Influence of Inoculation Date on the Colonization of *Xylella fastidiosa* and the Persistence of Almond Leaf Scorch Disease Among Almond Cultivars

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Abstract

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Field grown 2-year-old almond trees (*Prunus dulcis* cvs. Butte, Carmel, Mission, Ne Plus Ultra, Padre, Peerless, Price, Solano, Sonora, and Thompson) were mechanically inoculated with *Xylella fastidiosa* in the growing seasons of 2002 and 2003 to study the effect of inoculation date on the movement and colonization of *X. fastidiosa* and the overwintering persistence of almond leaf scorch disease (ALS) in these cultivars. *X. fastidiosa* was inoculated into the base of current-season growing shoots in April, May, June, July, August, September, and October. Almond trees inoculated in spring months developed more ALS-symptomatic leaves and more extensive within-plant spread of *X. fastidiosa* by the end of the current growing season compared with trees inoculated in July, August, September, and October. Trees inoculated in June developed the most severe ALS symptoms during the season in which they were inoculated. Trees inoculated in June and July 2002 had significantly higher disease ratings in 2003 than inoculations made in August and October 2002. Based on disease ratings observed in 2003, 1 year after inoculation, Sonora and Solano were the most ALS

susceptible, Mission and Price intermediate, and Carmel, Padre, Ne Plus Ultra, Butte, Peerless, and Thompson were the least susceptible cultivars for allowing *X. fastidiosa* to overwinter and cause disease the following year. Assessment of all trees in August 2004 indicated that trees inoculated in June and July 2002 had a significantly higher amount of ALS-infected branches than trees inoculated in other months. Butte, Carmel, Padre, and Thompson cultivars had no symptomatic branches, while *X. fastidiosa* infections persisted or colonized new branches in Sonora, Solano, Peerless, Price, Mission, and Ne Plus Ultra. Based on the 2004 assessment, Sonora was the most susceptible cultivar. Surveys of a diseased orchard in Chico, CA showed large differences in ALS incidence in four almond cultivars. Nonpareil and Peerless had significantly greater incidence of disease than Butte and Carmel over the 2 years surveyed. These data suggest that cultivar susceptibility and the time of *X. fastidiosa* infection are important factors in determining the persistence of ALS in almond trees.

Almond leaf scorch (ALS) is caused by *Xylella fastidiosa* Wells et al. (32), a gram-negative, xylem-limited, and insect-transmitted bacterium that also causes diseases in many other agriculturally important plants, including peach, grapevine, citrus, coffee, and plum (1,4-6,11,14,17), and urban trees and shrubs such as elm, sycamore, oak, maple, and oleander (25,27). ALS was first noticed on a few randomly scattered trees in the Antelope Valley of Los Angeles County in the early 1950s and shortly afterward in Contra Costa County in California. ALS, which was also previously referred to as "almond decline" or "golden death" (21), devastated several commercial almond orchards in Los Angeles County from 1966 to 1976, and it was observed in 10 different almond cultivars that represent 86% of the total almond acreage in California (20). The most characteristic symptom of ALS is leaf scorching followed by decreased productivity, general decline, and subsequent death of affected trees within a few years (20). A recent study demonstrated that ALS infection can significantly decrease nut yields in Sonora and Nonpareil (28), the two major cultivars that are cultivated in many California almond orchards.

X. fastidiosa is transmitted by xylem-sap-feeding insects such as sharpshooter leafhoppers and spittlebugs (26), and rarely by propagation practices such as budding and grafting (20). Mechanical inoculations of some ALS strains of *X. fastidiosa* into grape caused Pierce's disease (PD), and vice versa (2,6). However, almond orchards with ALS often occur adjacent to healthy vineyards, and

almond orchards adjacent to grape vineyards with PD usually do not have ALS (22). Molecular phylogenetic comparisons showed that most almond strains formed two groups separate from the grape strains. Some almond strains fell within the grape group but no grape strains were included in either almond group (2,3,12). Almeida and Purcell (2) reported that the almond genetic grouping isolates did not cause PD in inoculated grape but systemically infected grape with lower frequency and populations than grape strains. Isolates able to cause both PD and ALS developed 10-fold higher bacterial concentrations in grape than in almond. In the greenhouse, the grape isolates overwintered with higher efficiency in grape than in almond and the almond isolates overwintered better in almond than in grape (2).

Feil and coworkers (7) studied the effects of inoculation date on the within-plant movement and overwintering persistence of *X. fastidiosa* in grapevines. They found that *X. fastidiosa* inoculated in April and May persisted at higher percentages after the subsequent winter than inoculations made from June, July, and August. However, less is known about the ability of *X. fastidiosa* to overwinter in various almond cultivars (3). Several studies showed that grapevines infected with *X. fastidiosa* may emerge pathogen free following exposure to comparatively severe winter temperatures (7,23,24). However, such cold temperatures are not required for some almond cultivars infected with *X. fastidiosa* to emerge pathogen free after completing winter dormancy (15; this study).

In the current study, we tested the hypothesis that early-season infections of *X. fastidiosa* persist at a higher rate over the subsequent winter than late-season infections using 10 almond cultivars. The susceptibility of the 10 almond cultivars to *X. fastidiosa* infection was evaluated and the within-plant movement of *X. fastidiosa* was compared across the 10 almond cultivars. Surveys of ALS incidence and persistence in an almond orchard were conducted in 2002 and 2003 in Chico, CA in order to better un-

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derstand ALS epidemiology and varietal susceptibility in commercial orchards.

Materials and Methods

Bacterial strains, culture conditions, and inoculations.

