Occurrence and movement of *Xylella fastidiosa* strains causing almond leaf scorch from neighboring vegetation into almonds

Kent M. Daane,[1] Elaine B. Shapland,[1] Glenn Y. Yokota,[1] Christina Wistrom,[1] Joseph H. Connell,[2] Roger A. Duncan,[3] and Mario A. Viveros[4]

[1] Department of Environmental Science, Policy and Management, University of California, Berkeley; [2] University of California Cooperative Extension, Modesto, CA; [3] University of California Cooperative Extension, Oroville, CA; [4] University of California Cooperative Extension, Bakersfield CA

Research Objectives:

1. Characterize the *X. fastidiosa* "strains" (e.g., ALS strain, PD strain) found in almond leaf scorch samples and compare them with strains from nearby "alternate" host plants (e.g. grape, mustards, ornamental plum).

2. Collect insects in ALS-infected orchards and nearby vegetation, and the conduct laboratory analyses determine whether or not they carry *Xf.*

3. Conduct transmission experiments to determine vector efficiency.

4. Collect regional data on ALS epidemiology with respect to orchard management (e.g., irrigation practices) and the surrounding environmental conditions (e.g., nearby crop plantings) (see corresponding proposal).

Overview

Our research in the 2004 crop season covered a wide range of almond leaf scorch (ALS) related studies. Most of the work concerned the establishment or movement of *Xylella fastidiosa* in or near almond orchards. *Xylella fastidiosa* is a xylem-limited bacterium that causes ALS, Pierce's disease (PD) of grapevines, and other diseases. In this report, we focus on one aspect of this research – the ground cover (weeds) vegetation that may act as a reservoir for the bacteria. We surveyed ground vegetation in ALS-infected almond orchards in California's Central Valley. Plant tissue samples were collected throughout a 2 year period and processed for *X. fastidiosa* presence using restriction enzyme digestion of RST31-RST33 polymerase chain reaction (PCR) products and bacterial culture on selective media. Overall disease incidence was low in the ground vegetation species, only 63 of 1369 samples tested positive. Of the 37 species of common ground vegetation tested, 11 tested positive for *X. fastidiosa*, including such common species as Sheperd's purse (*Capsella bursa-pastoris*), filaree (*Erodium* spp.), cheeseweed (*Malva parvifolia*), burclover (*Medicago polymorpha*), annual bluegrass (*Poa annua*) London rocket (*Sisymbrium irio*), chickweed (*Stellaria media*). There was a seasonal component to bacterial presence, with positive samples found only between November and March. Both ground vegetation and almond trees were most commonly infected with the almond strain of *X. fastidiosa* (6 of 7 surveyed sites). ALS-infected almond samples had a *X. fastidiosa* concentration within reported ranges (1.84×10^6 - 2.15×10^7

CFU/g), however, we were unable to accurately measure *X. fastidiosa* titer in sampled ground vegetation for comparison. These results are discussed with respect to ground vegetation management for ALS control.

Introduction

Pierce's disease of grapes and almond leaf scorch are incurable plant diseases that threaten the profitable production of these crops in California. Both diseases are caused by the xylem limited, nutritionally fastidious bacterium *Xylella fastidiosa* (7,22,32,35). Leaf damage occurs when the bacteria grow to such high concentrations that water and nutrient transport systems become occluded, leading to water stress in the leaves (24). Leaf and stem damage can progressively worsen until photosynthesis and nutrient production are impaired, thus lowering the quality and quantity of fruit produced and, eventually, killing susceptible grape or almond cultivars (23). *Xylella fastidiosa* is also the causal agent of diseases in other crops, including citrus variegated chlorosis, oleander leaf scorch, plum leaf scald, alfalfa dwarf, and phony peach (4,28,29,30,31).

In California, much research has been conducted on the epidemiology of Pierce's disease (PD). Early surveys for the causal agent determined that the pathogen was spread by xylem-feeding sharpshooters, such as the bluegreen sharpshooter (*Graphocephala atropunctata*) (12,15,16). Subsequent studies confirmed that these and xylem-feeding hemipterans can acquire and transmit *X. fastidiosa* via their normal feeding on the xylem tissues (18,27,32). More recent studies have focused on PD epidemiology as influenced by the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say) (32,33). As important as these insect vectors is the plants that harbor *X. fastidiosa*. Surveys, in and near vineyards with PD in northern California and Florida, found numerous plants that can harbor *X. fastidiosa* in their tissues, without outward expression of susceptibility (30Nome et al. 1980; 6, 19). Similar surveys for the bacteria in plants growing near citrus groves with citrus variegated chlorosis have been conducted in Brazil (20).

