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Winter curing of *Prunus dulcis* cv ‘Butte,’ *P. webbii* and their interspecific hybrid in response to *Xylella fastidiosa* infections

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Abstract Clonal replicates of *Prunus dulcis* cv ‘Butte,’ *P. webbii* and their interspecific hybrid P 63-61 were inoculated with *Xylella fastidiosa* strain M23 and evaluated for almond leaf scorch disease and subsequent winter curing of infections during three growing seasons. Initial inoculations established greater than 90% infection in each of the accessions, based on PCR diagnoses from petiole tissues sampled near the inoculation site. Classic leaf scorch symptoms were evident in each population during the first growing season in a controlled greenhouse environment. Trees were removed from the greenhouse during the winters to accumulate chill hours and to provide the possibility of winter curing *X. fastidiosa* infections. Both PCR diagnostics and in vitro cultivation were used during the second and third growing seasons to determine the persistence of *X. fastidiosa*

in clones among the three populations. Tree survival and the degree of winter cured infections differed among the three populations, with *P. webbii* and P 63-61 demonstrating enhanced levels of survivorship over ‘Butte.’ After two cycles of ambient winter temperatures and subsequent growth, ‘Butte’ averaged 21.2% winter cured trees with 73.1% mean survival. Tree survival and winter cured infections were nearly 100% for both *P. webbii* and P 63-61, demonstrating the utility of *P. webbii* in almond breeding efforts aimed at reducing tree vulnerability to *X. fastidiosa* infections.

Keywords Almond leaf scorch · Breeding · *Prunus dulcis* · *P. webbii* · Winter curing · *Xylella*

Introduction

California fruit growers first learned of *Xylella fastidiosa* induced diseases in the mid-1880s when Pierce’s Disease was described on grapevines growing near Anaheim, CA (Pierce 1892). By the mid-1930s, a leaf scorch disease had been described on almond trees near Riverside, CA, and in the 1970s the disease was observed on almonds in 14 different California counties (Mircetich et al. 1976). Since that time, almond leaf scorch disease (ALSD) has been only a minor problem in California orchards until a re-emergence occurred at the end of the twentieth century. Nut yields are now known to be significantly

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less for ALSD affected almond trees, and cultivar differences exist with regard to the degree of yield reduction (Sisterson et al. 2008).

Xylella fastidiosa (*Xf*) is a Gram negative and nutritionally fastidious plant pathogenic bacterium. ALSD and Pierce's Disease strains of *Xf* were previously reported to be pathologically similar (Davis et al. 1981; Mircetich et al. 1976). Recent studies showed that *Xf* strains associated with ALSD in California are composed of two genetically and pathologically distinct groups (Almeida and Purcell 2003; Chen et al. 2005; Henderson et al. 2001). Based on single nucleotide polymorphisms (SNPs) at the 16S rDNA sequences, Chen et al. (2005) designated the *Xf* strains causing only ALSD as an A-genotype, and strains causing both ALSD and Pierce's Disease as a G-genotype. Traditionally, *Xf* in infected tissue has been identified by in vitro cultivation. However, cultivation of *Xf* in the laboratory is time consuming. More recently, polymerase chain reaction (PCR) based detection of bacterial DNA representing the bacterium has become popular. Several specific PCR primer sets are currently available for *Xf* detection including the most thoroughly tested RST31/33 primer set (Minsavage et al. 1994), derived from the RNA polymerase genomic locus. Additional primer sets, derived from 16S rRNA gene (Chen et al. 2005), have often been used as important tools for taxonomic classification of *X. fastidiosa* (Wells et al. 1987). Recently, Chen et al. (2008) developed and evaluated a PCR procedure to detect *Xf* from pulverized freeze-dried tissues (PFT-PCR) without the need for DNA extraction.