Strains of *X. fastidiosa*, Fetzner, Temecula, and Dixon were grown either in liquid PD3 (4) or PW media (5) at 28°C. Strain Fetzner was originally isolated from grapevine in Napa, and strain Dixon was isolated from almond in Solano County, CA (12). Strain Temecula was isolated in 1998 from a naturally infected grapevine in Temecula, CA (31). Long-term storage was at -70°C in PD3 broth including 15% glycerol for Fetzner and Temecula, and in PW broth plus 15% glycerol for Dixon. For bacterial inoculations, all three *X. fastidiosa* strains were grown separately either in PD3 or PW liquid media for 10 days on a rotary shaker at 28°C or on PD3 or PW agar media for 2 weeks at 28°C. Cells grown in liquid media were harvested by centrifugation at $7,000 \times g$ for 10 min at 4°C and the bacterial pellet was resuspended in 3 ml of sterile deionized water (sdH₂O). Strains grown on agar media were harvested by flooding the plate with 2 ml of sdH₂O. The concentration of each *X. fastidiosa* strain was adjusted to 10^8 CFU/ml as estimated by measuring optical density at 600 nm with a spectrophotometer. To ensure host infection, a mixture containing equal amounts of all three *X. fastidiosa* strains was used as the bacterial inoculum. Inoculations were made by placing 20 µl of bacterial suspension on the base of the current-season growing stems and pricking through the bacterial drop with a needle into the middle part of the stem (13). The inoculum was drawn into the xylem within a few seconds by the transpirational stream. Two inoculations, 10 to 20 mm apart, were carried out on the base of each current-season growing shoot.

Plant materials and disease evaluations. In February 2002, 88 1-year-old almond trees (*Prunus dulcis* (Mill.) D.A. Webb cvs. Butte, Carmel, Mission, Ne Plus Ultra, Nonpareil, Padre, Peerless, Price, Solano, Sonora, and Thompson), either in pots or with bare roots, were planted in the field at the Department of Plant Pathology of University of California (UC), Davis. Natural infection of ALS in almond planted at this UC Davis facility has never been observed during the past 30+ years. Eight trees of each cultivar were planted. Four cultivars (Ne Plus Ultra, Solano, Sonora, and Thompson) were on Lovell peach rootstocks. Three cultivars (Butte, Carmel, and Peerless) were on Nemaguard peach rootstocks. Two cultivars (Mission and Padre) were on Hansen hybrid rootstocks. Nonpareil and Price were on Deep purple and Marianna 2624 rootstocks, respectively. The 88 trees were fertilized and irrigated regularly to promote vegetative shoot growth. Bacterial inoculations were performed monthly from April to October. Due to delayed vegetative growth after planting, bacterial inoculations were only performed on 15 June, 15 July, 30 August, and 15 October 2002. In 2003, bacterial inoculations were performed on 15 April, 15 May, and 15 September. During each inoculation date, four current-season growing shoots were inoculated per tree per cultivar. In addition, an inoculation with sdH₂O was used as a negative control for each cultivar when inoculations were performed on 15 June 2002. Over the 3-year study, all of the Nonpareil trees died due to graft union incompatibility and were removed from analysis. Thus, the experiment was a completely randomized 8-by-10 factorial design with each of the four inoculated current-season growing shoots regarded as a replication. The number of ALS-symptomatic leaves that showed marginal scorching and a yellow halo between the healthy and scorched parts of the leaf were recorded every 2 weeks after inoculations. The distance from the most distant ALS symptomatic leaf to the point of inoculation (POI) was measured with a ruler.

The overwintering persistence of *X. fastidiosa* in inoculated shoots was determined by rating the inoculated shoots of 2002 during the entire growing season of 2003 at biweekly intervals starting from late June, using a four-scale disease rating system in which 0 represents healthy leaves without ALS symptoms, 1 = a few ALS symptomatic leaves to less than one-third of the leaves on the inoculated branch, 2 = one-third to two-thirds of symptomatic

ALS leaves on the inoculated branch, and 3 = over two-thirds of leaves showing symptoms on the inoculated branch.

Persistence and overwintering ability of *X. fastidiosa* in all 80 inoculated trees was assessed finally on 4 August 2004. In this assessment, all branches with ALS symptoms in each tree were recorded. The total number of branches recorded with ALS symptoms included both labeled branches, originally inoculated with *X. fastidiosa* in 2002 or 2003, and unlabeled branches, which were counted separately and considered to be new infections in which *X. fastidiosa* had moved and colonized noninoculated shoots from the originally inoculated branches within the same tree.

ALS assessment in a commercial orchard. In late August and early September 2002, an outbreak of ALS occurred in several orchards in Chico, a major almond-growing region of California. A section of one affected orchard consisting of 1,895 14-year-old almond trees was mapped and rated for ALS symptoms. Four cultivars were planted in the orchard, including 50% Nonpareil, 25% Peerless, 12.5% Butte, and 12.5% Carmel. ALS symptoms were rated on a 0-to-3 rating scale, where 0 = healthy tree, 1 = only one limb showing symptoms with ALS, 2 = more than one limb showing ALS symptoms, and 3 = all limbs within the tree were ALS symptomatic. Almond trees were mapped and rated in 2002 and again in 2003 to monitor disease progression in previously symptomatic trees and identify new infections within the orchard. Leaf samples from symptomatic trees were collected and assessed for the presence of *X. fastidiosa* by immunocapture polymerase chain reaction (IC-PCR) assay using healthy almond leaves as a negative control.

