orchards (3 orchards in Fresno and 2 orchards in Kern County) in 2003 and 2004 to assess/evaluate the relative importance of primary versus secondary patterns of *Xf* spread. Here again, formal results of these analyses are presented in the attached document (Groves et al. 2005. Spatial analysis of almond leaf scorch disease in the San Joaquin Valley of California: factors affecting pathogen distribution and spread. Plant Dis. 59:581-589). Briefly, two-dimensional maps of the spatial distribution(s) of diseased

trees observed in 2003 surveys were generated from each surveyed orchard and characterized by calculation of mean incidence of ALS-affected trees within and across rows. A sub-sample of *Xf* isolations plated on selective media from collections in 2003 were inoculated into susceptible almond cultivars in greenhouse and small-plot pathogenicity tests to fulfill Koch's postulates. Disease incidence varied among almond cultivars in each orchard with the highest mean infection rates and most severe symptoms present in the cultivar 'Sonora'. *Xylella fastidiosa* isolates consisted of mixtures of G- and A-genotypes present in surveyed orchards previously described. The *Xf* G-genotypes in each orchard were consistently associated with the most severely affected 'Sonora' trees in three of the four orchards. Both ordinary runs and simple randomization analyses revealed aggregations of ALS in three of the four orchards with a high frequency of disease clusters present in the outermost orchard rows. Geo-statistical analyses were used to further interpret the spatial patterns of ALS-affected trees to determine random versus aggregated patterns of disease incidence. Plots of semivariance in ALS incidence over distance varied in shape and magnitude among cultivars. Semivariance increased over distance in cultivars 'Sonora' and 'Carmel' indicating spatial dependence or aggregations of incidence best fit by a combination of spherical and linear models. These results document both random and aggregate patterns of ALS spatial distribution in selected orchards and further illustrate how cultivar susceptibility influences the distribution patterns of ALS incidence and the impact of *X. fastidiosa* genotype on symptom severity.

Spatial ALS patterns were again mapped in 2004 at each of the survey orchards in Fresno and Kern Counties and plans for surveys in 2005 are underway. Mapped ALS incidence over survey years 2003-05 will again be analyzed using combinations of spatial analyses to characterize the temporal patterns of disease progress. Quartile variance plots of these data over successive years will further be used to accurately determine spread patterns of ALS. Our findings indicate that clusters of diseased trees were associated commonly with field borders adjoining habitats known to support populations of potential vectors. Primary spread of *Xf* from outside inoculum sources would lead initially to random patterns of infected plants, which may or may not be followed by tree-to-tree movement, or secondary spread of the pathogen, resulting in disease clusters or foci. Over multiple seasons, successive waves of primary spread may account for the spatial patterns of ALS observed in our study to date where clusters of infected trees were often associated with field borders. Detailed surveys over successive seasons will be necessary to better understand the temporal patterns of ALS progress and the extent of secondary spread that may in fact occur. ALS-mapped orchards also provide us with the unique opportunity to evaluate patterns of *Xf* spread based upon genotype. Spatial and temporal analysis of disease incidence data focusing on separate genotype profiles will greatly improve our ability to accurately identify local inoculum sources and to comparatively determine if a single, or a mixed population of *Xf* genotypes are preferentially spreading within orchards over time.

The impact(s) of ALS on almond yield and quality was evaluated in Fresno and Kern County orchards in 2004. Tree characteristics including diameter, height, yield, and nut quality were measured from a sub-sample of ALS-affected and asymptomatic almond. Trees were individually shaken, nuts swept, and collected for yield and quality assessments across three almond cultivars collected at 3 survey locations (Table 1). The impact of ALS on overall tree productivity coupled with spatial and temporal patterns of disease spread (primary vs. secondary), will provide critical new information about the necessity for, or epidemiological importance of, rouging ALS-affected trees. Select harvests are again planned for 2005.

TABLE 1. 2003 yield summary for harvests of ALS-affected and asymptomatic almond.

County	Orchard	Cultivar	ALS Incid.	Treatment	Mean Kernal Wt (g)[1]	Shelling Percent[1]	Total lbs/ tree[1]
Fresno	Orch 1	Sonora	0.28	ALS	1.21 a	70.4 a	22.3 a
				Control[2]	1.55 a	75.1 a	40.5 b
	Orch 2	Sonora	0.11	ALS	1.12 a	69.2 a	12.4 a
				Control	1.19 b	68.4 a	26.8 b
		Carmel	0.15	ALS	0.88 a	41.5 a	14.8 a
				Control	1.11 a	46.9 a	26.5 a
Kern	Orch 3	Sonora	0.07	ALS	1.20 a	75.3 a	26.3 a
				Control	1.29 a	75.4 a	37.3 b
		Nonpareil	0.02	ALS	0.91 a	69.0 a	42.4 a
				Control	0.98 b	72.4 b	56.8 b

[1] Means not followed by the same letter in columns between treatments are significantly different (α=0.05) (Proc GLM: LSMEANS)

[2] Trees harvested as controls represent ALS-asymptomatic trees

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Two *Xylella fastidiosa* Genotypes Associated with Almond Leaf Scorch Disease on the Same Location in California

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ABSTRACT

Chen, J., Groves, R., Civerolo, E. L., Viveros, M., Freeman, M., and Zheng, Y. 2005. Two *Xylella fastidiosa* genotypes associated with almond leaf scorch disease on the same location in California. Phytopathology 95:708-714.

Almond leaf scorch disease (ALSD) has recently reemerged in the San Joaquin Valley of California threatening almond production. ALSD is caused by *Xylella fastidiosa*, a nutritionally fastidious bacterium. Single nucleotide polymorphisms (SNPs) in the 16S rRNA gene (16S rDNA) of *X. fastidiosa* strains were identified to characterize the bacterial population in infected trees. Genotype-specific SNPs were used to design primers for multiplex polymerase chain reaction assays of early passage cultures. Two genotypically distinct types of *X. fastidiosa* strains, G-type and A-type, coexist simultaneously in the same infected almond orchard. This was substantiated by restriction fragment length polymorphism analysis of a different genetic locus, RST31-RST33, which has previously been used to identify and differentiate *X. fastidiosa* strains. Furthermore, unique bacterial colony morphology was consistently associated with the A-type *X. fastidiosa* strains. To our knowledge, this is the first report of a mixed genotype infection of *X. fastidiosa* disease on the same location under natural environmental conditions. The concept of mixed genotype infection could affect the current epidemiological study based on the assumption that one genotype causes ALSD on one location and, therefore, the disease management strategy.

Almond leaf scorch disease (ALSD) has recently reemerged as a serious disease threatening almond production in the San Joaquin Valley of California (2). The bacterial etiology of ALSD was initially based on electron microscopic examination of symptomatic leaves from infected almond trees (18) and subsequently confirmed by fulfilling Koch's postulate (10). The pathogen is taxonomically classified as *Xylella fastidiosa*, a nutritionally fastidious, xylem-limited bacterium (29). Leaf scorching during the summer and fall is the most noticeable symptom of ALSD, although this symptom can be easily confused with those of other disorders such as salt toxicity. Disease confirmation relies on pathogen identification, often accomplished by enzyme-linked immunosorbent assay, specific polymerase chain reaction (PCR), and bacterial growth characteristic in axenic cultivation. These techniques identify the pathogen at the species level.

Genetic and pathogenic variation of *X. fastidiosa* is an important factor affecting disease epidemiology and control. Previous research on *X. fastidiosa* has focused on the etiology of a single pathotype present in a susceptible host. The ALSD pathogen was reported to cause Pierce's disease (PD) following insect vector transmission to grapevines (18). Based on reciprocal pathogenicity tests, Davis et al. (10) concluded that both ALSD and PD were caused by the same pathogenic strains. We consider each bacterial isolate as a strain. Recently, two studies using strains sampled from different locations in California identified that ALSD could be caused by two distinct genotypes, one could cause PD and the other could not (2,12). In these studies, all strains in the non-PD group were isolated from northern counties of the Central Valley of California. The strains identified in the PD group were from southern areas of the Central Valley.

The possibility of natural mixed infection by multiple genotypes in a single location as well as a single plant has not yet been demonstrated in the *X. fastidiosa* pathosystem, although *X. fastidiosa* strains have a very broad host range (11) with no reported vector specificity. It is assumed that plant hosts can be exposed to multiple *X. fastidiosa* strains. Recognition of mixed infection is important for disease study and control. In phloem-limited and leafhopper-vectored phytoplasma pathosystems, mixed genotype infections have been known to influence the disease symptomatology and epidemiology (1,15,25).

The lack of documentation of mixed genotype infections of *X. fastidiosa* is probably due to the lack of efficient research tools. Currently, *X. fastidiosa* strains are differentiated by pure culture-dependent, DNA polymorphism-based methods such as randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) analyses, etc. These techniques are not applicable to mixed genotype samples because of the lack of specificity to a genotype. There is a need for a technique that can simultaneously identify *X. fastidiosa* species and differentiate the genotypes. We propose that a conserved genomic region could be used for species recognition and that a single nucleotide polymorphism (SNP) within the conserved region could be used to differentiate genotypes below the species level.

In this study, we developed an SNP-based PCR to identify and differentiate *X. fastidiosa* strains collected from ALSD-affected almond. The 16S rRNA gene was chosen because (i) 16S rRNA gene sequences are highly conserved at the species level, making them good candidates for species identification (5,22); (ii) SNPs exist in the 16S rDNA sequences and are key elements in forming subspecies level clusters (7,16); and (iii) 16S rRNA genes are currently the most sequenced locus in bacteria. SNPs derived from a large number of sequences provide a higher level of reliability. Using the SNP-based PCR, we examined the mixed infection

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of *X. fastidiosa* genotypes from ALSD samples on the same location.

MATERIALS AND METHODS

Isolation of *X. fastidiosa* from ALSD samples. ALSD-affected almond trees were sampled in orchards in Fresno, Kern, Stanislaus, and Tulare counties in the San Joaquin Valley, and in Butte and Solano counties in the Sacramento Valley of California. Samples were collected between September and November 2003, when leaf scorching symptoms were the most visible. Small branches with symptomatic leaves were excised, placed in labeled plastic bags, and transported to the San Joaquin Valley Agricultural Sciences Center, Parlier, CA. Upon arrival, samples were stored at 4°C and processed for isolation within 24 h.

Petioles were separated from leaf blades and surface-sterilized in 1% sodium hypochlorite for 2 min followed by three rinses in sterile, de-ionized water. Two transverse incisions were made in each petiole equally spaced along the length of the petiole. Xylem sap was expressed aseptically with a pair of flame-sterilized, needle-nose pliers onto the sterile surface of a petri dish. A drop of periwinkle wilt (PW) broth (9) was immediately added and mixed with the sap. One loopful of the sap mixture was then streaked on PW medium solidified by Gel-Rite (PWG, 13) and incubated at 28°C. The appearance of opalescent colonies was monitored with a binocular microscope for up to 40 days. Candidate strains were transferred onto fresh PWG, their identity tested by PCR with primer set RST31-RST33 (17), and subjected to genotyping within three passages of subculturing. To obtain a pure strain from the early passage and presumably genotype-mixed culture, single colonies were triple-cloned. The reference strains of *X. fastidiosa*, Temecula and Dixon, were provided by A. Purcell (University of California at Berkeley).

Identification of SNPs and PCR primer designs. A total of 38 16S rDNA sequences from different sources (4,7,16,20,24,27, 29) were retrieved from the GenBank database. These sequences were aligned using the CLUSTAL-W software (26) through the network service of the European Bioinformatics Institute (EBI) (available online by EBI). SNPs were initially identified by comparison of the sequences from *X. fastidiosa* strains Temecula, Dixon, Ann-1, and 9a5c. The authenticity and reliability of SNPs was further substantiated by comparative analysis of multiple sequences from different sources (7,16,20,29). The nucleotide order in the 16S ribosomal RNA gene, or open reading frame PD0048, in the *X. fastidiosa* strain Temecula genome sequence (27) was used as a reference to standardize the nucleotide position. Strain Temecula was used to represent the PD-causing ALSD strains, and strain Dixon was used to represent non-PD-causing ALSD strains.