PD and ALS epidemiology requires more than the presence of susceptible crop varieties, insect vectors, and plant species that host *X. fastidiosa* and are suitable for feeding and/or breeding by these insect vectors. For example, after *X. fastidiosa* is acquired by a host plant, expression of disease symptoms depends on many factors including the plant's natural antibiotics (26), temperature (9), season (10), the *X. fastidiosa* strain (1, 5, 13), and *X. fastidiosa* concentration (2, 18). For this reason, a reservoir population of *X. fastidiosa* can reside in and around grape or almond orchards without the outward expression of plant disease. For disease epidemiology, these unseen reservoirs of the bacterial pathogen, in ground vegetation or cash crops, increases the likelihood that nearby susceptible crops will become infected. Removal of *X. fastidiosa* reservoir vegetation has, in fact, been an effective methods for controlling the spread of PD in California's coastal wine grape regions (26). As yet, there have not been similar studies of vegetation management for controlling the spread of ALS, which has been increasing in prevalence and severity in California's interior valleys. Currently, there is a lack of information about the epidemiology of *X. fastidiosa* reservoir vegetation in and around almond orchards, and whether this system closely parallels or is different from that in grapes.

The purpose of this study was to survey vegetation in northern and central California almond orchards, which report increased incidence of ALS, in order to determine the possible reservoir hosts of *X. fastidiosa* At each survey site, we documented the ground vegetation species present throughout the season, determined the presence or absence, concentration, and strain of *X. fastidiosa* in the sampled ground vegetation, and recorded the disease incidence and *X. fastidiosa* strain in almond trees near harvest-time. By identifying the seasonal presence and incidence of *X. fastidiosa* in common ground vegetation in or near almond orchards, weed control efforts can appended to also reduce reservoir *X. fastidiosa* host species and reduce the level of bacterial inoculum.

MATERIALS AND METHODS

Ground vegetation survey. Sample sites were selected based on grower reports of ALS disease incidence. Surveyed orchards included sites in the north Central Valley (Butte County, Glenn County), the middle of the Central Valley (Stanislaus County) and the south Central Valley (Kern County). Every 2-6 weeks, depending on the seasonal availability of ground vegetation, a visual survey for the four most abundant weed species was conducted in each orchard. After the common species were determined, samplers transected the length of four evenly spaced rows (300 – 400 m per row) and, in each transect, collected specimens (3 – 5 leaves) of each weed species, which were stored separately for each species and transect in a 3.8 liter plastic bag. Typically, 10 – 30 individual plants were sampled for each weed species in each transect. The collected material was stored in a cooler (ca. 7°C) and transported to the laboratory, where samples were processed within 2 days of field collection.

Bacteria presence. Each sample (plant species and orchard row) was processed separately for the presence of *X. fastidiosa*. We selected plant parts where sharpshooters are known to feed, such as the leaf petioles, succulent base of grasses, and plant stems (11). Subsamples of these plant sections, from each sample, were removed with a sterilized razor, as described by Hill and Purcell (18). The samples were assayed for the presence of *X. fastidiosa* using immunocapture DNA separation and PCR amplification with primers RST31-RST33, using a procedure developed by Hendson et. al (14).

For PCR amplification, all samples were prepared in sterile 0.6 mL micro centrifuge tubes with 12.5µL Taq master mix (Qiagen), 6.5µL PCR water, 1µL of each primer RST-31 and RST-33, and 4µL of DNA extract. PCR reactions were carried out in a Thermal Cycler according to the conditions described by Minsavage, et al. (21). PCR products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide and viewed under ultraviolet light. The presence of *X. fastidiosa* in the original sample was determined by a band at 733 kb (21).