Detection of *X. fastidiosa* by IC-PCR assay. Typical symptomatic ALS leaves were sampled in mid-September and October 2002 and in July 2003 for the presence of *X. fastidiosa* using the IC-PCR assay that was originally developed by Smart and coworkers (29). Petioles and leaf midribs (0.03 to 0.76 g) were removed from two ALS leaves or one ALS leaf plus a healthy leaf that was adjacent to the ALS leaf on the stem and placed in plastic sample mesh bags (Agdia Inc., Elkhart, IN). Then, 2 to 3 ml of modified succinate-citrate-phosphate buffer (MSCP) was added to the sample bags and the tissue was pulverized with a grinding machine (Bioreba AG, Switzerland). MSCP buffer contained disodium succinate at 1.0 g/liter, trisodium citrate at 1.0 g/liter, K₂HPO₄ at 1.5 g/liter, KH₂PO₄ at 1.0 g/liter, and polyvinyl-pyrrolidone (PVP-40; Sigma-Aldrich) at 25.0 g/liter, with 0.02 M L-ascorbic acid and pH adjusted to 7 just before use (19). Plant extract (1 to 1.4 ml) in MSCP was collected from each sample and centrifuged in a microcentrifuge tube at $14,000 \times g$ for 5 min. The supernatant was aspirated and the pellet was resuspended in 500 µl of 0.1× phosphate buffer saline (PBS) (10× PBS contains NaCl at 80 g/liter, KH₂PO₄ at 2 g/liter, Na₂HPO₄ at 11.5 g/liter, and KCl at 2 g/liter) plus 0.2% bovine serum albumin (BSA; Fraction V, minimum 96%, Sigma-Aldrich), centrifuged at $14,000 \times g$ for 2.5 min and the pellet resuspended in 500 µl PBS/BSA. Anti-*X. fastidiosa* rabbit antiserum (100 µl diluted 1:1000 in PBS/BSA) was added to the plant extract and incubated for 30 min at room temperature with gentle shaking. The plant extract was then centrifuged, the supernatant discarded, and the pellet resuspended in 1 ml of PBS/BSA. This solution was recentrifuged, and the supernatant was discarded and again resuspended in 1 ml PBS/BSA buffer. Sheep anti-rabbit antibody-coated beads (5 µl; Dynabeads M-280 Sheep Anti-Rabbit IgG; Dynal Biotech ASA, Oslo) were added to each sample and incubated at room temperature for 30 min with gentle shaking. The complex of the sheep anti-rabbit coated beads containing bound *X. fastidiosa* cells was isolated from the extract using a magnet. Captured beads were washed three times in 1.0, 1.0, and 0.5 ml of PBS/BSA buffer for 5 min, respectively. The beads were finally resuspended in 10 µl of sdH₂O and 5 µl of the suspension was used for the PCR test.

For the specific detection of *X. fastidiosa*, primers RST31 (5'-GCGTTAATTTTCGAAGTGATTTCGATTGC-3') and RST33 (5'-CACCATTTCGTATCCCGGTG-3') were used in the PCR reaction (19). Amplifications were performed in a 30-µl reaction volume containing 1× PCR buffer (including 15 mM MgCl₂), 0.5 µM each

forward and reverse primer, 0.15 mM each dNTP, 1 U of Platinum *Taq* DNA polymerase, and either 5 µl of sample bead suspension or 60 ng of purified *X. fastidiosa* genomic DNA (positive control) or 5 µl of sdH₂O (negative control). A DNA Thermal Cycler (Mastercycler Gradient, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) was used, with 1 cycle of 1 min at 95°C; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and a final extension of 72°C for 10 min.

PCR products were resolved on 1% agarose gels using 1× Tris-acetate-EDTA buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3) at 72 V for 70 min. The gels were then stained with ethidium bromide (0.5 µg/ml) and visualized under UV light for amplicons using a UV transilluminator (White/UV transilluminator, UVP).

Data analysis. Data were analyzed for statistical significance using the general linear model procedure (Statistical Analysis System; SAS Institute, Cary, NC). When appropriate, square-root and log transformations (8) were applied to the raw data before subjecting it to analysis of variance (ANOVA). In all cases, least-square means were used to compare treatments, and both the transformed and untransformed means are presented. For ordinal data such as disease ratings, the SAS CATMOD procedure was adopted for statistical significance test and pairwise contrast. The SAS RELIABILITY procedure was used for testing the hypothesis of equality among percentile data, and the SAS MULTTEST procedure was used for further testing significance of pairwise contrast among percentiles.

Results

ALS symptom development and *X. fastidiosa* movement following inoculation. Leaves with typical ALS symptoms were first observed on inoculated shoots 4 to 10 weeks post inoculation (Fig.

Fig. 1. Almond leaf scorch (ALS) development in the growing seasons of 2002 and 2003 following inoculations with *Xylella fastidiosa* in the same year. On each inoculation date, four current-year shoots were inoculated on one tree of each cultivar, for a total of 40 inoculated branches. **A**, Average numbers of leaves showing ALS symptoms averaged over 40 branches per inoculation date. **B**, Distance (mm) of the farthest ALS leaf to the point of inoculation averaged over 40 branches per inoculation date. Error bars represent one standard error.

1A). The number of ALS leaves resulting from the July 2002 inoculation increased more gradually compared with inoculations made in April and May 2003 and June 2002. The late-August inoculation in 2002 produced the fastest onset of symptomatic ALS leaves at the end of September 2002, which was 4 weeks post inoculation. No symptomatic ALS leaves developed from September 2003 or October 2002 inoculations in the same growing season following inoculation. Inoculations made in April and May 2003 and June 2002 produced the greatest number of ALS-symptomatic leaves in the same growing season among all inoculations (Fig. 1A). No symptomatic leaves occurred on trees inoculated with sdH₂O.

The movement of *X. fastidiosa* in the xylem following inoculation was estimated by measuring the distance from the POI to the farthest ALS-symptomatic leaf (Fig. 1B). In general, the first ALS leaf appeared a few centimeters above the POI and, thereafter, more ALS leaves appeared above the first ALS leaf as the growing season progressed. In some cases, ALS leaves were observed on the whole length of shoot by the end of the growing season but only in a few cases were ALS leaves observed below the POI (*data not shown*). There was a high correlation between the observation of typical ALS symptoms and detection of *X. fastidiosa* by PCR, as discussed below.

X. fastidiosa movement in response to different inoculation dates showed a pattern similar to the increasing number of symptomatic leaves (Fig. 1B). The fastest extension of *X. fastidiosa* upward from POI occurred from mid-July to mid-September (Fig. 1B). *X. fastidiosa* inoculated in June moved the greatest distance, followed by the April and May 2003 inoculations (Fig. 1B). For the 10 almond cultivars inoculated on 15 June 2002, maximum *X. fastidiosa* movement among the four inoculated branches of each cultivar was 0 to 430, 513 to 1,000, and 700 to 1,120 mm observed in the middle of August, September, and October 2002, respectively (Table 1). The longest *X. fastidiosa* movement in all inoculated branches was 1,120 mm from the POI in Mission observed on 15 October 2002 (Table 1).