Besides ALSD and Pierce's Disease, *Xf* is an important pathogen of many ornamental trees and shrubs growing in areas with mild winter climates. A colder environment limits *Xf*-induced disease development, and cold treatments have been shown to reduce *Xf* populations in both propagation materials and potted grapevines (Feil and Purcell 2001). Grapevines with Pierce's Disease have been successfully eliminated of *Xf* over the course of a dormant season when low temperature duration and severity was sufficient to kill established *Xf* populations in overwintering canes and trunk tissues. The concept has been termed 'winter curing,' and is among the factors that limit secondary spread of *Xf*-induced diseases in regions environmentally suboptimal to *Xf*.

Almond is a widely grown tree nut produced throughout California's Central Valley with current estimated inshell production approximating slightly over 500,000 t (Anon 2007). While the cultivar 'Nonpareil' predominates in orchard area and production, dozens of cultivars are grown under wide-ranging management practices and orchard conditions. The 'Butte' cultivar, introduced in 1963 (Brooks and Olmo 1972), has become increasingly popular in recent years and now represents just under 10% of total almond receipts (Anon 2007). It has demonstrated itself as being among the highest yielding almond cultivars in the central and southern San Joaquin Valley (Lampinen et al. 2002).

'Butte' has been used as a parent in developing new self-compatible almonds at the Agricultural Research Service almond breeding program in Parlier, CA due to its homozygous sweet status at the kernel flavor locus. Enhanced breeding efficiency is achieved by using 'Butte' in hybridizations with self-compatible 'Tuono,' or other self-compatible almonds heterozygous for kernel flavor. Hybrids between 'Butte' and the non-domesticated *Prunus webbii* are also being evaluated for phenological and morphological characteristics. Currently, we are interested in the response of 'Butte'/*P. webbii* hybrids to *Xf* infections, as 'Butte' has been shown to be the least susceptible cultivar to this bacterial pathogen among the California almonds (Daane and Purcell 2005). Non-domesticated species have been used successfully in interspecific hybridizations to introduce disease resistance into domesticated crops (Leppik 1970). With the interspecific hybrid and parental trees readily available for screening, our objective in this current study was to examine the degree of initial ALSD susceptibility and subsequent winter curing in almond cultivar 'Butte,' *P. webbii* and their interspecific hybrid throughout three growing seasons.

Materials and methods

Plant materials

On-site trees of *P. dulcis* cv 'Butte,' *P. webbii* (Accession No. DPRU 197, F8 15-33 from the USDA/ARS National Clonal Germplasm Repository, Davis, CA) and hybrid P 63-61 provided scion materials for dormant grafting. P 63-61 is a 'Butte' ×

P. webbii hybrid created in 1994 as part of a kernel flavor study (Ledbetter and Pyntea 2000). This hybrid has similarities with both ‘Butte’ (edible kernels) and *P. webbii* (pubescent leaves), and is more vigorous vegetatively as compared with either parent.

One-year old ‘Nemaguard’ peach seedlings (Reisner Nursery, Visalia, CA) of 6.4 mm (1/4”) caliper were used as rootstocks for the dormant scions. Dormant rootstocks were planted in 15.6 l pots filled with # 4 Sunshine Mix (SunGro, Seba Beach, AB, Canada), providing sufficient volume for root development during the three season growing period. Three weeks after planting, rootstocks were whip-grafted (65 trees, each accession) in mid-January 2006, and allowed to develop and break bud in the ambient environmental conditions. Growing grafted trees were then moved to a climate controlled greenhouse (growing season temp: min 15°C, max 33°C) in early-March. Scions were trained to a single growing bud. Cultural practices including irrigation, fertilization and insecticides were administered as needed to maintain an optimal environment for plant growth.

Trees were maintained in the greenhouse during the normal growing seasons (March–October), and moved outside to the ambient environment for the onset of chill accumulation. The potted trees accumulated chill hours until vegetative bud swell, after which they were returned to the greenhouse environment. Tree mortality was tabulated as trees returned to the greenhouse, and surviving trees were pruned back to the primary overwintered stem, approximately 15 cm above the graft union. Vegetative buds pushed quickly in response to the warmer greenhouse environment, and surviving trees were trained to a single shoot for the next season’s growth and development.