Bacteria strain. After gel electrophoresis, a preliminary strain difference analysis was carried out according to Minsavage, et al. (21). The PCR product from all positive samples was subjected to restriction enzyme digestion with *Rsa*1 (10 µL PCR product, 0.2 µL *Rsa*1, 0.2 µL BSA, 2.0 µL Buffer C, 7.6 µL water) at 37°C for 2 hours. The RST31-RST33 PCR products from *X. fastidiosa* strains of oak, oleander, peach, plum, and all but three ALS strains are cleaved into two fragments, about 233 bp and 500 bp, while the PCR products from all PD strains as well as the ALS strains ALS1, Manteca, and Tulare are not digested by *Rsa*1 (14).

Bacteria titer and incidence. Attempts were made to culture *X. fastidiosa* from all weed samples collected as well as from symptomatic almond trees in each orchard. For weed species, location and sample date shown to be positive for the presence of *X. fastidiosa*, using immunocapture DNA separation and PCR amplification, fresh samples were collected. Similarly, near harvest-time (September) we surveyed each orchard for trees showing classical symptoms of ALS (22) and collected symptomatic almond leaf and petiole samples. Both ground vegetation and almond samples were processed for bacterial culture on selective media within 24 hours.. We used a series of culture medias to provide a rudimentary indication of *X. fastidiosa* strain. Grape strains of *X. fastidiosa* grow on both PWG (17) and PD3 (8), but almond strains grow only on PWG (1). Samples were prepared for culture according to procedures described by Hill and Purcell (17,18). In this way we could determine both the strain and titer of bacteria in the tree.

Data analysis. Results for *X. fastidiosa* presence in vegetative ground cover species are presented as a qualitative "positive" or "negative" to express the potential role of these plant species as alternative hosts.

Average bacterial concentration in media cultures is presented as CFU/g, with average concentrations among vineyards compared using Analysis of Variance, and means separated using Tukey's HSD comparison. Linear regression was used to compare the percentage positive *X. fastidiosa* in ground vegetation species with sample size for each plant species.

RESULTS

Ground vegetation Survey. From June 2003 to April 2005, 58 collection trips were made, about 10 trips to each of the six sampled orchards. There were 37 species of ground vegetation commonly found (Table 1), with most material collected in winter and spring when ground vegetation was common. Between August and October, it was difficult to find live ground vegetation within the almond orchards, a result of almond management practices for applied water and harvest operations. During this period, fewer weed species were available to sample and we often pooled samples across the orchard rows transected to produce a single sample for each plant species and orchard.

Bacteria presence. We processed 1369 samples from the six orchards, from which 63 samples were positive for *X. fastidiosa* (4.6%). Of the 37 species of common ground vegetation, we recovered *X. fastidiosa* from 11 species, including 5 species from which it had not previously been recovered in the field (Table 1). There was a strong seasonal component to bacterial presence in ground vegetation, with no *X. fastidiosa* positive samples found between April and mid-October during the two years of the study. Standard orchard management practices require the ground under the almond trees to be completely free of vegetation prior to harvest in August and applied irrigation is also discontinued, so that the nuts can be shaken from the trees and dried on the bare ground. Because all orchards followed these practices, there was little or no vegetation to be sampled from August to October (Figure 1, data from both years are combined).

Bacteria presence and strain. Results from both restriction enzyme digestion of RST31-RST33 PCR products with *Rsa*1 and bacterial culture on selective media showed that almond trees in 6 of 7 experimental orchards were infected with the almond strain of *X. fastidiosa*. At one site (Zeering Rd in Stanislaus County), a grape strain of *X. fastidiosa* was isolated from all weeds and almond trees sampled (Table 2). At each site, tissue samples from both almond trees and surrounding weeds gave the same result: each contained either grape or almond strain of *X. fastidiosa*, but never both.

Bacteria titer and incidence. Petioles from ALS infected almond trees at Zeering Rd containing the grape strain of *X. fastidiosa* had an average concentration of 2.15×10^6 CFU/g, which is significantly greater than the concentrations at other sites sampled ($P = 0.014$). Our results agree with previous findings that the average *X. fastidiosa* titer in ALS-symptomatic almond leaves (2) is lower than the average *X. fastidiosa* titer in PD symptomatic grapes (18). The goal of assessing the titer of *X. fastidiosa* in weeds was met with much frustration, as every sample was contaminated with other bacteria before *X. fastidiosa* presence could be determined (results not shown). Previous researchers have also encountered this difficulty when attempting to culture such a slow growing bacteria from field samples (20,36).