Confirmation of *X. fastidiosa* within-plant movement by IC-PCR. The IC-PCR assay was sensitive enough to detect *X. fastidiosa* in a single leaf midrib weighing as little as 30 mg. In mid-September 2002, IC-PCR was used to confirm infection of the farthest ALS leaf from the POI for all trees inoculated on 15 June 2002. Among the 38 shoots with ALS leaves induced by inoculations on 15 June 2002, 87% of them were IC-PCR positive. ALS leaves from the 15 July 2002 inoculations were confirmed with IC-PCR in October 2002. In contrast to the IC-PCR results obtained in September, 72% of the 29 shoots were IC-PCR positive when tested in October. In July 2003, 13 ALS leaf samples were collected from 11 cultivars (1 sample from NonPareil) which were inoculated in June to October 2002. In all, 12 of 13 samples tested positive by IC-PCR; the 1 negative sample was from cv. Thompson inoculated in August 2002.

Table 1. Distance *Xylella fastidiosa* moved (mm) in 10 almond cultivars following mechanical inoculation on 15 June 2002^z

Cultivar	Measurement date following inoculation			
	30 July	15 Aug	15 Sep	15 Oct
Butte	0	385	705	905
Carmel	0	25	570	780
Mission	0	122	1,000	1,120
Ne Plus Ultra	0	300	553	900
Padre	0	100	660	1,035
Peerless	0	0	808	1,040
Price	0	430	610	710
Solano	0	87	672	980
Sonora	0	120	513	700
Thompson	0	340	670	965

^z Distance measurements were made on four dates in July, August, September, and October 2002 following inoculation in June. Only the longest moving distance of the four inoculated branches is presented.

ANOVA of the *X. fastidiosa* movement data which were confirmed with IC-PCR assay showed that inoculation dates but not cultivar had a significant effect on *X. fastidiosa* movement (Table 2); that is, the average movement of *X. fastidiosa* within the xylem was significantly ($P < 0.0025$) longer in trees that were inoculated on 15 June (mean distance $\pm$ standard error = 402 ± 41 mm) than those inoculated on 15 July 2002 (239 ± 42 mm). None of the 10 cultivars differed significantly in their ability to inhibit *X. fastidiosa* movement in the xylem; Price had the shortest average *X. fastidiosa* movement (221 mm) and Peerless the longest (674 mm) (data not shown). It should be noted that *X. fastidiosa* movement was scored on the appearance of typical foliar symptoms; it is likely that nonsymptomatic leaves distal to the last symptomatic leaf may have contained populations of *X. fastidiosa* that were too low to induce typical disease symptoms. Thus, our estimations of the relative movement and colonization by *X. fastidiosa* were conservative estimates based on pathogen populations that were great enough to induce foliar symptoms.

Persistence of *X. fastidiosa* over the following winters. Following inoculations made in the growing season of 2002, the first ALS-symptomatic leaves were observed at the end of June 2003 (Fig. 2). A rapid increase of ALS disease ratings occurred during a 2-month period from mid-July to mid-September (Fig. 2A). Inoculations made in mid-June 2002 resulted in the highest disease ratings, while inoculations made in late August 2002 resulted in the lowest disease ratings (Fig. 2A). Interestingly, the mid-October 2002 inoculation resulted in higher disease ratings than inoculation in August 2002 (Fig. 2A). Sonora and Solano had the highest disease ratings and Thompson had the lowest disease ratings among the six almond cultivars that developed ALS disease in the growing season of 2003 (Fig. 2B).

Statistical analysis using the SAS CATMOD procedure on the disease rating data obtained in October 2003 demonstrated highly significant effects of inoculation dates ($\chi^2 = 52.27$, $P < 0.0001$) and cultivars ($\chi^2 = 41.43$, $P < 0.0001$). The 2003 disease ratings resulting from the mid-June inoculations in 2002 were significantly higher than those made in late August and mid-October (Table 3). The 2003 disease ratings of the mid-July inoculations of 2002 were significantly higher than those of late August 2002 but were not significantly different from those of mid-June and mid-October (Table 3). Surprisingly, the 2003 disease ratings from the late August 2002 inoculations were the lowest and even significantly lower than those from inoculations made in October 2002 (Table 3).

Based on disease severity ratings of 16 branches for each cultivar, the overwinter persistence of *X. fastidiosa* was significantly higher in Sonora and Solano compared with Thompson, Peerless, Butte, Ne Plus Ultra, Padre, and Carmel but was not significantly different from Price and Mission (Table 4). The overwinter persistence of *X. fastidiosa* was not significantly different between Solano and Sonora (Table 4). Based on disease severity ratings, Mission and Price did not differ in their ability to host *X. fastidiosa* for overwintering but disease severity ratings were significantly greater in Mission and Price than in Thompson, Peerless, and Butte (Table 4). Based on disease severity ratings, among the 10 almond

cultivars tested, Solano and Sonora were most susceptible, Mission and Price were intermediate, and Carmel, Padre, Ne Plus, Butte, Peerless, and Thompson were the least susceptible cultivars for hosting overwintering populations of *X. fastidiosa* (Table 4).