Xylella inoculations

Xylella fastidiosa strain ‘M23,’ previously isolated from ALSD affected almond trees in Kern County of California, was used for all tree inoculations. Strain M23 is a G-genotype and pathogenic to both almond and grape. Bacteria were grown in periwinkle (PW) medium as described by Davis et al. (1981). Log phase bacterial cells (10–14 days) were harvested following previously described procedures (Chen et al. 2008). Scion inoculations were accomplished by needle penetrations at the basal portion (5–10 cm) of

current season growth on stem tissue of approximately 4–6 mm diameter. Approximately 10 µl of inoculum was expressed for each inoculation and two inoculations were performed on each tree at each of two locations, spaced 1–2 cm apart. Immediately after inoculation, scions were pruned to a uniform height, 10–15 cm above the inoculation site.

Diagnostic sampling

Sample collection and preparation

Almond leaf samples were collected with intact petioles from all growing trees at designated sampling dates. Initial samples were collected between the inoculation site(s) and pruning cut at 10 weeks after needle inoculation. Additionally, stem samples were collected approximately 10–15 cm below growing apices at this initial date. During the first growing season, samples were only used for diagnostic PFT-PCR. In subsequent growing seasons, leaf samples were first used for bacterial isolation from petiolar tissue, with the same piece then later used for PFT-PCR.

Bacterial isolation and cultivation

Procedures used for isolation and cultivation of *Xf* were described by Chen et al. (2005). Briefly, approximately 2 cm of petiole was separated from the leaf and surface sterilized. An incision was made in the center of the petiole. Xylem sap was expressed aseptically using a pair of flame-sterilized, needle-nose pliers onto the sterile surface of a Petri dish. An approximately 30 µl droplet of PW broth (Davis et al. 1981) was immediately added and thoroughly mixed with the sap. One 5 µl loopful of sap mixture was then streaked onto Gelrite (Sigma-Aldrich, Inc. St. Louis, Mo) solidified PW medium (PW-G) and incubated at 28°C. The appearance of opalescent colonies was monitored using a binocular microscope for up to 40 days following isolation attempts. Candidate isolates were transferred onto new PW-G and confirmed as *X. fastidiosa* by PCR using 16S rDNA primers (Chen et al. 2005).

Freeze-drying and sample pulverization

Almond leaves or petiole leftovers from *Xf* isolations were placed in labeled paper envelopes and freeze-

dried using a Freezone 2.5 Freeze Dry System (Labconco Corp., Kansas City, Mo). Almond petioles were sufficiently dry with an overnight (>12 h) treatment following the manufacturer's recommendation (temperature <−40°C; and vacuum <1.33 × 10^{−3} mBAR). The dried samples were used immediately or stored at 4°C in plastic bags. Individual freeze-dried leaf petioles (approximately 2 cm in length) were placed in 2 ml microtubes with sterile ceramic beads and pulverized to a fine powder with a 20 s treatment in a Fast-Prep machine (FP120, Qbiogene, Inc., Carlsbad, CA). The pulverized freeze-dried tissue (PFT) was suspended in 500 µl of 1% TE buffer and used for PCR after 1:100 dilution with 1% TE buffer.

PCR procedures

PCR reaction (25 µl) was carried out using the TaKaRa Taq™ (Hot Start Version) kit (Takara Bio Inc., Seta 3-4-1, Otsu, Shiga, 520-2193, Japan). The reaction mixture contained: 2.5 µl of 10× DNA polymerase buffer, 2.5 µl of dNTPs (2.5 mM of each dNTP), 0.5 µl of each of the 10 µM forward and reverse primers, 1 µl of diluted petiole suspension, 0.2 µl of *Taq* DNA polymerase (5 U/µl) and 18.3 µl of H₂O. The multiplex PCR procedure (Chen et al. 2005) was used for *X. fastidiosa* detection. Briefly, primers Teme150fc (5' tctaccttat cgtgggggac 3') and Teme454rg (5' aacaactagg tattaaccaa tgcc 3') specific to G-genotype, and primers Dixon454fa (5' cctttgttg gggaagaaaa 3') and Dixon1261rg (5' tagctcacc tcgcgagatc 3') specific to A-genotype, were used for PCR amplification in an MJ Research Tetrad II DNA engine with an initial denaturing 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 stored at 4°C. The amplified DNAs were resolved through 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