Primer 3 software (21) was used to facilitate primer design. The desired T_m of all primers was set to 60 ± 3°C. The strategy for primer design is illustrated in Figure 1. In principle, a specific primer was designed by placing an SNP at the 3′ end as previously described (3). Two multiplex PCR formats were developed. For the three primer format, primers Teme150fc and Teme454rg matched the nucleotides from strain Temecula at the SNP positions. This primer set generated a calculated 348-bp amplicon for *X. fastidiosa* strain Temecula, but not strain Dixon due to the two 3′ mismatches. Considering the possibility of no amplification, where PCR failure could be implied, a third primer, XF16s1031r, conserved to all known *X. fastidiosa* strains, was added (Fig. 1). In the presence of a non-Temecula-type strain, the alternative primer set, Teme150fc-XF16s1031r, has only one mismatch to the DNA template. Under this circumstance, the amplicon could still be made but in the size of 700 bp (Fig. 1). The amplification serves as a positive control for a successful PCR reaction and the 700-bp amplicon size indicates the presence of a non-Temecula strain genotype.

In the four primer format, two primer sets were used. In addition to Teme150fc-Teme454rg as in the three primer format, the other primer set, Dixon454fa-Dixon1261rg, generated an 847-bp amplicon from strain Dixon. In fact, primer Dixon454fa and primer Teme454rg overlapped the same SNP position (Fig. 1). To check for specificity, each primer sequence was compared with the nonredundant, GenBank bacterial sequence database through the BLAST network service at the National Center for Biotechnology Information (provided online by NCBI). To test a primer pair, the two primers sequences were concatenated with 40 arbitrarily selected ambiguous nucleotides in between. The concatenated sequence was used for BLASTn search.

Analysis of RST31-RST33 locus. Primer set RST31-RST33 (17) was used for PCR confirmation of *X. fastidiosa* at the species level. For further strain differentiation, RST31-RST33 amplicons were digested with *Rsa*I for 1 h at 37°C (17). RFLPs were identified by 1.5% agarose gel electrophoresis and ethidium bromide staining. To further analyze the DNA polymorphism at the nucleotide sequence level, sequences flanked by RST31 and RST33 from the completely sequenced genome of the four *X. fastidiosa* strains were identified through a BLASTn search using the sequence from strain Temecula as query and the result was pre-

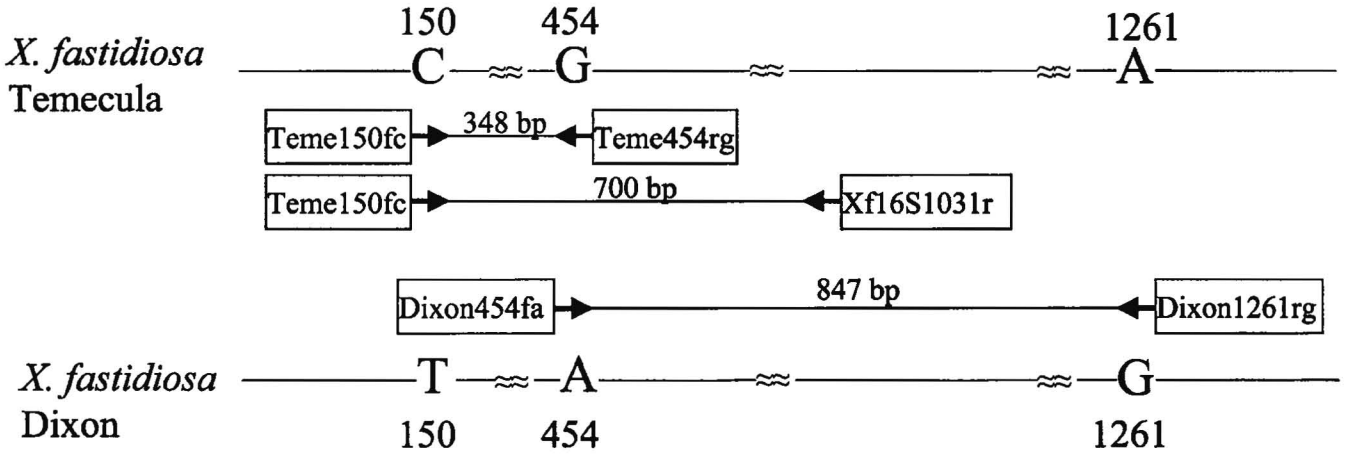

Fig. 1. Primer designs based on single nucleotide polymorphisms in 16S rDNA sequences from *Xylella fastidiosa* strains Temecula (top) and Dixon (bottom). Three single nucleotide polymorphic positions (150, 454, and 1261) are indicated; ≈ represents multinucleotides. Primers and their amplicons are presented. Teme150fc = 5′ tctaccttat cgtgggggac 3′, Teme454rg = 5′ aacaactagg tattaaccaa ttgcc 3′, Xf16s1031r = 5′ aaggcaccaa tccatctctg 3′, Dixon454fa = 5′ ccttttgttg gggaagaaaa 3′, and Dixon1261rg = 5′ tagctcaccc tcgcgagatc 3′.

sented in a multiple alignment format. Nucleotide variations were identified based on the sequence alignment.

PCR amplification. DNA templates were prepared by suspending a loopful of cell culture in PWG in 100 µl of sterile water. PCR (25 µl) was carried out using the Takara *Taq* (Hot Start Version) kit (Takara Bio Inc., Otsu, Japan). The reaction mixture contained 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 100 µM each dNTPs; 400 mM of each primer; 1 unit of *Taq* DNA polymerase; and 1 µl of cell suspension. Amplification was conducted in a thermocycler (Model PTC-100; MJ Research, Waltham, MA) with an initial denature at 96°C for 10 min followed by 30 cycles consisting of denaturing at 96°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The amplification products were then stored at 4°C. The amplified DNAs were resolved through 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Culture morphology. Bacterial cultures were streaked to single colonies on PWG. Ten to twenty days was usually required for optimum visualization of some colony characteristics. Bacterial colony morphology was examined under a stereo binocular microscope with fiber-optic bifurcated lights. Images were recorded with a DP12 microscopic digital camera (Olympus Optical Ltd., Tokyo, Japan).

RESULTS

Isolation for *X. fastidiosa*. A total of 303 isolations were attempted. Of these, 178 samples from all six counties were positive for *X. fastidiosa*. The number of bacterial colonies varied considerably ranging from fewer than 5 colonies per plate to more than 250 colonies per plate. Growth of presumptive *X. fastidiosa* colonies required at least 3 days or, typically, 5 to 10 days. In some cases, this required 20 days. The longer incubation period was most often associated with fewer (<10) bacterial colonies, an indication of low bacterial titer in the samples. In the isolation-negative plates, prolonging the incubation time to 40 days did not result in additional bacterial growth. Most of the early isolations were from a small number (<8) of ALSD samples in various orchards. The *X. fastidiosa* strains were later proved to be either A-type or G-type. To prove the presence of mixed genotypes in the same orchard, four orchards in Fresno and Kern counties were subjected to more systematical analysis with a larger number of sampling (Table 1).

One common method of *X. fastidiosa* isolation from petioles is to blot expressed plant sap directly onto medium (14). Because of the soft surface of PWG and the limited amount of xylem sap expressed, a sap-blotted surface was often indented, interfering with later bacterial colony observation. Blotting the xylem sap on the sterile surface of a petri dish and streaking the sap mixture on a PWG plate eliminated problems associated with colony observation.

Identification of SNPs and primer designs. Four SNPs were identified from the alignment of 38 16S rDNA sequences, and the three SNPs related to ALSD strains used in this study were shown in Figure 1. The cytidine in position 150 and guanosine in position 454 separated all of the eight grape and a mulberry strain sequences from the others (data not shown). These nine sequences originated from four separate laboratories (7,16,20,27). The two SNPs were chosen as markers for the strain Temecula genotype and designated as G-type, referring to the grape representation as well as the guanosine in position 454. The guanosine at position 1261 was characteristic of a group of *X. fastidiosa* strains from a wide range of hosts. One of them was the Dixon strain causing ALSD. We designated strains in this group as A-type, referring to the almond representation as well as the adenosine in position 454, which was later used for ALSD primer design.

The BLASTn search result from each primer sequence identified *X. fastidiosa* as the most, or one of the most, similar bacteria. No single bacterium, with the exception of *X. fastidiosa*, simultaneously matched to two primers or a primer set, i.e., Teme150fc-Teme454rg, Teme150fc-Xf16s1031r, and Dixon454fa-Dixon1261rg (data not shown). The specificity of the three primer sets was further demonstrated when the primer concatenated sequence was used for BLASTn search where *X. fastidiosa* was the only bacterial species to have hits for both primers (data not shown).

In addition to species specificity of the PCR primers, the SNPs at the 3′ end of these primers allowed for the differentiation of genotypes, or A-type and G-type, of *X. fastidiosa*. Since Teme131fc-Teme478rg has no base variation in the G-type strains, the primer set detected this genotype with a 348-bp amplicon (Fig. 2A). Under the PCR stringency used, the two 3′ end mismatches were sufficient to block the specific DNA amplification from non-G-type strains, or to yield a substantially weaker amplification (Fig. 2A, strain Dixon). The other primer combination, Teme131fc-Xf16s1031r, was however able to generate a 700-bp DNA amplicon identifying a non-G-type strain (Fig. 2A).

Based on the available information (2,12), the non-G-type detected by the three primer PCR format was presumably an A-type. The four primer multiplex PCR format was used to confirm this assumption. In the four primer multiplex PCR format, primer set Teme131fc-Teme478rg generated a 348-bp DNA amplicon as expected and primer set Dixon435fa-Dixon1128rg produced an 847-bp amplicon (Figs. 1 and 2B). For the strains examined in this study, results from the two multiplex PCR formats always agreed.

Analysis of RST31-RST33 locus. PCR using primer set RST31-RST33 generated an expected 721-bp amplicon, confirming the *X. fastidiosa* identity of the ALSD strains studied (Fig. 2C). RST31-RST33 amplicons from G-type strain could not be digested by *Rsa*I; yet, for A-type strains, two DNA fragments (149 and 572 bp) were evidenced (Fig. 2D). These results were expected and consistent with the SNP analyses. In Figure 2D, incomplete *Rsa*I digestion of A-type strain amplicons was apparent, but this did not interfere with the interpretation of strain differentiation. Overnight *Rsa*I incubation, rather than 1 h, resulted in complete DNA digestion (data not shown). It should be noted that the genomic locus flanked by RST31-RST33 was physically independent from the two identical *rrn* loci in the *X. fastidiosa* genome. Based on the information from the whole genome sequence annotation (24,27), the RST31-RST33 DNA sequence has 502 bp in the 3′ end of gene *rpo*D, coding for an RNA polymerase sigma-70 factor, and 219 bp in the downstream intergenic region. An SNP within an *Rsa*I site covering position 572 (G in strain Temecula and A in strain Dixon) was responsible for the RFLP difference between the G-type (Temecula) and the A-type (Dixon) strains.

Sequence comparisons further demonstrated that two bases downstream of the SNP was a repeat sequence of two 14 bp units in the CVC-9a5c genome (data not shown). Such a repeat does not occur in strains Temecula, Dixon, and Ann-1. Because of the sequence repeat, the RST31-RST33 amplicon from CVC-9a5c should be 735 bp compared with the 721 bp in strains Temecula and Dixon. The size of the RST31-RST33 amplicon from strain Ann-1 was 723 bp. The original publication reported a 733-bp

TABLE 1. Summary of *Xylella fastidiosa* isolation and genotyping from almond leaf scorch disease samples from selected orchards in the San Joaquin Valley of California

County	Orchard	Sample size	Cultures positive	RST31/33 positive	Genotype	
					G-type	A-type
Fresno	A	22	4	4	1	3
Fresno	B	22	7	7	5	2
Kern	C	16	7	7	7	0
Kern	D	83	67	67	18	49

amplicon from a Florida PD strain (17). In all case, these nucleotide variations were too small to be differentiated in agarose gel electrophoresis.