DISCUSSION

All previous field surveys for *X. fastidiosa* in alternate host plants have focused on PD management. With the recent increase of ALS in California, there was an even greater need to survey plants in almond orchards for *X. fastidiosa*. We showed the presence of *X. fastidiosa* grape and almond strains, which are the causal agents for ALS, were present in 29.7% of the common ground vegetation species sampled. Numerous studies have documented the survival of *X. fastidiosa* in different plant species (3,26,36). However, fewer have included field surveys (but see 6,19,31), and this is the first reported survey for the

season-long incidence of *X. fastidiosa* in non-symptomatic host plants in almond orchards. In comparison, one of the first and more extensive plant surveys as yet undertaken, looked at the ability of insect vectors to transfer *X. fastidiosa* (at the time unknown) from PD-infected grapes to suspected host plants to clean grapes (11). Despite the broad range of host plants included in that survey, conclusions about disease transmission were made based solely on symptom development in the clean grape plant. Subsequent studies have shown that plants can harbor *X. fastidiosa* at concentrations high enough for insect acquisition and transmission, but without exhibiting symptoms of disease (26). In addition, the symptoms of PD and alfalfa dwarf that Freitag (11) used as diagnostics can be misidentified with water or nutrient stress (22).

We used the more sensitive detection method of immunocapture DNA separation followed by PCR to survey for *X. fastidiosa* in vegetation in almond orchards for two years. In another recent field survey, riparian plants in the Napa River Valley were processed for *X. fastidiosa*, using ELISA as a detection method, and found a number of perennial trees and shrubs, as well as ground covers, were positive for the bacterium (31). Costa et al. (6) also used ELISA as a detection method, due to its low cost and lower time investment than PCR, to screen large numbers of potential host plants for *X. fastidiosa* in vineyard ground covers. While immunocapture DNA separation and PCR are more sensitive detection methods for finding *X. fastidiosa* in plant material than is ELISA (21), all established modern DNA detection methods are reliable for finding *X. fastidiosa* in plant material. Results from our survey found *X. fastidiosa* in many of the same ground cover species as the Costa et al. (6) field survey and Wistrom and Purcell (36) glasshouse study (Table 1).

The sampled habitat in our study (almond fields in the Central Valley) provided clear differences from the earlier surveys (vineyards and riparian aeas). Similarities between almond and grape crops include winter dormancy, late summer harvest, and overlap in growing regions. However, differences in the management of these crops result in very different ground management, which could result in different patterns of disease spread. For example, the rows between grapevines are often seeded with annual or perennial ground covers to improve the quality of the soil, or to serve as a trap crop for insect pests. This vegetation is left to grow the whole year, with only occasional mowing required for worker mobility. In contrast, in mid-summer the almond floor is striped of all vegetation in preparation for the August harvest. The trees are shaken to drop the nuts onto the ground, where they dry in the sun for 2 weeks before being collected with a vacuum. This practice is reflected in our survey results, which are based on vegetation collected during all months except for August, when no vegetation was available to be collected. Other differences arise from the shape and size of the crop plants. Grapes are planted in discrete rows, where adjacent vines often intertwine. Insects of all types can easily travel from one plant to anther, which may account for much of the observed vine to vine spread in insect vectored diseases of grape. Almonds are planted in a grid pattern, with trunks separated by 3 to 6 meters. If adjacent plants do touch, it is in the canopy, high off the ground and less accessible to any insect vectors feeding on ground vegetation. This arrangement could be one explanation of the lack of obvious tree-to-tree spread that we observed in the surveyed almond orchards (Daane and Shapland, unpublished).

An interesting result is that of the *X. fastidiosa* positive plant species in our survey, 9 of the 11 were present in the orchards on most of the sampling dates and thus comprised the largest sample sizes of all ground vegetation species. There was a positive and significant relationship between the number of samples taken per plant species and the percentage of samples positive for *X. fastidiosa* ($y = 0.0553x - 0.2074$, $r^2 = 0.8935$). One explanation is that insect vectors that prefer the more commonly found plant species have moved the bacterium amongst these plants. In contrast, insect vectors that prefer the less common ground vegetation species, at least in our sampled orchards, were not attracted to these sites and, therefore, did not move the bacterium among plants. For example, some of the less common plant species in the sampled orchards, were common hosts of *X. fastidiosa* in other surveys, but were negative in our two year survey (Table 1). Thus the feeding behavior of these insects could be a more important factor in

controlling the spread of ALS. We are currently conducting transmission tests in the greenhouse, using insects found via sweep net surveys in these same orchards.