Assessment of ALS symptoms in August 2004 showed that, of the 70 trees inoculated with *X. fastidiosa* in 2002 and 2003, 24 trees had persistent infections and showed ALS symptoms on at least one branch. The 2004 assessment demonstrated that 54 branches, which were originally inoculated with *X. fastidiosa* in 2002 or 2003, showed typical ALS symptoms in August 2004, and 34 branches, which were not originally inoculated with *X. fastidiosa* in 2002 or 2003, also showed ALS symptoms in 2004. Among the 54 ALS branches recorded in August 2004, inoculations made in mid-June and mid-July 2002 resulted in significantly higher percentages of *X. fastidiosa* overwintering persistence through the winters of 2002–03 and 2003–04 than inoculations made in August 2002 and September 2003 (Table 5). Among these 34 newly infected ALS branches, inoculations made in mid-June and mid-July 2002 resulted in significantly higher numbers of newly infected ALS branches compared with inoculations made in August 2002 and September 2003 (Table 5). Among all 80 ALS branches recorded in August 2004, inoculations made in mid-June and mid-July 2002 produced significantly higher percentages of ALS branches in 2004 than inoculations made in August 2002 and September 2003 (Table 5). Surprisingly, the October inoculation of

Fig. 2. Disease rating in 2003 in 10 almond cultivars inoculated with *Xylella fastidiosa* in 2002. **A**, Disease ratings in 2003 produced by inoculation dates in 2002. On each inoculation date, four current-year shoots were inoculated on one tree of each cultivar, for a total of 40 inoculated branches. Each data point is an average of disease ratings of 40 branches per inoculation date and error bars represent one standard error. **B**, Disease ratings in 2003 analyzed by cultivar. Four current-year shoots on one tree of each cultivar were inoculated on each inoculation date in 2002, for a total of 16 shoots per cultivar in 2002. Data points shown are an average of 16 branch ratings with 1 standard error. For simplicity, only the six cultivars that had overwintering infections are shown (Solano, Sonora, Price, Ne Plus Ultra, Peerless, and Thompson).

Table 2. Analysis of variance for square-root-transformed distance of the farthest almond leaf scorch (ALS) leaf to the point of inoculation 3 months post inoculation, which was confirmed by immunocapture polymerase chain reaction

Source	df	Mean square	$P > F$
Inoculation date (I) ^y	1	932.76	<0.0025
Cultivar (C) ^z	9	178.96	>0.0672
I $\times$ C	9	96.17	>0.4288
Error	60	93.56	

^y Testing for the effects of inoculation dates on *Xylella fastidiosa* movement within xylem.

^z Testing for the susceptibility of cultivars to *X. fastidiosa* movement within xylem.

2002 also yielded significantly higher percentages of ALS branches observed in 2004 than the inoculations made in August 2002 and September 2003 (Table 5). Inoculations made in June, July, and October 2002 had the most *X. fastidiosa* infections that were able to survive the winters over the following 2 years. Inoculations made in April and May 2003 had fewer branches with surviving infections (Table 5). Although inoculations made in June and July 2002 resulted in more persistent infections, these infections only persisted in Sonora, Solano, Peerless, Price, Mission, and Neplus (Table 6). ALS symptomatic branches were not observed in Butte, Thompson, Carmel, and Padre in 2004 (Table 6).

ALS assessment of four almond cultivars in a commercial orchard. Overall ALS incidence within the Chico orchard was approximately 17% based on the visual disease ratings for 2002 and 2003 (Table 7). All 18 leaf samples collected from this orchard in 2002 were PCR positive for detection of *X. fastidiosa*. Ratings in 2002 found that Peerless had the significantly highest ALS incidence (32.4%); next was Nonpareil (16%). Butte and Carmel only had 2.3 and 0.4% ALS incidence, respectively, but the difference between Butte and Carmel was not significant (Table 8). In 2003, a similar pattern emerged in that Peerless had the highest disease incidence of the four cultivars, even after 105 Peerless trees were removed over the winter of 2002–03 (Table 8). Again in 2003, cvs. Butte and Carmel had very low disease incidence: 2 and 0.5%, respectively (Table 8). These data indicated that Peerless and Non-

pareil were more susceptible to *X. fastidiosa* infection than Butte and Carmel in the commercial orchard.

Discussion

The most striking difference between overwintering success of *X. fastidiosa* infections of almond plants versus grapevines is that curing occurs in infected almond at much warmer winter temperatures than are needed for the cold-curing phenomenon to occur in grapevines (7,18,21,24). For example, an extensive study of the cold-curing requirement for PD of grapevines using both field and growth-chamber environments showed that relatively low rates of PD curing occurred at the same UC Davis field location where all of this research was conducted on winter curing of ALS disease (18). This indicates that the absolute magnitude of cold winter temperatures is not as important as time of inoculation and cultivar susceptibility for winter curing to occur in almond compared with *Vitis vinifera* grapevine cultivars.

In this study, the two most important factors that affected systemic infection of *X. fastidiosa* within almond trees were time of *X. fastidiosa* infection and cultivar susceptibility. Other abiotic factors, such as the magnitude and duration of cold winter temperatures, might also affect the ability of *X. fastidiosa* to successfully overwinter and cause ALS the following growing season; however, the effect of winter temperature was not evaluated in this study. Biotic factors such as the presence of fungal or bacterial endosymbionts might also affect pathogen survival but there is no data to support this possibility. Susceptible cultivars infected with *X. fastidiosa* in the spring or early summer likely have enough time to establish infections at favorable temperatures that allow *X. fastidiosa* to achieve populations high enough to be able to survive over the winter and further colonize the tree the next growing season. Results from this study support the importance of inoculation date and cultivar susceptibility in establishing chronic ALS disease.