Detection of different *X. fastidiosa* genotypes in ALSD samples. Both G-type and A-type strains were identified from samples collected from ALSD-affected orchards within the San Joaquin Valley in 2003 as shown in Table 1. The two orchards (A and B) in Fresno County were located approximately 4 km apart and each contained both strain types of *X. fastidiosa*. In Kern County, sampling from orchard C yielded only the G-type. However, results from the larger, systematically sampled orchard D, which was approximately 2.4 km from orchard C, showed the presence of both A-type and G-type of *X. fastidiosa*. Among the 67 isolates from 83 samples, 18 were classified as G-type while 49 were determined to be A-type.

Colony morphology. Detailed microscopic examination of *X. fastidiosa* strains using direct light from different angles revealed significant colony morphology variations. In general, there were two major colony morphotypes, smooth and "pit"-like, namely. The smooth type was circular or occasionally slightly irregular, convex with entire margins (Fig. 3A). All of the observed G-genotype strains showed this colony morphotype. The "pit"-like morphotype was characterized by its granular rough appearance on the outskirt colony surface. The central portion was largely convex or pulvinate (Fig. 3B). The two-steps or umbanate morphology could give the appearance of pit formation when observed through the base of the petri dish under a stereo binocular microscope with fiber-optic bifurcated lights. The colony form ranged from circular to spindle shaped and the spindle shape seemed to be associated with streak marks on the medium surface.

All of the "pit"-like colony strains were further confirmed to be genomically A-type by PCR using both the 16S rDNA SNP primers and RST31-RST33. The "pit"-like colony morphotype was the predominant type among all A-type strains from our ALSD samples. A small group of A-type strains showed a non-"pit"-like colony morphology. They were in a somewhat irregular form and convex with undulate margin (Fig. 3D). The Dixon strain, which was a high passage culture, belonged to this type under our cultivation conditions.

Figure 3C and D representatively shows the presence of mixed colonies from two individual petioles from orchard D (Table 1). However, one colony type was always predominant, estimated to be at least 99% of the colonies on the primary isolation plates. This may partially explain why both the three and the four primer multiplex PCR consistently detected only one genotype, either G-type or A-type, in our assays. The DNA templates from the predominant genotype suppressed those from the minority genotype during the PCR process. Although a weaker DNA band of the corresponding genotype could sometimes be visualized (Fig. 2A, strain Dixon; Fig. 2B, strain Kern R 76), we interpret that these resulted from nonspecific amplifications.

DISCUSSION

In prokaryotes, the 16S rDNA loci are commonly regarded as highly conserved and are of taxonomic value for a species de-

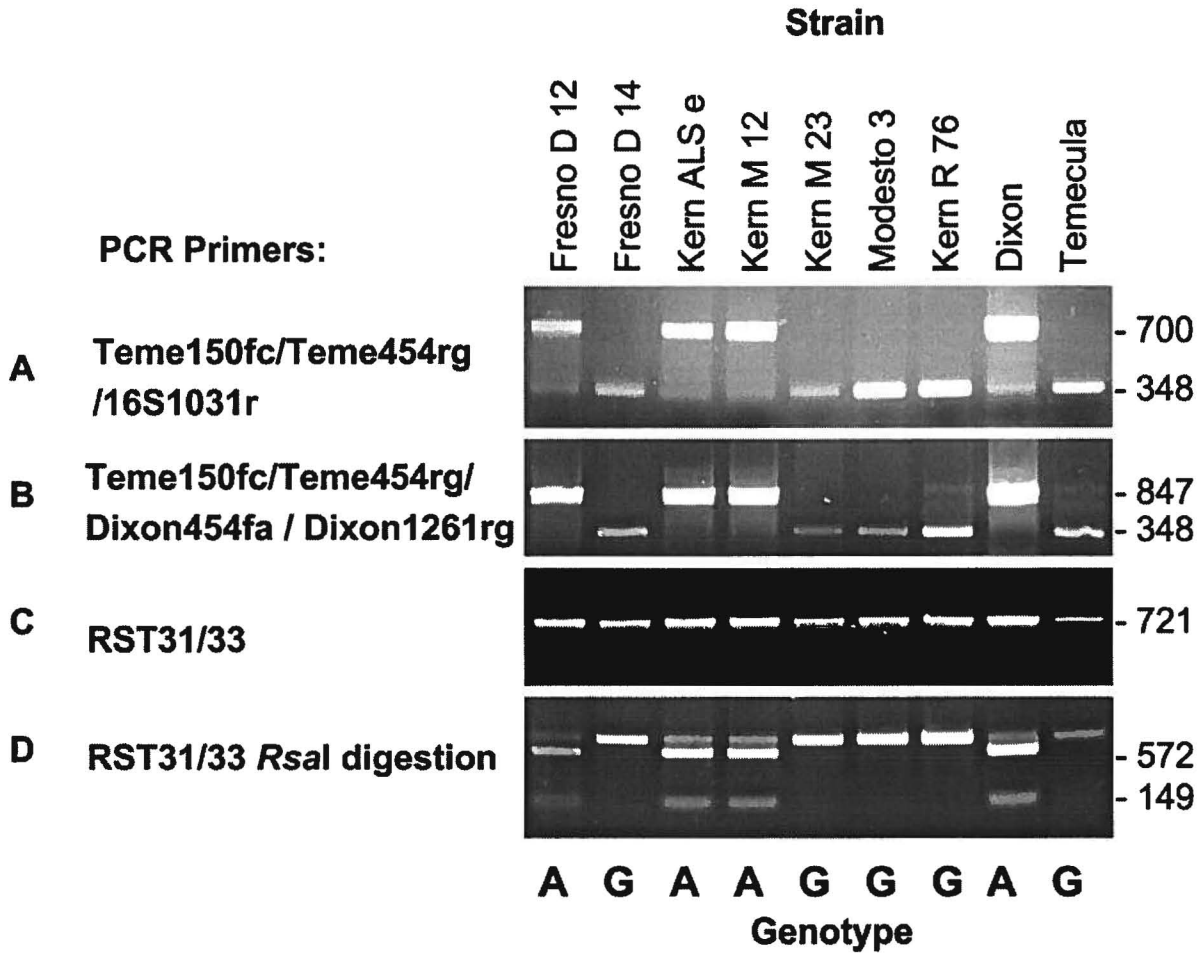

Fig. 2. Polymerase chain reaction (PCR) amplifications of almond leaf scorch isolates from San Joaquin Valley of California using the **A,** three primer format, **B,** four primer format, and **C,** RST31-RST33 primer set. Panel **D** is the *RsaI* digestion of RST31-RST33 amplicons. Numbers on the right are DNA fragment size in base pairs.

scription. It has, however, been a general perception that 16S rDNA sequences are not suitable for the classification of bacteria below the species level because of the limited amount of DNA polymorphic data. For example, four SNPs could only account for 0.25% of the 1,545-bp rDNA sequence in *X. fastidiosa*. As more genome sequences are identified, particularly those multiple coverage sequences from whole genome sequencing efforts, SNPs of high taxonomic and evolutionary value are being discovered. Therefore, SNPs in 16S rDNA are valuable for strain differentiation.

One major concern is sequence errors. In other words, the SNPs must not be an artifact. The source of sequence variations has been discussed extensively by Clayton et al. (8), which include laboratory error, biological source variations, within- and between strain variations, and interoperon differences. Many previously submitted 16S rDNA sequences in the GenBank database were possibly single passed sequences with a potentially high error rate. This study was not designed to identify the source of variations in all the rDNA sequences. Rather, our effort was to identify important SNPs based on sequences from the complete genome sequencing projects and substantiate these SNPs by extracting information from other 16S rDNA sequences of different sources, and to test these SNPs by screening a large number of strains from ALSD samples.

Our objective was to determine if both G-type and A-type or non-G-type *X. fastidiosa* strains simultaneously occurred in ALSD orchards in California. Results from the 16S rDNA SNP typing were further substantiated by RFLP analysis of the RST31-RST33 locus (Fig. 2) and by correlation with the previously undescribed colony morphology of *X. fastidiosa* ALSD strains (Fig. 3). It is also noted that multisequence comparisons identified position 1338 as an SNP separating the CVC group from other *X. fastidiosa* (data not shown), suggesting a possibility of performing a similar SNP analysis on this economically important strain in the future.

ALSD was previously reported to be caused by the same *X. fastidiosa* strain or pathotype as the one causing PD of grapevine (10,18). The report by Mircetich et al. (18) is based on vector

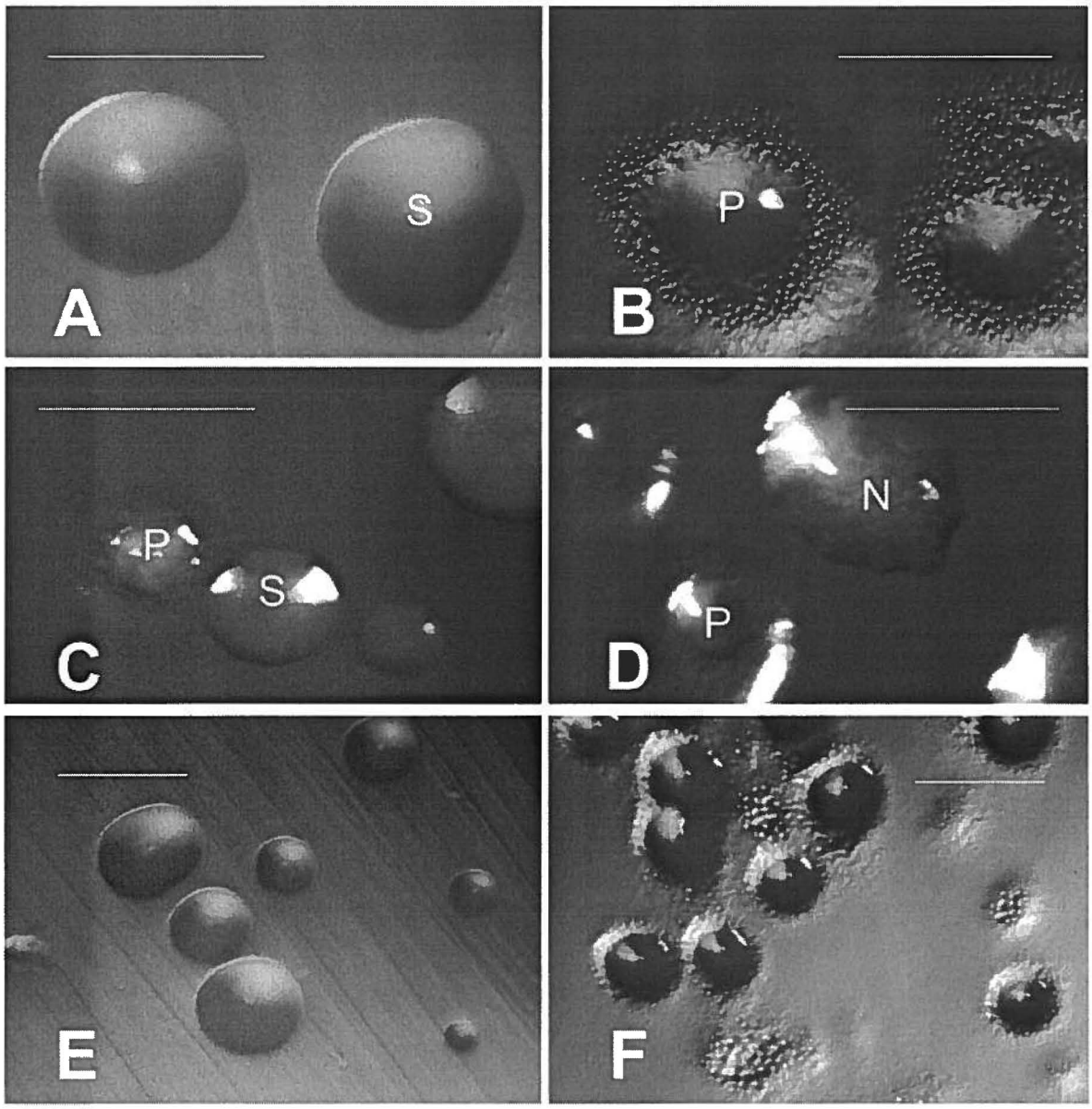

Fig. 3. Microscopic colony morphology of different *Xylella fastidiosa* genotypes isolated from almond leaf scorch disease samples. **A,** Smooth type; **B,** "pit"-like type; **C,** "pit"-like and smooth type; **D,** "pit"-like and non-"pit"-like A-type; and **E and F,** triple-cloned colonies from a single almond petiole (**E,** smooth G-type; and **F,** "pit"-like A-type). Bar scale = 0.5 mm.

transmission experiments and no strain variation information was described. Using PD2 medium, Davis et al. (10) isolated the causal agents of PD and ALSD, but strain variation was not investigated although the bacterial strains were shown to cross-infect each host. Based on pathogenicity experiments and RAPD analysis of total genomic DNA, Almeida and Purcell (2) recently reported that ALSD was in fact caused by at least two *X. fastidiosa* genotypes, a grape type and an almond type. Their data also suggested that there could be subgroups within the almond type; yet, the issue of mixed genotype infection in ALSD was not discussed.