Perhaps most important for the almond-vector-pathogen epidemiological relationship for ALS and resident ground vegetation is that we detected *X. fastidiosa* in weeds only during the cooler months, between October and April. This is in contrast to most previous field surveys that were conducted primarily during the growing season and in which *X. fastidiosa* was detected during the warmer summer months (6,11,20,36). Seasonality and temperature is important for ALS or PD epidemilogy as *X. fastidiosa* survives best in the plants at a moderate temperature (9) and plants inoculated on leaf tissue late in the growing season may not develop chronic disease symptoms (10). We hypothesize that the ground vegetation in the surveyed orchards best harbored *X. fastidiosa* at a temperature that was most consistent during the winter months, and when these fall/winter ground covers were newly formed and in good condition. During the late spring and summer months, most ground vegetation in the almond orchard, in contrast to seeded cover crops in vineyards, were small and in poor condition. These results suggest further investigation of the seasonal presence and concentration of *X. fastidiosa* in ground covers with the seasonal presence and abundance of potential insect vectors. Unlike in vineyards where a clear edge effect has been found with PD incidence (26), most previous work has not revealed any clear spatial patterns with ALS (25, but see 13).

We found the almond strain of *X. fastidiosa* was most common in the surveyed ALS-infected orchards. Recent studies on the biology of different strains of *X. fastidiosa* have shown varying abilities to infect different hosts: grape strains will cause disease symptoms in almond, grape (1), and oleander (28), but neither almond strains (1,2) nor oleander strains (28) will cause disease in grape. A recent study near Fresno, California, showed that characteristics of different varietals of almonds as well as strain type (almond or grape) result in differing severity of ALS (13). A parallel study found both the almond and grape genotypes of *X. fastidiosa* in the same plant, pointing out the presence of a less virulent strain does not preclude the existence of a more virulent strain (5). We found the highest concentration of *X. fastidiosa* in almond petioles containing the grape strain, with the average titer significantly larger than at other sites ($p<0.014$). This is consistent with previous findings that grape strain can colonize almond petioles to a higher extent than can *X. fastidiosa* of the almond strain.

The survey results showed that common ground vegetation can harbor *X. fastidiosa* on the almond floor. Together these results suggest that perhaps a year-long weed management strategy over the whole orchard would be an important component, along with rouging infected trees or tree limbs, for management of *X. fastidiosa*.

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Table 1: Weeds and alternate hosts collected from almond orchards and processed for the presence of *Xylella fastidiosa* using immunocapture DNA extraction and PCR.

Scientific Name	Common Name	*X.fastidosa* This study	*X.fastidosa* Previous studies	Reference
Amaranthus spp.	Pigweed	-	-	6
			+	36*
Amsinckia spp.	Fiddleneck	-	-	6
Anagallis arvensis	Scarlet pimpernel	-	-	6
Avena fatua	Wild oat	-	+	11
Brassicaceae spp.	Mustards	-	+	6
Capsella bursa-pastoris	Shepherd's purse	+	None	
Chamaesyce maculate	Spotted spurge	-	None	
Chenopodium album	Lambsquarter	-	None	
Claytonia perfoliata	Miner's lettuce	-	+	30,31
Conyza bonariensis	Fleabane	-	None	
Conyza canadiensus	Horseweed	-	+	36*
Coronopus didymus	Lesser swinecress	-	None	
Cyperus esculentus	Yellow nutsedge	-	+	11
Echinochloa crus-galli	Barnyard grass	-	+	17* 36*
			+	
			+	11*
Erodium spp.	Filaree	+	-	6
			+	36*
Escallonia montevidensis	Escallonia	-	+	11
Festuca spp.	Fescue grass	-	None	
Helianthus spp.	Sunflower	-	+	6*
Hordeum murinium	Hare barley	-	+	11
Lactuca serriola	Prickly lettuce	-	-	30,31
			+	36*
Malva parvifolia	Cheeseweed	+	-	11*
			-	6
Medicago polymorpha	Burclover	+	+	11
Poa annua	Annual bluegrass	+	+	11
Portulaca oleracea	Common purselane	-	-	11*
			+	36*
Ranunculus spp.	Buttercup	-	None	
Rumex crispus	Curly dock	-	+	11
Salsola tragus	Russian thistle	-	None	
Senecio vulgaris	Common groundsel	+	None	
Sisymbrium irio	London Rocket	+	None	
Sonchus oleraceus	Annual sowthistle	-	+	36*
Sonchus spp.	Sowthistle	+	-	6
Sorghum halepense	Johnson grass	-	+	11*
			-	30,31
Stellaria media	Chickweed	+	None	
Typha spp	Cat tail	-	None	
Urtica urens	Burning nettle	+	None	
Veronica persica	Speedwell	+	None	
Xanthium strumarium	Cocklebur	-	+	36*