Generally, it took 8 to 10 weeks for *X. fastidiosa* to multiply within the xylem of the inoculated shoots and produce incipient ALS symptoms (Fig. 1). This latency period was very similar to that reported by Ledbetter et al. (15) for cv. Butte; however, in a smaller study, almond shoots inoculated in March and June took more than 3 months to show symptoms (6). Similarly, a 3-month period was required for grapevines that were inoculated with *X. fastidiosa* in April or May to develop incipient symptoms of marginal leaf scorching in late summer (7). Our data showed that *X. fastidiosa* inoculated in June moved and produced ALS foliar symptoms an average of 163 mm farther than when it was inoculated in July 2002. *X. fastidiosa* movement was generally much

Table 3. Pairwise comparisons evaluating the influence of inoculation date on *Xylella fastidiosa* overwinter persistence in 10 almond cultivars based on a 0-to-3 scale disease ratings assessed in 2003 following inoculation made in 2002

Date of inoculation ^z	15 June 1.42 ± 0.18	15 July 1.00 ± 0.15	30 August 0.28 ± 0.07
15 October 0.73 ± 0.10	$\chi^2 = 12.13$ $P = 0.0005$	$\chi^2 = 2.34$ $P = 0.1262$	$\chi^2 = 13.53$ $P = 0.0002$
30 August 0.28 ± 0.07	$\chi^2 = 37.59$ $P < 0.0001$	$\chi^2 = 19.23$ $P < 0.0001$	...
15 July 1.00 ± 0.15	$\chi^2 = 3.39$ $P = 0.0656$	...	...

^z For easy comparisons of the effect of inoculation date on almond leaf scorch overwinter persistence, the mean disease ratings of 40 branches ± standard error of each inoculation date, which were observed in October 2003 following inoculations made in 2002, are presented under each inoculation date. Both χ^2 and P values are presented for pairwise contrasts among the four inoculation dates according to the SAS CATMOD procedure.

Table 4. Pairwise comparisons evaluating *Xylella fastidiosa* overwinter persistence in 10 almond cultivars based on disease ratings made in October 2003 following inoculations made in 2002

Cultivar ^z	Sonora 1.56 ± 0.27	Solano 1.56 ± 0.30	Mission 1.06 ± 0.25	Price 1.06 ± 0.25	Carmel 0.75 ± 0.14	Padre 0.69 ± 0.15	Ne Plus Ultra 0.56 ± 0.16	Butte 0.40 ± 0.13	Peerless 0.40 ± 0.16
Thompson 0.33 ± 0.13	$\chi^2 = 17.80$ $P < 0.0001$	$\chi^2 = 15.03$ $P = 0.0001$	$\chi^2 = 7.27$ $P = 0.0070$	$\chi^2 = 7.20$ $P = 0.0070$	$\chi^2 = 5.05$ $P = 0.0246$	$\chi^2 = 3.48$ $P = 0.0622$	$\chi^2 = 1.38$ $P = 0.2398$	$\chi^2 = 0.14$ $P = 0.7041$	$\chi^2 = 0.11$ $P = 0.7379$
Peerless 0.40 ± 0.16	$\chi^2 = 14.23$ $P = 0.0002$	$\chi^2 = 12.22$ $P = 0.0005$	$\chi^2 = 5.27$ $P = 0.0217$	$\chi^2 = 5.27$ $P = 0.0217$	$\chi^2 = 2.76$ $P = 0.0968$	$\chi^2 = 1.79$ $P = 0.1807$	$\chi^2 = 0.55$ $P = 0.4586$	$\chi^2 = 0.00$ $P = 1.0000$	...
Butte 0.40 ± 0.13	$\chi^2 = 15.70$ $P < 0.0001$	$\chi^2 = 13.29$ $P = 0.0003$	$\chi^2 = 5.90$ $P = 0.0151$	$\chi^2 = 5.90$ $P = 0.0151$	$\chi^2 = 3.45$ $P = 0.0633$	$\chi^2 = 2.22$ $P = 0.1363$	$\chi^2 = 0.67$ $P = 0.4117$	...	...
Ne Plus Ultra 0.56 ± 0.16	$\chi^2 = 10.72$ $P = 0.0011$	$\chi^2 = 9.18$ $P = 0.0024$	$\chi^2 = 3.07$ $P = 0.0800$	$\chi^2 = 3.07$ $P = 0.0800$	$\chi^2 = 0.82$ $P = 0.3643$	$\chi^2 = 0.35$ $P = 0.5532$	...	...	...
Padre 0.69 ± 0.15	$\chi^2 = 8.39$ $P = 0.0038$	$\chi^2 = 7.16$ $P = 0.0075$	$\chi^2 = 1.77$ $P = 0.1838$	$\chi^2 = 1.77$ $P = 0.1838$	$\chi^2 = 0.10$ $P = 0.7569$	...	...	...	...
Carmel 0.75 ± 0.14	$\chi^2 = 7.37$ $P = 0.0066$	$\chi^2 = 6.27$ $P = 0.0123$	$\chi^2 = 1.25$ $P = 0.2628$	$\chi^2 = 1.25$ $P = 0.2628$	...	...	...	...	...
Price 1.06 ± 0.25	$\chi^2 = 1.95$ $P = 0.1629$	$\chi^2 = 1.74$ $P = 0.1877$	$\chi^2 = 0$ $P = 1.0000$	...	...	...	...	...	...
Mission 1.06 ± 0.25	$\chi^2 = 1.95$ $P = 0.1629$	$\chi^2 = 1.74$ $P = 0.1877$	...	...	...	...	...	...	...
Solano 1.56 ± 0.30	$\chi^2 = 0.00$ $P = 1.0000$	...	...	...	...	...	...	...	...

^z For easy comparisons of cultivar susceptibility to almond leaf scorch, the mean disease ratings of 16 branches ± standard error of each cultivar, which were observed in October 2003 following inoculations made in 2002, are presented under each cultivar. Both χ^2 and P values are presented for pairwise contrast among the 10 almond cultivars according to the SAS CATMOD procedure.

greater than that observed by Davis et al. (6), in which *X. fastidiosa* moved only 100 to 300 mm above the inoculation site. Almond trees used in the Davis et al. (6) experiment were mature and established trees. Young trees were also used in the Ledbetter et al. (15) study but they did not report the distance symptomatic leaves occurred from the POI. Faster symptom development, as well as the greater *X. fastidiosa* movement found in our study, may be attributable to the use of younger trees with rapidly growing shoots and high rates of transpiration. In general, younger almond trees could

be more susceptible to initial *X. fastidiosa* infection, although the ability of *X. fastidiosa* to survive over the winter is more dependent on cultivar susceptibility.