Our data clearly indicate that at least two genotypes of *X. fastidiosa* coexisted in the ALSD samples collected from selected orchards within California. The strongest evidence comes from one ALSD survey in an orchard in Kern County in October 2003 (Table 1, orchard D). In this survey, the approximately 14.6-ha orchard was systematically evaluated for the presence of ALSD symptoms. Eighty-three symptomatic trees were identified and samples were collected accordingly. Out of the 67 isolates collected from 83 symptomatic samples, 49 were classified as A-type (73%) and the remainder classified as G-type.

Second, at least in one case, two genotypes of *X. fastidiosa* were found in the same infected leaf petiole or almond tree. Because one population was predominant over the other, the multiplex PCR in the current format could not conclusively determine the presence of both genotypes from the same culture sample. However, the simultaneous presence of two genotypes in the same orchard, plus the lack of evidence for vector specificity, prompted us to investigate the scenario of mixed genotypes in the same tree. Detailed microscopic observation of early passage culture (presumably mixed or not pure) from the same leaf petiole revealed the presence of at least two colony types of *X. fastidiosa* (Fig. 3C and D). Efforts were made to separate and triple-clone the two colony types (Fig. 3E and F), and PCR results further confirmed the association of colony types with 16S rDNA SNP types and RFLP types of the RST31-RST33 locus (data not shown).

Description of *X. fastidiosa* colony morphology has been limited. Early reports included both smooth and rough colony types on PW agar with no association to specific strains (9,29). A PWG plate is essentially the same as a PW agar plate, but agar is replaced by Gel-Rite in the former, making the medium much more transparent for microscopic observation. The "pit"-like A-type colony morphology is significantly different from the non-"pit"-like A-type (Fig. 3D, E, and F). However, only 1 out of the 49 A-type strains isolated from orchard D in Table 1 was predominantly non-"pit"-like. Further studies are needed to determine whether these two phenotypes are genetically distinct. The persistence of colony morphology in high passage subculture and different types of media also needs to be evaluated. In two previous reports (2,12), a genotype other than that of the Dixon strain was found, but no description of colony morphology was provided.

The biological implication of SNPs used in this study is not known. Yet, they can serve as valuable genetic markers for pinpointing a genotype from the bacterial population. Previous ecological discussions have implied that SNPs could lead to, or be correlated with, niche expansion that form specialized strains and even novel biological species (19,28). The combination of SNP profiles groups the 38 sequences (4,7,16,20,24,27) into four genotypes. While the variations of some strain sequences need further clarifications, the SNP grouping was, in general, consistent with the reports based on nearly complete 16S rDNA sequence analysis (7,16,20), i.e., a grape PD group, a CVC group, a group of mixed host origins including almond, and a genetically distinct group represented by a pear leaf scorch strain from Taiwan. A scheme of subspecies of *X. fastidiosa* has recently been proposed for the PD group, CVC group, and the group of mixed host origins (23). SNPs in the 16S rDNA were not used in the study. A more comprehensive SNP analysis in 16S rDNA may lead to the establishment of a standardized frame-work assisting *X. fastidiosa* subspecies grouping.

It is worth mentioning that the SNP profile of a single California ALSD strain belonged to the 16S rDNA CVC group previously reported from Brazil (20). In that report, sequence analysis from both 16S rDNA and *Gyr*B loci clustered the almond strain with CVC strains. The same almond strain was not included in this study. In an RFLP study, Chen et al. (6) grouped the same almond strain into the grape PD strain cluster. Serological tests also suggested this strain to be more related to PD strain (29). Further study is needed to clarify such differences.

In summary, we report the simultaneous presence of at least two genotypes of *X. fastidiosa* within ALSD samples collected from orchards in the San Joaquin Valley of California. The interaction of different *X. fastidiosa* genotypes and the almond host as related to disease development remains to be studied. Understanding the variations of cultivar susceptibility to different *X. fastidiosa* strains could influence the study of ALSD epidemiology and resistant germplasm collections. The existence of mixed phenotypes also addresses the need for assurance of culture purity in *X. fastidiosa* strain characterization. The method described in this manuscript can be further modified for strain quantification and in vivo detection.

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Spatial Analysis of Almond Leaf Scorch Disease in the San Joaquin Valley of California: Factors Affecting Pathogen Distribution and Spread

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ABSTRACT

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Almond leaf scorch (ALS) disease has emerged as a serious threat to almond (*Prunus amygdalus*) production areas throughout California's San Joaquin Valley. This disease is caused by the xylem-limited bacterium *Xylella fastidiosa,* and this pathogen is transmitted by xylophagous insects including sharpshooter leafhoppers (Hemiptera: Cicadellidae) and spittlebugs (Hemiptera: Cercopidae). Among four orchards surveyed, enzyme-linked immunosorbent assay (ELISA) and bacterial isolation followed by polymerase chain reaction (PCR) were equally effective in detecting *X. fastidiosa* from ALS-symptomatic trees. Disease incidence varied among almond cultivars in each orchard, with the highest mean incidence and most severe symptoms frequently encountered in 'Sonora'. *X. fastidiosa* isolates consisted of mixtures of grape or "G-genotype" and almond or "A-genotype" strains present in surveyed orchards. The *X. fastidiosa* G-genotypes characterized from each orchard were associated with the most severely affected 'Sonora' trees in three of the four orchards. Both ordinary runs and simple randomization analyses revealed aggregations of ALS in three of the four orchards. Clusters of ALS-affected trees frequently occurred in the outermost orchard rows. Plots of semivariance in ALS incidence over distance varied in shape and magnitude among cultivars. Semivariance increased over distance in 'Sonora' and 'Carmel', indicating spatial dependence or aggregations of incidence best fit by a combination of spherical and linear models. These results document both random and aggregate patterns of ALS spatial distribution in selected orchards and further illustrate how cultivar susceptibility influences the distribution patterns of ALS incidence. Following the recent introduction and establishment of the glassy-winged sharpshooter, *Homalodisca coagulata*, the impact upon the epidemiology and spread of ALS is unknown.

Additional keywords: Pierce's disease

Almond leaf scorch (ALS) disease has emerged recently as a serious threat to almond production areas throughout California's San Joaquin Valley (5,36). This disease is caused by the xylem-limited bacterium *Xylella fastidiosa,* which also causes several plant diseases in California including Pierce's disease (PD) of grape, oleander leaf scorch (OLS), and alfalfa dwarf (AD). *X. fastidiosa* bacterial strains have a diverse host range (5,8,10,30), are genetically diverse, and generally cluster within groups associated with different host species (2,6,7,22,23). Recent studies on the genetic relationships of different *X. fastidiosa* strains support the hypothesis that the bacterial species consists of more than one subspecies or pathovar (5,8,15). Knowledge of the genetic diversity of *X. fastidiosa* strains associated with ALS in the central San Joaquin Valley of California, especially as it relates to disease epidemiology, is not well understood.

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The pathogen is transmitted by xylem-feeding sharpshooters (Cicadellidae) and spittlebugs (Cercopidae) (4,18,28,29,31,33). In California, there are at least 20 species of sharpshooters or spittlebugs capable of transmitting *X. fastidiosa* (31); however, only four species are considered to be epidemiologically important in transmission of *X. fastidiosa* to grapes (18,29). The vector(s) of *X. fastidiosa* associated with ALS, however, has not been well documented. Nevertheless, some leafhopper and spittlebug species have been implicated as possible vectors of *X. fastidiosa*–ALS strains (28,29).

While *X. fastidiosa* has long been present in the San Joaquin Valley of California (24,32), the incidence of ALS appears to have emerged as a significant threat in numerous locations throughout much of the almond-producing region. This increase in ALS is reported to be widely distributed in the affected areas and often appears to be associated with large acreages of adjoining permanent pasture or irrigated alfalfa forage crops (36). Many vineyards that are recurrently affected by Pierce's disease in this region of the San Joaquin Valley are associated with similar habitats that harbor *X. fastidiosa* vectors (17,29). The spatial pattern of PD incidence in susceptible grape in these areas decreases over distance from inoculum sources and often is associated with the edges of vineyards (16,26,27). Tubajika et al. (35) recently demonstrated that infections of PD were not only aggregated in clusters of diseased vines close to field edges, but were also clustered in disease foci over successive years, suggesting that vine-to-vine, or secondary spread of *X. fastidiosa* by *Homalodisca coagulata* (Say), was an important mechanism for *X. fastidiosa* spread. In coastal California, the blue-green sharpshooter, *Graphocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae), is responsible for primary spread of the pathogen from outside inoculum sources in the early spring (14,27).

Limited information is currently available on the distribution and spread of *X. fastidiosa* infections resulting in ALS in the San Joaquin Valley of California. ALS-affected orchards were previously characterized as having few symptomatic trees, which were randomly and widely distributed throughout affected orchards without any association to known vector dispersal habits (28). In recent years, the area-wide incidence of ALS has increased in portions of the southern San Joaquin Valley of California in regions where it has not previously been documented (25,36). Analyses of the spatial patterns of ALS in these newly affected areas will provide new information regarding the relative importance of primary inoculum sources, patterns of *X. fastidiosa* movement into and among susceptible almond cultivars, and the necessity for, or epidemiological importance of, roguing ALS-affected trees.

The objectives of this study were to determine the spatial pattern of ALS incidence in managed almond orchards naturally infected with *X. fastidiosa,* to characterize the patterns of disease spread between ALS genotypes, and to describe the differential patterns of susceptibility to disease among affected almond cultivars.

MATERIALS AND METHODS

Study areas. ALS surveys were conducted at two locations each in Fresno and

Kern counties in the central and southern portions of the San Joaquin Valley of California in 2003. Fresno County orchard 1 was a 24-year-old orchard consisting of almond, *Prunus amygdalus* (Mill.) D.A. Webb (Rosaceae) 'Price', 'Norman', and 'Nonpareil', planted 9.1 m between rows and 7.9 m within rows. The planting pattern consisted of alternating, four-row blocks of cultivars arranged 'Nonpareil', 'Norman', 'Nonpareil', 'Price', respectively, and oriented in an east-west direction. In each four-row block, 'Nonpareil' represented 50% of the trees planted, while 'Norman' (25%) and 'Price' (25%) equally constituted the remainder. Orchard 2 in Fresno County was planted in 1988 and also consisted of alternating, four-row blocks of 'Nonpareil' (50%), 'Sonora' (25%), and 'Carmel' (25%) planted 6.7 m between rows and 7.9 m within rows in an east-west orientation. The two survey orchards in Fresno County were separated by approximately 9.2 km. Kern County orchards 3 and 4 were planted in 1995 and 1996, respectively, and were arranged as alternating, four-row blocks of 'Nonpareil' (50%), 'Sonora' (25%), and 'Fritz' (25%) planted 7.3 m between rows and 6.1 m within rows in a north-south orientation. Survey orchards in Kern County were separated by a distance of approximately 7.7 km.