Results are compared against previous field surveys near vineyards and riparian areas, except for references marked * which refer to greenhouse studies.

Site (County)	Strain in almonds	Strain in ground vegetation	*X. fastidiosa* titer[2]
Butte	Almond	Almond	1.84×10^6 ab
Butte	Almond	Almond	2.30×10^6 b
Glenn	Almond	Almond	1.19×10^7 a
Glenn	--	Almond	N/A
Stanislaus	Grape	Grape	2.15×10^7 c
Stanislaus	Almond	Almond	6.27×10^6 ab
Kern	Almond	Almond	1.08×10^7 a

[1] Results based on PCR and restriction enzyme digestion with RsaI.
[2] Average CFU/g cultured from ALS symptomatic petioles, July 27 and October 14, 2004.
[3] Different letters after each mean indicate a significance difference ($P < .05$), Tukey's pairwise comparison.

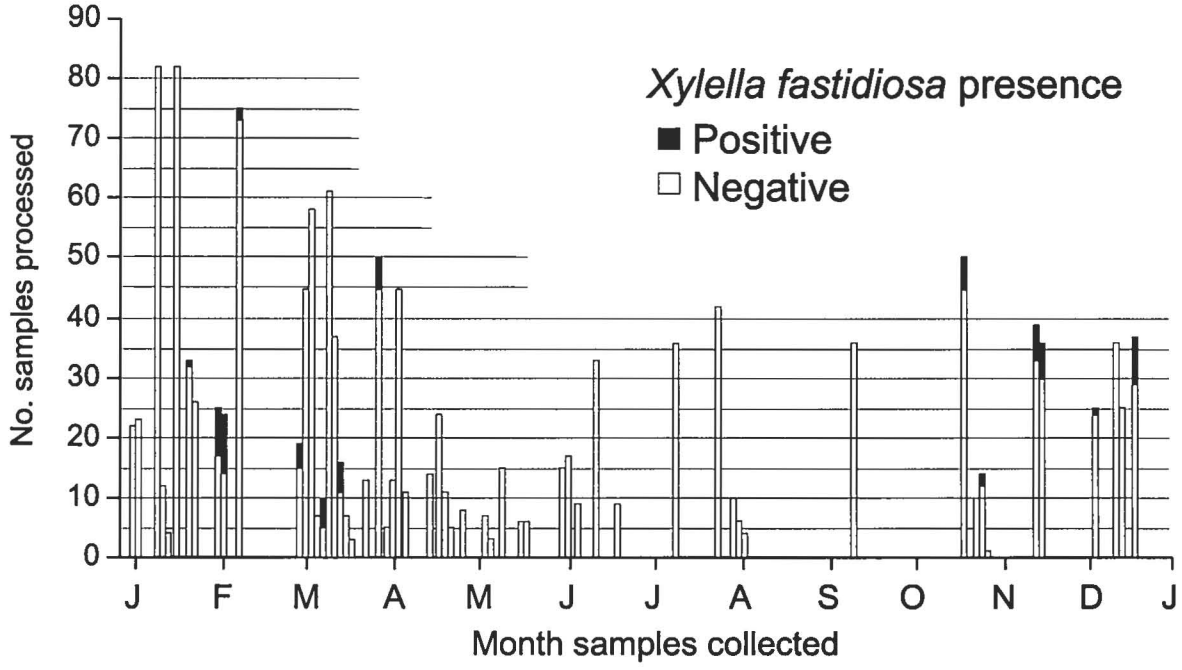

Figure 1: Survey of vegetation in almond orchards for *X. fastidiosa*. Data show results from six almond orchards in Butte, Glenn, Stanislaus, and Kern Counties from June 2003 to April 2005.