Except for trees inoculated in September and October, all trees showed some symptom development on inoculated shoots within the same growing season they were inoculated. However, the overwinter persistence of *X. fastidiosa* infection was significantly dependent on inoculation dates and cultivar susceptibility. The significantly greater movement of *X. fastidiosa* within the almond

Table 5. Assessment in 2004 of almond leaf scorch (ALS) symptoms in almond trees inoculated with *Xylella fastidiosa* in 2002 and 2003 as a function of time of inoculation^a

Inoculation date	Branches with ALS symptoms in 2004				
	Inoculated		Uninoculated		
	No. of branches ^o	Fraction of branches ^p	New symptoms ^q	Fraction of branches ^r	Fraction of trees ^s
15 June 2002	15 ^t	15/54 a	11	11/34 a	5/10 a
15 July 2002	14 ^u	14/54 a	12	12/34 a	6/10 a
15 October 2002	10 ^v	10/54 ac	9	9/34 ab	4/10 a
30 August 2002	2 ^w	2/54 bc	0	0/34 c	2/10 a
15 April 2003	5 ^x	5/54 ac	1	1/34 bc	2/10 a
15 May 2003	6 ^y	6/54 ac	1	1/34 bc	4/10 a
15 September 2003	2 ^z	2/54 bc	0	0/34 c	2/10 a
Total	54	54/54	34	34/34	24/70

^a On each inoculation date, four branches on one tree of each cultivar were inoculated for a total of 40 branches on 10 trees inoculated. Fractions followed by the same letter in the same column are not significantly different at $P < 0.05$ according to SAS MULTTEST procedure.

^o Number of branches in 2004.

^p Fraction of branches for each date.

^q Branches with new ALS symptoms in 2004.

^r Fraction of newly infected branches for each date.

^s Fraction of trees with ALS in 2004.

^t Symptoms were recorded in Sonora, Solano, Mission, Price, and Peerless only but not in other cultivars.

^u Symptoms were recorded in Sonora, Solano, Ne Plus Ultra, Mission, Price, and Peerless only but not in other cultivars.

^v Symptoms were recorded in Sonora, Solano, Ne Plus Ultra, and Peerless only but not in other cultivars.

^w Symptoms were recorded in Sonora and Ne Plus Ultra only but not in other cultivars.

^x Symptoms were recorded in Sonora and Solano only but not in other cultivars.

^y Symptoms were recorded in Sonora, Solano, Price, and Peerless only but not in other cultivars.

^z Symptoms were recorded in Mission and Peerless only but not in other cultivars.

Table 6. Assessment in 2004 of almond leaf scorch (ALS) symptoms in inoculated and uninoculated branches on 10 almond cultivars inoculated with *Xylella fastidiosa* in 2002 and 2003^x

Cultivar	Branches inoculated in 2002	Branches inoculated in 2003	Fraction of branches with ALS symptoms ^y	Uninoculated branches with ALS symptoms ^z	Total infected branches observed in 2004	Fraction of trees with remaining ALS symptoms	Fraction of trees with ALS that spread from inoculated branches
Sonora	16	12	17/28 a	13	30	6/7 a	5/7 a
Solano	16	12	10/28 ab	10	20	5/7 ab	2/7 ab
Peerless	16	12	11/28 ab	5	16	5/7 ab	2/7 ab
Price	16	12	6/28 ac	2	8	3/7 ab	1/7 ab
Mission	16	12	7/28 ac	0	7	3/7 ab	0/7 b
Ne Plus Ultra	16	12	3/28 bc	4	7	3/7 ab	2/7 ab
Butte	16	12	0/28 c	0	0	0/7 b	0/7 b
Carmel	16	12	0/28 c	0	0	0/7 b	0/7 b
Padre	16	12	0/28 c	0	0	0/7 b	0/7 b
Thompson	16	12	0/28 c	0	0	0/7 b	0/7 b

^x Fractions followed by the same letters in the same column are not significantly different ($P < 0.05$) according to the pairwise contrast using the SAS MULTTEST procedure.

^y Remaining infections from original inoculations in 2002 and 2003.

^z New infection: Branches which were not originally inoculated with *X. fastidiosa* in 2002 and 2003 but showing ALS in 2004.

Table 7. Incidence of almond leaf scorch (ALS) in a Chico orchard containing Nonpareil, Peerless, Butte, and Carmel cultivars in 2002

Disease rating	No. of trees (%) ^z				
	Nonpareil	Peerless	Butte	Carmel	Total
Healthy	794 (84)	329 (67.6)	217 (97.7)	240 (99.6)	1,580 (83.4)
1	63 (6.7)	53 (10.9)	3 (1.4)	0	119 (6.3)
2	54 (5.7)	26 (5.3)	2 (0.9)	0	82 (4.3)
3	34 (3.6)	79 (16.2)	0	1 (0.4)	114 (6.0)
Total ALS trees	151 (16)	158 (32.4)	5 (2.3)	1 (0.4)	315 (16.6)
Total	945	487	222	241	1,895

^z Percentiles within parentheses are the proportions of healthy or diseased trees in each cultivar.

branches and significantly higher disease ratings in 2003 and 2004 from inoculations made in June suggests that late spring or early summer is the optimal time for *X. fastidiosa* to infect and develop persistent ALS disease.

Interestingly, the October 2002 inoculation led to a higher proportion of overwintering infections than the August 2002 inoculation. Although it is unclear why this occurred, we hypothesize that high outdoor temperatures may have deleteriously affected *X. fastidiosa* viability within the trees at this time of year. Furthermore, it was unclear why April and May inoculations in 2003 were not as effective as inoculations in June and July 2002. All trees inoculated in April and May 2003 had been established in the field for an extra full year, and perhaps this additional tree maturity slowed the movement of *X. fastidiosa* and, consequently, the ability of *X. fastidiosa* to overwinter.