Disease assessments. Almond leaf scorch disease incidence surveys were conducted 30 October 2003 at the two Fresno County orchards and 7 November 2003 at each of the two Kern County locations. All trees in each orchard were assessed visually for symptoms of ALS, and a symptom severity score was assigned to all trees rated on a scale of 1 to 4: (1 = asymptomatic, healthy trees; 2 = symptoms present on 1 scaffold; 3 = symptoms present on >1 scaffold; and 4 = symptoms on all scaffolds). Trees rated >1 for ALS symptoms were individually marked with colored-flagging for subsequent leaf collections and pathogen detection. Characteristic ALS symptoms often occurred at leaf tips and margins and ranged in appearance from irregular light green or gray-green regions to leaves with tan-colored, marginal scorch that included a characteristic yellow or chlorotic band separating the scorched and green leaf tissues. To confirm the presence of *X. fastidiosa* in ALS-symptomatic trees, approximately five small branch sections (ca. 12.5 cm) with symptomatic leaves were removed from each flagged tree on the date of the survey, placed in labeled plastic bags, and transported in a cooler to the laboratory in Parlier, CA. Leaf samples were stored at 4°C in labeled bags and processed for pathogen isolation and detection within 24 h. Two-dimensional maps of the spatial distribution of *X. fastidiosa*–infected trees were generated for each orchard following diagnostic confirmation.

***X. fastidiosa* culture and detection.** Double-antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) and bacterial isolation attempts followed by polymerase chain reaction (PCR) were used to detect *X. fastidiosa* from trees expressing ALS symptoms. Isolation of *X. fastidiosa* from leaf petioles onto solid media was used to confirm the presence of viable bacteria and for subsequent strain characterization. Leaf petioles were aseptically removed from leaf blades and surface-sterilized in 1% sodium hypochlorite for 2 min followed by three successive rinses in sterile, distilled water. Petioles were then subdivided into three equally spaced regions (each ca. 5 to 7.5 mg), and xylem fluid was expressed aseptically from each petiole section onto the sterile surface of a petri dish using a pair of flame-sterilized, needle-nose pliers. A 25-µl droplet of periwinkle-wilt (PW) broth was added and mixed with the expressed xylem fluid. One 5-µl inoculation loop of the expressed sap mixture was streaked onto periwinkle-wilt solid media modified with Gelrite (PWG) (19) at 28°C, incubated for a period of 10 days, and subsequently held for an additional period not exceeding 40 days. The appearance of opalescent colonies was monitored using a binocular microscope, and candidate isolates were transferred onto new PWG solid media.

Triple-cloned, single colonies were genotyped using primers designed from single nucleotide polymorphisms (SNPs) in the 16S rRNA gene of *X. fastidiosa* using a four-primer PCR format (9). Primer set Temecula 150fc (5′tctaccttatcgtgggggac3′)–Temecula 478rg (3′ccgttaaccaattatggatcaacaa5′) generated a 348-bp DNA amplicon, and primer set Dixon 454fa (5′ccttttgttggggaagaaaa3′)–Dixon 1261rg (3′ctagagcgctcccactcgat5′) produced an 847-bp amplicon. Taken together, the two PCR assays discriminated between both grape and almond genotypes of *X. fastidiosa* by generating an amplicon representative of the *X. fastidiosa* 'Temecula' or an amplicon representative of the *X. fastidiosa* 'Dixon', respectively. Briefly, DNA templates were prepared by suspending a loopful of cell culture transferred from PWG solid media into 100 µl of sterile water. PCR was carried out in a 25-µl reaction volume containing 1 µl of cell suspension (ca. 10 to 25 ng of genomic DNA template) in 1× reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$) with the addition of: 0.2 mM of dNTPs, 1 U of *Taq* DNA polymerase (TaKaRa taq, Hot Start Version, Takara Bio Inc., Otsu, Shiga, Japan), and 0.2 µM each of forward and reverse primers. DNA amplification was carried out in an MJ Research Thermocycler (Model PTC-100) programmed with an initial denature at 96°C for 10 min, followed by 30 PCR amplification cycles consisting of denaturing at 96°C for 30 s, annealing at 55°C for 30 s, with a final extension at 72°C for 30 s. A 15-µl volume of each amplification product was separated through a 1.5% agarose horizontal gel electrophoresis at 10 V cm^{-1} for 30 min in 1× TAE buffer (0.1 M Tris-HCl, pH 8.1, 0.2 M glacial acetic acid, and 2 mM EDTA). The gel was stained and visualized with ethidium bromide (0.5 mg ml^{-1}). A 100-bp DNA ladder was used as a size marker, and individual samples were scored as positive for the presence of *X. fastidiosa* if the corresponding DNA band was visualized with UV light.

In addition to bacterial isolation attempts and subsequent PCR, serological assays of petiole tissue samples collected from ALS-symptomatic trees were also conducted using a commercial DAS-ELISA kit (Agdia Inc., Elkhart, IN) according to the instructions provided by the manufacturer. From each symptomatic tree, approximately 50 to 75 mg of leaf petiole tissue was homogenized in 1× phosphate-buffered saline using a Homex 6 tissue extractor (Bioreba Inc., Reinach, Switzerland). One hundred microliters of prepared tissue was collected from each sample and dispensed into replicate wells, and all plates included both positive and negative controls. ELISA results were recorded with a Multiskan MCC/340 microplate reader (ThermoLabsystems Corp., Vantaa, Finland) at a wavelength of 490 nm. Plants were scored positive for *X. fastidiosa* if the optical density (OD) of test wells was greater than that of the mean OD of noninoculated, healthy control plants of the same plant cultivar plus 3 standard deviations.

Data analyses. Almond leaf scorch disease incidence analyses were completed using SAS, version 7 (SAS Institute, Cary, NC). Methods of *X. fastidiosa* detection including DAS-ELISA and bacterial isolation were compared within and among almond cultivars at each surveyed orchard location using a χ^2 analysis of positive ratios for ALS symptomatic tissues. Mean ALS disease incidence, ALS disease severity rating, and mean proportions of *X. fastidiosa* genotypes were compared among almond cultivars at each experimental orchard using a randomized, stripped plot analysis of variance, general linear models procedure (PROC GLM; LSMEANS) using the repeating four-row planting pattern as experimental replicates. In Fresno County, orchards 1 and 2 contained 32 and 16 rows, respectively, resulting in 8 and 4 experimental replicates; whereas orchards 3 and 4 in Kern County contained 64 and 56 rows, resulting in 16 and 14 experimental replicates. Comparisons of mean ALS disease incidence and disease severity rating were conducted after an initial $\log_{10}$ data transformation for normalization of variance and means separated using PROC GLM (LSMEANS). Comparisons of the mean proportion of *X. fastidiosa* genotypes

among almond cultivars were initially arcsine square transformed and means separated by F tests ($\alpha = 0.05$). All means presented in tables and figures were back-transformed.

For each survey location, ALS distribution was evaluated within and across cultivars using a distribution free test, or ordinary "runs" analysis, to test for clustering or a random distribution of diseased trees (21). The analyses were conducted by cultivar (within rows) and across cultivars (across rows) for each row or column on the respective disease assessment dates. The percentage of rows with clustered patterns of disease was calculated, and the location of rows or columns with clustered patterns of ALS was noted. Only ALS-symptomatic trees testing positive for the presence of *X. fastidiosa* by either DAS-ELISA or bacterial isolation on solid media were used in this and the following distribution analyses.

To test for "edge effects" in the distribution of ALS-affected trees, a simple permutation procedure was constructed (34). Each orchard was first subdivided into equal-sized quadrants consisting of 4 adjacent rows (all cultivars represented) of 4 trees per row. A new variable (I_d) was then generated for each quadrant as the sum of the observed disease incidence plus one (I_n + 1) multiplied by the distance (d_m) in meters from a particular field edge. Next, disease incidence data were randomly rearranged and quadrant products summed for 500 separate iterations relative to each of the four field edges in each orchard. The null hypothesis evaluated in this test, that ALS incidence is randomly distributed within an affected orchard and not associated with a particular field border, can then be rejected if the true test statistic (ΣI_d) is less than 95% of the random values generated for each respective field border. Tests were constructed and analyzed by cultivar individually and across cultivars simultaneously relative to field borders.

Geostatistical analysis of ALS within fields. Latitude and longitude coordinates of the four experimental orchards initially were transformed into plane coordinates using a Universal Transverse Mercator (UTM) projection in ArcView, Geographic Information Systems (GIS), Version 3.3 (Environmental Systems Research Institute, Redlands, CA). The UTM coordinates were used to conduct empirical semivariogram analyses on the spatial patterns of ALS incidence, disease severity, and *X. fastidiosa* genotype in each orchard and among cultivar within orchards. Plots of modeled covariance lines were calculated as:

$$\gamma(h) = \left[1\left(2\left|n(h)\right|\right)^{-1}\right]\sum_{i=1}^{n(h)}(z_i - z_{i+h})^2$$

and represent the average of squared differences in values z between grid cells i and j separated by a lag distance h. The plot of $\gamma(h(j))$ against distance h over all directions results in an omnidirectional semivariogram that typically increases in value with increasing distance (11,37). Parameters used to characterize the semivariogram plot include the nugget, which represents the value of $\gamma(h)$ at $h = 0$ or measurement error; the range, or sample distance between points beyond which little or no autocorrelation among variables occurs; and the sill, which corresponds to the overall variance for data greater than the range. Semivariogram shapes were fit to circular, exponential, linear, and spherical models using a nonlinear, least squares optimization weighted by the number of distance pairs. The best model fit was selected based upon the lowest error mean square value. The degree of anisotrophy, or directional spatial dependence, was examined for ALS incidence, disease severity rating, and genotype within rows (zero degrees azimuth) and across rows at directions of 22.5, 45, 67.5, 90, 112.5, 135, and 157.5° azimuth.

RESULTS

***X. fastidiosa* detection.** *X. fastidiosa* was diagnosed in each of the four orchards surveyed in Fresno and Kern counties where ALS symptoms were observed. Averaging over the four surveyed orchards, DAS-ELISA and bacterial isolation on solid media were equally effective (χ^2 = 0.47, df = 1, P = 0.7339) in detecting *X. fastidiosa* from ALS-symptomatic trees, averaging 67.3% (134/199 attempts) and 56.7% (94/166 attempts) positive detection, respectively. In Fresno County orchard 1, *X. fastidiosa* was detected more frequently by DAS-ELISA than by bacterial isolation in almond cultivars 'Price' and 'Norman' (χ^2 = 8.904, df = 1, P = 0.0094; χ^2 = 6.77, df = 1, P = 0.0188, respectively), whereas no differences in detection frequencies were observed in 'Nonpareil' (χ^2 = 2.66, df = 1, P = 0.2013) (Table 1). In Fresno County orchard 2, both methods were equally efficient in detecting *X. fastidiosa* within 'Sonora' (χ^2 = 2.14, df = 1, P = 0.2891) and 'Nonpareil' (χ^2 = 2.37, df = 1, P = 0.2331), and again in Kern County orchard 3, detection frequencies were similar among the three cultivars, 'Sonora', 'Fritz', and 'Nonpareil' (χ^2 = 1.75, df = 1, P = 0.3818; χ^2 = 1.86, df = 1, P = 0.3218; χ^2 = 1.19, df = 1, P = 0.4539, respectively) (Table 1). In Kern County orchard 4, both methods were comparable for 'Sonora' (χ^2 = 1.24, df = 1, P = 0.4011) and 'Fritz' (χ^2 = 5.18, df = 1, P = 0.1318) (Table 1), and no "Nonpareil' trees were observed with ALS symptoms. Three asymptomatic trees of each almond cultivar were selected randomly from each orchard for serological analyses and isolation on solid media. Neither detection method yielded a positive assay for the presence of *X. fastidiosa* among 36 samples collected from asymptomatic almond cultivars over all survey locations (χ^2 = 0.0, df = 1, P = 1).

Disease assessments. Incidence of ALS disease varied among cultivars in each of four surveyed orchards in Fresno and Kern counties in California. Among 709 trees in Fresno County orchard 1, 32 trees had characteristic ALS symptoms. Combining the results of both ELISA and bacterial isolation, *X. fastidiosa* was confirmed in 19 (1.6%) symptomatic trees. Incidence varied more (F = 8.77, df = 2, 14, P = 0.0110) among cultivars with higher disease incidence ('Norman' [2.2%] and

Table 1. Comparative frequencies of *Xylella fastidiosa* detection in almond leaf scorch–symptomatic cultivars using double-antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) and isolation on solid periwinkle wilt (PW) media

County	Location	Cultivar	DAS-ELISA[w]	*X. fastidiosa* isolation[x] (no.+/total)
Fresno	Orchard 1	'Price'	8/14 (57.1%) a[y]	4/14 (28.6%) b
		'Norman'	6/14 (42.9%) a	3/14 (21.4%) b
		'Nonpareil'	3/4 (75.0%) a	2/4 (50.0%) a
	Orchard 2	'Sonora'	11/16 (68.8%) a	9/16 (56.3%) a
		'Carmel'	21/26 (80.7%)	N/A[z]
		'Nonpareil'	6/11 (54.6%) a	4/11 (36.4%) a
Kern	Orchard 3	'Sonora'	45/62 (72.6%) a	49/62 (79.0%) a
		'Fritz'	2/5 (40.0%) a	1/5 (20.0%) a
		'Nonpareil'	20/30 (66.7%) a	17/30 (56.7%) a
	Orchard 4	'Sonora'	8/11 (72.7%) a	7/11 (63.6%) a
		'Fritz'	4/6 (66.7%) a	2/6 (33.3%) a
		'Nonpareil'	0	0

[w] Optical densities at 492 nm ranged between 0.108 and 0.577 in *X. fastidiosa*–infected tissue and between 0.059 to 0.088 in asymptomatic, healthy tissue. Plant tissue was considered *X. fastidiosa*–infected if the optical density of test wells exceeded the mean optical density of asymptomatic controls of the same plant variety plus 3 standard deviations.

[x] Early passage, single colonies isolated on PW solid media were confirmed as *X. fastidiosa* using primers designed from single nucleotide polymorphisms in the 16S rRNA gene.

[y] Detection frequencies not followed by the same lowercase letter within rows are significantly different, while frequencies not followed by the same uppercase letter in columns by orchard are significantly different by χ^2 analyses ($\alpha = 0.05$).

[z] N/A: not available; petiole samples were not collected from 'Carmel' for bacterial isolation assays and are not reported.

'Price' [2.9%]) than in 'Nonpareil' (0.5%) (Fig. 1A). Similarly, the proportion of *X. fastidiosa*–infected trees classified in three disease severity categories differed among cultivars ($F = 8.03$, df = 4, 23, $P = 0.0099$). Affected 'Norman' trees were the least severely affected (rating = 2), while nearly all 'Price' trees had ALS severity ratings 3 (50%) and 4 (43%). The few 'Nonpareil' trees were distributed nearly equally among the three disease rating categories. In Fresno County orchard 2, 53 trees exhibited ALS symptoms. *X. fastidiosa* was confirmed in 41 (5.8%) of the symptomatic trees, and disease incidence differed ($F = 7.81$, df = 2, 8, $P = 0.0378$) among cultivars, with the highest incidence in 'Carmel' followed by 'Sonora' and 'Nonpareil', averaging 10.5, 6.5, and 1.6%, respectively (Fig. 1B). The proportion of *X. fastidiosa*–infected trees in each severity category did not differ ($F = 3.82$, df = 4, 13, $P = 0.0773$) among almond cultivars in orchard 2. Nearly all 'Nonpareil' trees (>90.0%) were rated in the least severe category (disease rating = 2).

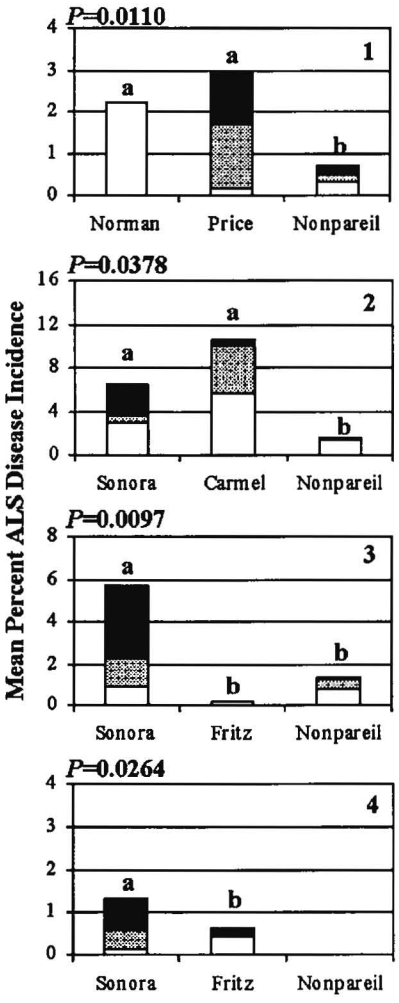

Fig. 1. Mean incidence of almond leaf scorch (ALS) among almond cultivars in orchards in Fresno (1 and 2) and Kern (3 and 4) counties in California illustrating the proportion of ALS-affected trees in each disease category: (open bar = R2, symptoms present on 1 scaffold; gray bar = R3, symptoms present on >1 scaffold; and black bar = R4, symptoms on all scaffolds). Probabilities of a difference in mean ALS incidence among almond cultivars at each orchard are provided ($P = 0.05$) (PROC GLM). Column means by variety with dissimilar letters are significantly different by PROC GLM, LSMEANS ($\alpha = 0.05$).

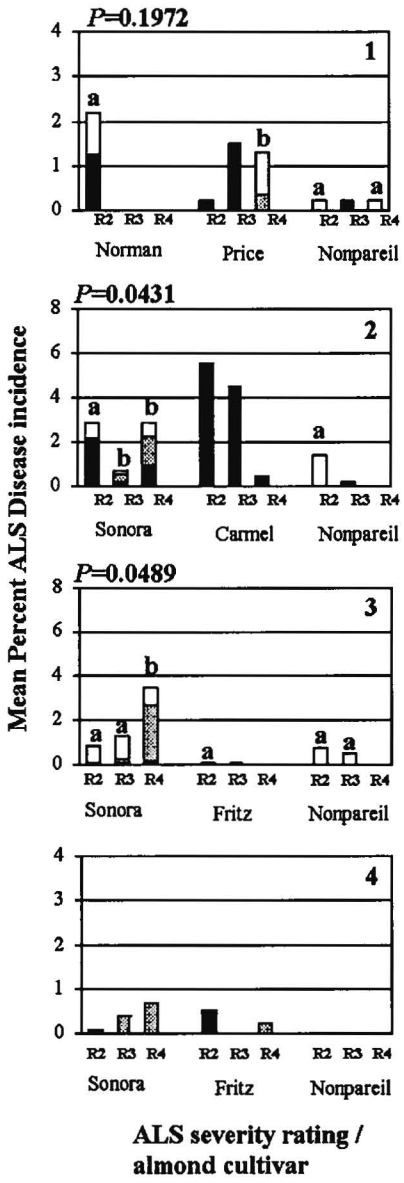

Fig. 2. Mean incidence of almond leaf scorch (ALS) within almond cultivars in orchards in Fresno (1 and 2) and Kern (3 and 4) counties in California illustrating the proportion of A-genotypes and G-genotypes of *Xylella fastidiosa* (open bar = A-type; gray bar = G-type; and black bar = unclassified) in each disease category (R2 = symptoms present on 1 scaffold; R3 = symptoms present on >1 scaffold; and R4 = symptoms on all scaffolds). Probabilities of a difference in the proportion of *X. fastidiosa* genotypes among cultivars are provided ($P = 0.05$) (PROC GLM). Column means by orchard with dissimilar letters contain significantly different *X. fastidiosa* genotype proportions by PROC GLM, LSMEANS ($\alpha = 0.05$).

In Kern County orchard 3, 97 trees possessed characteristic ALS symptoms, from which *X. fastidiosa* was confirmed in 75 (2.1%) trees. Incidence among cultivars differed ($F = 10.09$, df = 2, 28, $P = 0.0097$), with the highest incidence recorded in 'Sonora' (5.7%) followed by 'Nonpareil' (1.3%) and 'Fritz' (0.2%) (Fig. 1C). The proportion of trees classified in each severity category varied among cultivars ($F = 13.66$, df = 4, 26, $P = 0.0103$), with 62% of all 'Sonora' classified in the highest disease rating category and the remaining trees rated in categories 2 (15%) and 3 (23%). All *X. fastidiosa*–infected 'Fritz' trees surveyed were rated in the least severe category, whereas the majority (>90%) of 'Nonpareil' rated in severity categories 2 (61%) and 3 (36%). In Kern County orchard 4, 17 trees exhibited characteristic ALS symptoms from which *X. fastidiosa* was confirmed in 13 trees (0.5%). Incidence of *X. fastidiosa* differed ($F = 5.09$, df = 2, 26, $P = 0.0264$) among cultivars (Fig. 1D), with the highest incidence observed in cultivar 'Sonora' (1.3%) followed by 'Fritz' (0.6%). No ALS-symptomatic trees were observed in 'Nonpareil' at orchard 4. The proportion of *X. fastidiosa*–infected trees in each disease severity category did not differ between cultivars ($F = 3.61$, df = 1, 26, $P < 0.1954$) 'Sonora' and 'Fritz'.

***X. fastidiosa* genotype relationship to ALS incidence.** Mixtures of *X. fastidiosa* genotypes were observed in three of the four surveyed orchards. Almond genotypes (A-genotype) were the most prevalent (70%) among positive isolations ($N = 55$), whereas only 30% ($N = 23$) were documented as grape-genotypes (G-genotype). In Fresno County orchard 1, all positive isolations obtained from cultivars 'Norman' ($N = 3$) and 'Nonpareil' ($N = 2$) were A-genotypes, whereas one of four 'Price' isolates was G-genotype (Fig. 2A). No differences ($F = 4.76$, df = 1, 2, $P = 0.1972$) in genotype proportions were observed among almond cultivars at this location. In orchard 2, the proportion of *X. fastidiosa* genotypes varied ($F = 322.68$, df = 1, 2, $P = 0.0327$) among almond cultivars (Fig. 2B). Only A-genotypes were associated with 'Nonpareil', whereas 'Sonora' contained a mixture of both A- ($N = 5$) and G-genotypes ($N = 4$). Within 'Sonora' alone, genotype proportions varied ($F = 9.84$, df = 1, 4, $P = 0.0231$) among severity ratings where all symptomatic trees rated 2 were characterized as A-genotype and 50 and 67% of trees rated 3 and 4 were classified as G-genotypes, respectively. In Kern County, a mixture of G- ($N = 18$) and A-genotypes ($N = 49$) occurred in orchard 3, and the relative proportion of both genotypes differed ($F = 167.83$, df = 1, 2, $P = 0.0489$) among cultivars (Fig. 2C). All 18 G-genotypes (27%)

occurred in 'Sonora', whereas only A-genotypes were isolated from both 'Fritz' (N = 1) and 'Nonpareil' (N = 17). In 'Sonora', the proportion of genotypes again differed (F = 8.13, df = 1, 12, P = 0.0109) among severity ratings. In orchard 4, all positive *X. fastidiosa* isolations were characterized as G-genotypes in 'Sonora' (N = 9) and 'Fritz' (N = 2) (Fig. 2D).

Spatial patterns of ALS. Ordinary runs analyses revealed aggregations of *X. fastidiosa*–infected trees in three of the four almond orchards surveyed in 2003. Among the four orchards surveyed, the proportion of disease aggregates within rows of specific cultivars was determined to be greater than the proportion of aggregates across rows. In survey orchard 1, the proportion of rows with significant (α = 0.05) aggregations ranged between 0.12 and 0.06 with clusters detected in 'Price' and 'Norman', respectively (Table 2). The clustering of *X. fastidiosa*–affected trees in 'Price' was present in two orchard rows (rows = 25, 29) and in 'Norman' in only a single row (row = 27). All aggregates were located within five of the outermost rows of the surveyed orchard along the orchard's southern border (Fig. 3A). No significant aggregations of diseased trees were detected within rows of 'Nonpareil', which had the lowest overall incidence, nor were any disease clusters noted across rows. In orchard 2, the frequency of occurrence of *X. fastidiosa* clusters within rows ranged between 0.20 and 0.40 for 'Sonora' and 'Carmel', respectively (Table 2). Clusters of affected 'Sonora' trees were detected in only a single row (row = 3), which corresponded to the outermost row oriented parallel to the northern field boundary (Fig. 3B). Disease aggregates within rows of 'Carmel' were also marginally distributed and associated with outer orchard rows parallel to both northern (row = 14) and southern (row = 2) boundaries (Fig. 3B). Significant aggregations of *X. fastidiosa*–infected trees across rows were observed in the two outermost rows (rows = 1, 2) along the orchard's western boundary (Table 2).