The overall evaluation of cultivar susceptibility, based on the 2003 disease ratings and the 2004 assessment of movement and symptom development with inoculated trees, showed that Sonora and Solano were the most susceptible cultivars. Mission, Price, Peerless, and Ne Plus Ultra had intermediate susceptibility and Carmel, Padre, Butte, and Thompson were the least susceptible cultivars for maintaining *X. fastidiosa* over the winter.

Our results with cv. Butte, in which none of the inoculated trees showed ALS symptoms in 2004, were considerably different than the results reported by Ledbetter et al. (15), in which only 21.2% of their Butte trees experienced winter curing. Their experimental design was considerably different from that used in this study; they used potted rather than field-grown trees and their trees were returned to a greenhouse upon budbreak, whereas the trees in this study remained in the field. Ledbetter et al. also cut back their main stems to 15 cm above the graft union each spring whereas the trees in our study were left unpruned. None of the trees, including Butte, used in this study experienced any mortality compared with the 27% mortality Butte experienced in the Ledbetter study. In addition to the very different cultural practices used in both studies, the strains of *X. fastidiosa* used to inoculate almond trees were different.

Almeida and Purcell (2) examined a number of *X. fastidiosa* grape and almond strains for their ability to cause either PD or ALS when mechanically inoculated into potted Cabernet sauvignon grapevines and NonPareil almond trees. Two of the strains they evaluated, Dixon (almond) and Temecula (grape), were the same as two of the three strains used as *X. fastidiosa* inoculum in this study. They found that Temecula, like all of the other grape strains evaluated, caused ALS foliar symptoms in the summer after being inoculated earlier in April. Similarly, all of the almond strains tested also caused ALS foliar symptoms the summer following inoculation. They allowed the diseased, potted almond trees to undergo natural dormancy through November, after which they were moved to a 4°C cold chamber for 1 month. The trees were then pruned, transferred to a greenhouse, and rated for foliar symptoms the next summer. All of the almond plants inoculated with the Dixon strain caused ALS the next summer whereas none of the almond plants inoculated with Temecula showed symptoms after undergoing the cold treatment. This result suggests that Temecula was not one of the strains that overwintered and caused disease in our study. At the time our study was undertaken, there was no

quick way to differentiate almond from grape strains of *X. fastidiosa* and, because the primary objective of this study was to evaluate almond cultivar susceptibility to ALS, we did not genetically characterize the strains that overwintered and caused disease in our study. Alemedia and Purcell (2) also conducted an ALS overwintering field trial on mature NonPareil almond trees growing at the same UC Davis facility where our study was conducted. Similar to their potted tree experiment, they found that both grape and almond strains caused ALS symptoms in mature trees in the summer following inoculations made in the spring. They also found that five of the six infections that resulted from inoculation with *X. fastidiosa* almond strains successfully overwintered and caused foliar symptoms the next summer. Surprisingly, 24 of 57 infections caused by grape strains overwintered and caused ALS symptoms 1 or 2 years following inoculation. Thus, our original rationale for using a mixture of almond and grape strains as inoculum for our study, which was initiated before the results of the Alemedia and Purcell (2) study were known, turned out, in retrospect, to be a feasible method for achieving the results we desired (i.e., evaluate almond cultivar susceptibility to ALS).

It is important to note that comparatively low disease severity ratings were observed in cv. Peerless in the summer of 2003 following inoculations made in 2002. However, in the summer of 2004, the severity of symptoms and the systemic movement of *X. fastidiosa* into many Peerless branches that were not initially inoculated shows that this cultivar was quite susceptible and supported overwintering populations of *X. fastidiosa*. This conclusion is also supported by the high disease incidence of Peerless trees in the commercial orchard. These results suggest that, although disease severity ratings made 1 year following inoculation may provide some insight into a cultivar's susceptibility, as was the case with Sonora and Solano, additional evaluations made 2 years following inoculation would be a better predictor of a cultivar's susceptibility over time.

Results of the Chico survey supported the conclusion that certain almond cultivars are better hosts for overwintering populations of *X. fastidiosa* than others, making them more susceptible to developing systemic ALS. Both the Chico orchard survey and our inoculation study showed that Butte and Carmel were more resistant, with lower disease ratings and fewer new infections in the second year the commercial orchard was surveyed. The Chico survey showed that Peerless had the highest occurrence of new infections as well as the most trees removed due to severe ALS symptoms. Additional evaluations of almond orchards in San Joaquin Valley and Kern County further support our results. In these studies, Nonpareil, Sonora, and Peerless were reported as most susceptible while Butte, Carmel, Mission, Fritz, and Thompson were considered moderately resistant (9,10,30).

The current study has revealed clear differential resistance of almond cultivars to *X. fastidiosa*. However, the rootstocks for the 10 almond cultivars were not the same and could have had a very minor influence on almond cultivar susceptibility to ALS. Peach or peach-almond hybrid rootstocks used by commercial almond tree nurseries in California are not primary inoculum sources for *X. fastidiosa*-induced diseases (16) and could be conferring minor resistance. However, all of the trees in the Chico survey were grafted on peach rootstocks; therefore, if there is any effect of rootstock on ALS development, it is likely very minor.

The current control method for ALS-infected trees is removal when nut yield becomes unacceptably low (28). Information from this research identified almond cultivars with greater resistance that could be planted in orchards that are adjacent to fields that harbor high populations of *X. fastidiosa* vectors. Additional research is needed to identify the mechanism or mechanisms that mediate resistance or susceptibility in different almond cultivars.

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Table 8. Statistical comparison of disease incidence (percentages of diseased trees over total number of trees surveyed) in 2002 and 2003

Cultivar	Incidence (%) ^z	
	2002	2003
Peerless	32.4 a	29.7 a
Nonpareil	16.0 b	18.5 b
Butte	2.3 c	2.0 c
Carmel	0.4 c	0.5 c

^z Percentiles followed by the same letters in the same column are not significantly different ($P < 0.005$) according to the pairwise contrast using the SAS CATMOD procedure.

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