Clusters of *X. fastidiosa*–infected trees were detected along field boundaries in Kern County orchard 3. In 'Nonpareil' and 'Sonora', the frequency of rows containing disease clusters ranged between 0.04 and 0.24, respectively (Table 2). Disease aggregates within rows included the first four 'Sonora' rows parallel to the western field boundary (rows = 1, 5, 9, 13) and only a single 'Nonpareil' row (row = 20) located closer to the field interior. No significant aggregations of diseased trees were observed within rows of 'Fritz'. Across row aggregations of *X. fastidiosa*–infected trees were detected among four of the six southernmost border rows (rows = 59, 60, 61, 62) in Kern County orchard 3 (Fig. 3C). Orchard 4 in Kern County had the lowest overall ALS incidence among each of the four selected orchard locations, and no within or across row clusters of diseased trees were detected.

The spatial patterns of *X. fastidiosa*–infected trees relative to specific orchard boundaries were investigated further using a simple permutation, or randomization procedure. Similar to the results obtained from the ordinary runs analysis, a significant edge effect was observed in 'Price' in 22 out of 500 (P = 0.044) iterations of the approximate randomization procedure associated with the southern orchard boundary in Fresno orchard 1 adjoining currently nonirrigated, fallow land (Fig. 3A). In Fresno orchard 2, the true ALS test statistic for 'Sonora' was significantly different (P = 0.03) among 13 of 500 randomized estimates relative to the eastern orchard boundary, which adjoined approximately 2.8 ha of irrigated, permanent pasture (Fig. 3B). At the same orchard, 27 of 500 approximate randomizations resulted in a significant (P = 0.05) western edge association observed in 'Carmel', which adjoined a larger (ca. 22.8 ha) set of subdivided, irrigated permanent pasture blocks (Fig. 3B). Diseased trees were marginally distributed in Kern County orchard 3, where 25 of 500 iterations of the randomization procedure resulted in a significant (P = 0.05) edge effect in 'Nonpareil' associated with the southern border of the orchard adjacent to irrigated alfalfa (Fig. 3C). No significant edge effects were observed in orchard 4 of Kern County using the approximate randomization procedure. Including all almond cultivars simultaneously in the randomization, no significant ALS edge effects were observed relative to orchard boundaries at any of the four survey orchards.

Geostatistical analysis of ALS distribution. Plots of semivariance in ALS incidence over distance varied in shape and magnitude among cultivars individually and across cultivars simultaneously at each of the four survey locations (Fig. 4). In Fresno County orchard 2, where ALS incidence was highest (6.5%), semivariance increased over distance in 'Sonora' and 'Carmel', indicating spatial aggregations of ALS incidence (Fig. 4B). The shape of the 'Sonora' semivariogram was best fit by a spherical model with an estimated sill and effective range value of 7.42 and 39.17 m, respectively (Table 3). A linear model best fit the 'Carmel' semivariance plot with a lower estimated sill value of 4.91 and an increasing, nonzero slope (P = 0.04). Averaging over all cultivars simultaneously, semivariance increased over distance and was best fit to a spherical model with an estimated sill of 2.04 and an effective range of 27.57 m (Table 3). No detectable spatial dependence was observed in 'Nonpareil' in orchard 2. Spatial aggregations of ALS incidence were observed in the cultivar 'Sonora' and across all cultivars simultaneously in Kern County orchard 3 (Fig. 4C). In both instances, spherical models best approximated the semivariance plots over distance with sill estimates of 3.07 and 2.09, respectively, and corresponding range values of 31.88 and 19.65 m (Table 3). No spatial dependence in ALS incidence was observed at either of the remaining orchards (1 and 4) in Fresno and Kern counties where the lowest mean disease incidences were recorded (1.9 and 0.6%, respectively) (Table 3). Because no directional variograms differed from omnidirectional semivariance plots with respect

Table 2. Ordinary runs analysis of almond leaf scorch (ALS) disease aggregations in selected almond orchards of Fresno and Kern counties, CA

County	Orchard	Cultivar	ALS incidence	Proportion of tests with significant Z value (aggregate rows of ALS-infected trees)	
				Within rows[y]	Across rows[z]
Fresno	Orchard 1	'Price'	0.029	0.12 (25, 29)	
		'Norman'	0.022	0.06 (27)	0.00
		'Nonpareil'	0.005	0.00	
	Orchard 2	'Sonora'	0.065	0.20 (4)	
		'Carmel'	0.105	0.40 (2, 14)	0.04 (1, 2)
		'Nonpareil'	0.016	0.00	
Kern	Orchard 3	'Sonora'	0.057	0.24 (1, 5, 9, 13)	
		'Fritz'	0.002	0.00	0.06 (59, 60, 61, 62)
		'Nonpareil'	0.013	0.04 (20)	
	Orchard 4	'Sonora'	0.013	0.00	
		'Fritz'	0.006	0.00	0.00
		'Nonpareil'	0.000	...	

[y] Reported values are the proportion of tests with significant aggregations of ALS-affected trees by almond cultivar within rows (α = 0.05). Parenthetical values indicate the within-row position(s) where significant ALS aggregations were observed. Almond cultivars in Fresno County orchards (1 and 2) were planted in a (west-east) orientation with ascending row numbers (north–south) and ascending across-row columns (west-east). Kern County orchards (3 and 4) were planted in a (north-south) orientation with corresponding ascending row numbers (west-east) and ascending across-row columns (north-south).

[z] Reported values are the proportion of tests with significant aggregations averaging among cultivars across rows (α = 0.05). Parenthetical values indicate across-row position(s) of significant ALS aggregations.

to ALS incidence, only omnidirectional plots are illustrated.

Few spatial aggregates of ALS severity rating and *X. fastidiosa* genotype were detected among the four orchards surveyed. In orchards 2 and 3 of Fresno and Kern counties, where ALS incidence was greatest, spatial dependence of *X. fastidiosa*–infected 'Sonora' trees rated in the highest disease severity category (rating = 4) was observed. Semivariance plots of these 'Sonora' trees over distance were best fit to spherical and linear models at orchards 2 and 3, respectively (Table 3). Semivariance gradually increased at orchard 2 to an estimated sill value of 0.49 with a corresponding range estimate of 24.77 m. In orchard 3, a linear model best fit the steadily increasing semivariance over distance ($P = 0.0239$) and possessed a higher estimated sill value of 1.43 (Fig. 5). Spatial dependence among *X. fastidiosa* G-genotypes was observed at orchard 3 in Kern County, whereas no corresponding A-genotype aggregations were detected (Fig. 6). Specifically, semivariance plots of the less frequently occurring G-genotype of *X. fastidiosa* were best fit to an exponential model where semivariance rose sharply to an estimated sill value of 0.98 with an estimated range not exceeding 18.33 m (Table 3). Directional plots of semivariance did not differ from omnidirectional plots and are not illustrated for either ALS symptom severity or *X. fastidiosa* genotype.

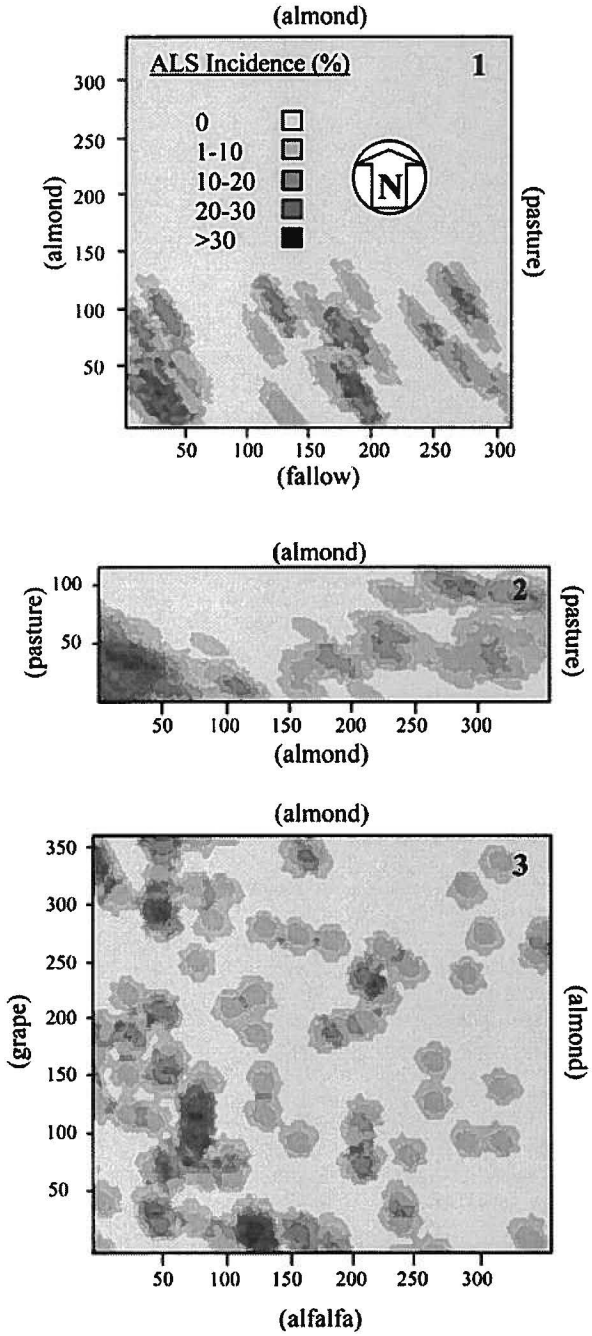

Fig. 3. Distribution of almond leaf scorch (ALS) incidence using universal kriging interpolations illustrating adjoining land uses at survey orchards in Fresno (1 and 2) and Kern (3) counties in California.

DISCUSSION

X. fastidiosa was isolated regularly from leaf petioles of trees exhibiting ALS symptoms in each of the surveyed orchards. Detection frequencies were similar using ELISA and bacterial isolation techniques with the exception of only a single survey location where methods differed somewhat between two of the cultivars surveyed. Such differences may result more from *X. fastidiosa* distribution patterns within affected almond trees than from the relative sensitivity or efficiency of either assay. Although little is known about the colonization patterns of *X. fastidiosa* in almond, recent work by Almeida and Purcell (4) demonstrated that bacterial populations in experimental and naturally infected almond were 10- to 100-fold lower than populations in susceptible grape. They proposed that xylem vessel structure may, in part, limit the uniform distribution of the pathogen within the trees, and prolonged infections may be required for widely disseminated infections to result. Similarly, Mircetich et al. (24) observed that the colonization rates (10 to 15%) of xylem vessels in affected almond were considerably less in comparison to infected grape, where nearly 20% of vessels were occluded.

Differences in ALS susceptibility among almond cultivars have been observed previously (25,32). Historically, incidence was most severe in cultivars 'Long IXL', 'IXL', and 'Jordanolo' with characteristic foliar leaf scorching symptoms, stem pitting on many secondary branches, and a large proportion of dead spurs followed by dieback of terminal growth. More recently, additional almond cultivars have been added to a growing list of ALS-susceptible cultivars and include 'Sonora', 'Peerless', and an increasing number of affected 'Nonpareil' (36). The incidence of ALS in other cultivars, including 'Butte', 'Mission', 'Aldrich', and Padre', has been reported to be rare or very infrequent (25,32). In the present study, differences in ALS incidence and severity were observed among the cultivars examined. Hopkins (20) demonstrated that virulence in Pierce's disease strains of *X. fastidiosa* was associated with increased bacterial populations. More recently, it has been determined that for grape strains of *X. fastidiosa* that were pathogenic to both grapes and almonds, the number and distribution of living bacterial cells within plants can be influenced greatly by host plant species (3,12). In a recent study, Almeida and Purcell (4) observed variable populations of *X. fastidiosa* in ALS-affected trees among

five field locations sampled in 2002 ranging between 1.3×10^5 and 9.5×10^7, although no information was provided about the particular cultivars evaluated. In our study, variations in ALS incidence and disease severity among cultivars may be influenced by differences in bacterial populations present in each almond cultivar.

Observed differences in ALS incidence among cultivars may be linked partially to infection by specific *X. fastidiosa* genotypes. In our study, the largest proportion of *X. fastidiosa*–infected trees was observed in 'Sonora' ($N = 73$). Among these infected trees, over 41% ($N = 30$) were infected by the G-genotype of *X. fastidiosa*. Earlier surveys of ALS throughout central and northern portions of California's Central Valley reported the disease to be only sporadic and widely distributed (28), and the emergence of the disease into southern portions of the Central Valley has been considered quite recent (5,36). Moreover, the occurrence of grape genotypes isolated from susceptible almond in this region of the San Joaquin Valley was originally reported to be only a rare occurrence due to accidental infections by infective vectors (28). Our field survey results illustrate that G-genotypes of *X. fastidiosa* were encountered regularly in orchards within the region and may help to explain the observed increase in disease incidence among susceptible cultivars.

The distribution and abundance of ALS in affected almond orchards of California has not been well documented and has previously been characterized as often low in overall incidence with few symptomatic trees scattered randomly or in patches in a manner inconsistent with the dispersal habits of known insect vectors (4). Further, disease progress within affected orchards was characterized as slow, impacting the productivity of individual orchards in 10 to 15 years (24,32). The spatial distribution patterns and movement of *X. fastidiosa* resulting in Pierce's disease of susceptible grapes has been well documented in California (18,27,35) and the southeastern United States. (1). Patterns of PD distribution in the north coast vineyards of California result from immigrating populations of the blue-green sharpshooter, *G. atropunctata* (27). In this important wine grape region of California, Pierce's disease incidence has been described as marginally distributed along field borders or riparian buffers resulting primarily from the spread of *X. fastidiosa* into vineyards from outside inoculum sources with only very limited secondary spread of the pathogen. In

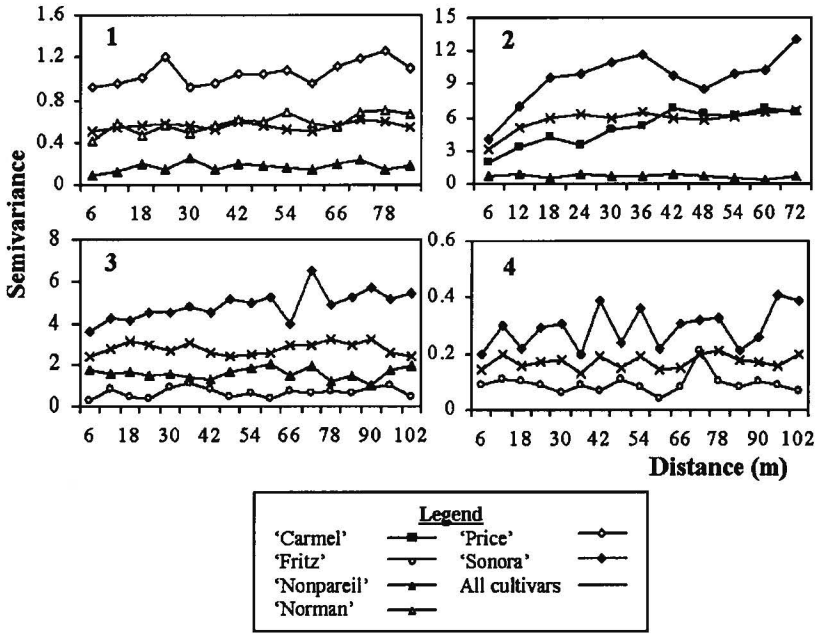

Fig. 4. Semivariogram plots of almond leaf scorch incidence in Fresno (1 and 2) and Kern (3 and 4) county survey orchards depicting spatial dependence among all almond cultivars and each cultivar independently.

Table 3. Semivariogram attributes and selected model parameters of almond leaf scorch (ALS) disease incidence, severity rating, and *Xylella fastidiosa* genotype in surveyed almond orchards of Fresno and Kern counties in California, 2003

Variable	County	Orchard	Almond cultivar	Model[w]	Semivariogram parameters[v]			R^2	P^x
					C_0	C_1	a (m)		
Incidence	Fresno	1	'Price'	Linear	0.97	1.08	...	0.79	0.1338
			'Norman'	Linear	0.44	0.52	...	0.88	0.1901
			'Nonpareil'	Linear	0.19	0.23	...	0.93	0.5387
			All	Linear	0.52	0.58	...	0.90	0.6991
		2	'Sonora'	Spherical	2.91	7.42	39.17	0.85	...
			'Carmel'	Linear	1.83	4.91	...	0.84	0.0421
			'Nonpareil'	Linear	0.54	0.69	...	0.91	0.6188
			All	Spherical	2.04	5.52	27.57	0.77	...
	Kern	3	'Sonora'	Spherical	3.07	4.27	31.88	0.69	...
			'Fritz'	Linear	0.61	0.67	...	0.86	0.0976
			'Nonpareil'	Linear	1.63	1.55	...	0.81	0.4713
			All	Spherical	2.09	2.41	19.65	0.64	...
		4	'Sonora'	Linear	0.26	0.28	...	0.79	0.2279
			'Fritz'	Linear	0.1	0.11	...	0.83	0.7345
			All	Linear	0.15	0.16	...	0.90	0.5557
Rating[y]	Fresno	2	'Sonora'	Spherical	0.11	0.48	30.72	0.89	...
	Kern	3	'Sonora'	Linear	0.28	1.47	...	0.85	0.0239
Genotype[z]	Kern	3	'Sonora'	Exponential	0.09	0.98	18.33	0.81	...

[v] C_0 = experimental error or the semivariance at the $\gamma(h)$ intercept (lag distance = 0); C_1 = estimated sill value or covariance estimate which remains unchanged with increasing distance (h); a = range value or distance (h) beyond which covariance remains unchanged.

[w] Omnidirectional, semivariance plots fit to the best linear or nonlinear model using a weighted, least-squared analysis and model selection based on lowest error mean square.

[x] Overall F test probability values ($\alpha = 0.05$) for H_o: slope = 0.

[y] Omnidirectional semivariance plots for ALS severity rated trees classified as rating = 4.

[z] Omnidirectional semivariance plots for ALS G-genotypes.

Florida, where the highly mobile and polyphagous glassy-winged (*H. coagulata*) and black-winged (*Oncometopia nigricans* (Walker)) sharpshooters are native and ubiquitous, vine-to-vine, or within-field secondary spread of *X. fastidiosa* among grapes, occurs regularly throughout the growing season (1). Following the recent establishment of *H. coagulata* in portions of the southern San Joaquin Valley, the spatial patterns of PD incidence were described as nonrandom or occurring as large, elongate clusters of infected vines indicative of patterns of secondary spread that reflected the feeding patterns of the newly introduced vector (35).

In this study, the spatial patterns of ALS incidence, disease severity, and *X. fastidiosa* genotype were nonrandomly distributed in only a portion of the almond orchards surveyed. The existence and degree of spatial dependence was influenced further by the particular almond cultivar evaluated. These differences may result from one or a combination of factors including dissimilar rates of overwinter survival, seasonal variations in bacterial populations, and feeding preference by xylophagous insect vector(s) (13,14,19). Our findings illustrate that disease incidence was greater in 'Sonora' and 'Carmel' than in the remaining cultivars, which were presumably less susceptible to or conversely more tolerant of infection by *X. fastidiosa*. Using geostatistics to illustrate the distribution of *X. fastidiosa* genotype, only G-genotypes of affected 'Sonora' trees at Kern County orchard 3 were observed to be clustered, while the remaining A-genotypes were randomly distributed (Fig. 6A and B). This is the first documented concomitant occurrence of multiple *X. fastidiosa* genotypes present in the same almond orchard. Moreover, with the diagnosis of PD in grapes adjoining orchard 3 (results not illustrated), these results further document the simultaneous association of both diseases in southern portions of the San Joaquin Valley where previously this occurrence was not reported. Almeida and Purcell (5) hypothesized that the rare cases of ALS in these areas were due to accidental infections by infective vectors emigrating from grape into susceptible almond. This pathway may help to partially explain the G-genotype aggregations observed in this study. However, further investigations are warranted to better understand the *X. fastidiosa* genotypes present within alfalfa, which occurs regularly in this region and flanked orchard 3 (and the adjoining PD-affected grape) on two borders.

The principal insect vectors of ALS have not been clearly identified in areas where this disease has been problematic historically, although presumably xylophagous sharpshooters responsible for movement of PD into grapes (28,29) and numerous spittle bugs (33) are probable vectors. Our findings indicate that clusters of diseased trees were associated commonly with field borders adjoining habitats known to support populations of potential vectors. Primary spread of *X. fastidiosa* from outside inoculum sources would lead initially to random patterns of infected plants, which may or may not be followed by tree-to-tree movement, or secondary spread of the pathogen, resulting in disease clusters or foci. Over multiple seasons, successive waves of primary spread may account for the spatial patterns of ALS observed in our study where clusters of infected trees were often associated with field borders. Detailed surveys over successive seasons will be necessary to better understand the temporal patterns of ALS progress and the extent of secondary spread that may in fact occur. The occurrence and recent increase in incidence of ALS in the southern San Joaquin Valley of California does not directly reflect the introduction and establishment of the newly introduced vector, *H. coagulata*. This recently established vector species may, however, dramatically change the epidemiology of ALS in the future by exposing almonds to a more diverse population of *X. fastidiosa* and increasing the likelihood of secondary spread within orchards in mid- to late-summer when bacterial populations are highest (4).

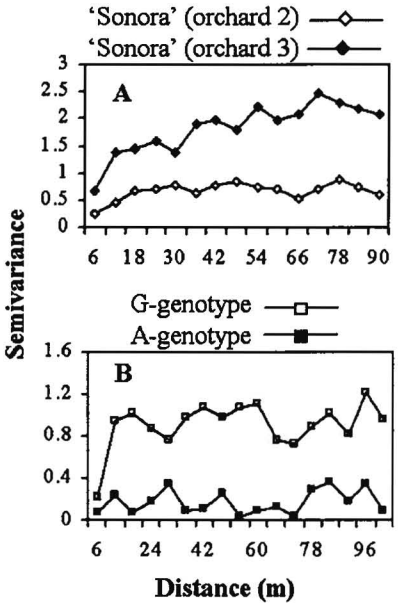

Fig. 5. Semivariogram plots of almond leaf scorch severity level = 4 within 'Sonora' in Fresno and Kern county survey orchards 2 and 3, respectively (**A**) and plots of *Xylella fastidiosa* genotypes within 'Sonora' in Kern County survey orchard 3 (**B**).

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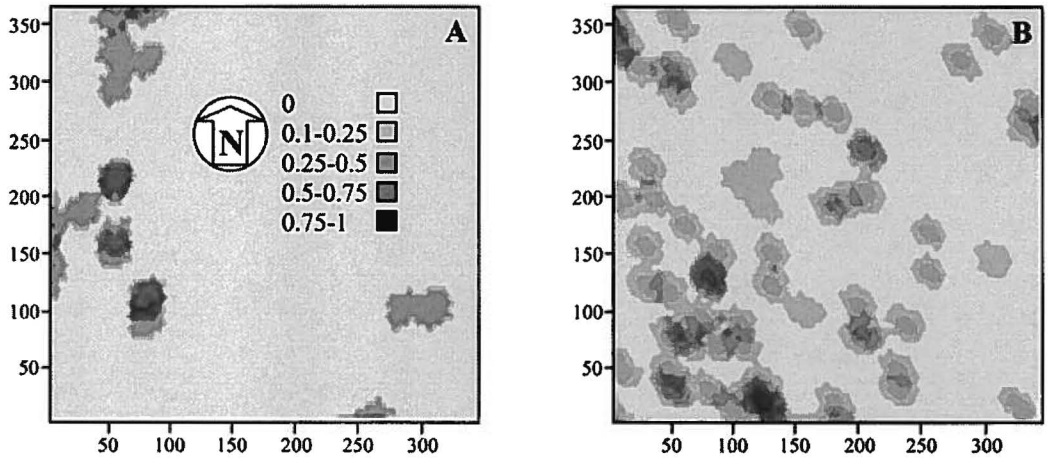

Fig. 6. Distribution of *Xylella fastidiosa* G-genotypes (**A**) and A-genotypes (**B**) using indicator kriging interpolations in Kern County survey orchard 3.

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