Statistical procedures

ANOVA was used to test the effects of accession on levels of symptomatic leaves and primary axis die-back during the first growing season. If a significant ($P \leq 0.05$) *F*-test statistic was obtained, Tukey's Honestly Significant Different test was used to com-

pare differences among means. Pearson pairwise correlations were constructed to evaluate the degree of correspondence between diagnostic methods (symptomatic leaves, positive PCR reactions, successful cultivation of *Xf*) during the second and third growing seasons. All data were managed using JMP stats (Cary, NC, SAS, Version 7.0).

Results

First season disease development

Almond leaf scorch disease symptoms were first observed in the eighth week after inoculation. Symptoms were first noticed in *P. webbii* replicates, but by week 10, classic ALSD symptoms became apparent to varying degrees, progressing acropetally, in each of the three accessions. Observed ALSD symptom expression began on leaves a few nodes above the inoculation site. Leaf blade color dulled, and then began to yellow irregularly in patches at the leaf margins. As the yellowed leaf mesophyll tissue lost moisture, affected leaf blade portions became a tan color until they were eventually dry and necrotic. 'Butte,' *P. webbii* and P 63-61 trees differed significantly ($P \leq 0.05$) in the extent of symptomatic leaves present 10 weeks post inoculation (Table 1). *P. webbii* trees had a much higher percentage of ALSD symptomatic leaves as compared with 'Butte' or P 63-61. However, the three accessions had similar proportions (96.2% for 'Butte' and *P. webbii*, 91.9% for P 63-61) of *Xf* positive petiole samples near the inoculation site based on PCR analyses. Detection frequencies of *Xf* in stem samples collected near meristematic apices varied greatly amongst the three groups of scions. Specifically, positive PCR signals from stem tissues were obtained from 94.1% of 'Butte' scions whereas only 62.7% of the P 63-61 scions were scored as positive (Table 1). Of the 167 trees evaluated 10 weeks post inoculation, only a single P 63-61 tree failed to produce a positive PCR signal in either the petiole or stem sample. This single P 63-61 tree (no ALSD symptoms) was removed from the test at the end of the first growing season.

Symptoms continued to develop on scions of all three accessions throughout the growing season. ALSD symptoms differed slightly in *P. webbii* as

compared with symptoms on ‘Butte’ and P 63-61. Relative to the described ALSD symptoms of almond, *P. webbia* exhibited a more general leaf yellowing, with fewer necrotic patches on leaves or leaf margins. Defoliation was more prominent on *P. webbia* as compared with ‘Butte’ and P 63-61. By October, many of the *P. webbia* scions were nearly completely defoliated. In contrast, late season ‘Butte’ and P 63-61 scions were only partially defoliated, with scorched leaves held firmly in place throughout the top halves of the scions’ canopies.

The inoculated trees were evaluated for primary axis dieback at the beginning of November, just prior to moving trees outdoors for winter chill accumulation. Significant ($P \leq 0.05$) differences were noted in the percentage of primary axis dieback between the three groups of scions. Primary axis dieback was significantly ($P \leq 0.05$) higher in ‘Butte’ as compared with either *P. webbia* or P 63-61 (Table 1), and one of the ‘Butte’ scions died back completely to the graft union during October (Fig. 1). Dieback of the primary axis was never observed in scions unless they were first expressing leaf scorch symptoms. While scorch symptoms were the primary indication of an initial *Xf* infection, the appearance of collapsed meristematic tissues, spreading basipetally, indicated a more advanced development of ALSD.

Scions were left unpruned for the winter, and experienced ambient outdoor temperatures from 3 November 2006 until 16 February 2007. During that period the potted trees received a total of 1,039 chill hours ($<7^{\circ}\text{C}$), with a temperature maximum occurring on 7 November (25.9°C) and a minimum (-5.7°C) on 16

January (Fig. 2a). Prior to resumption of growth in the greenhouse environment, scions were again pruned back to a single 8–10 cm segment of primary axis.

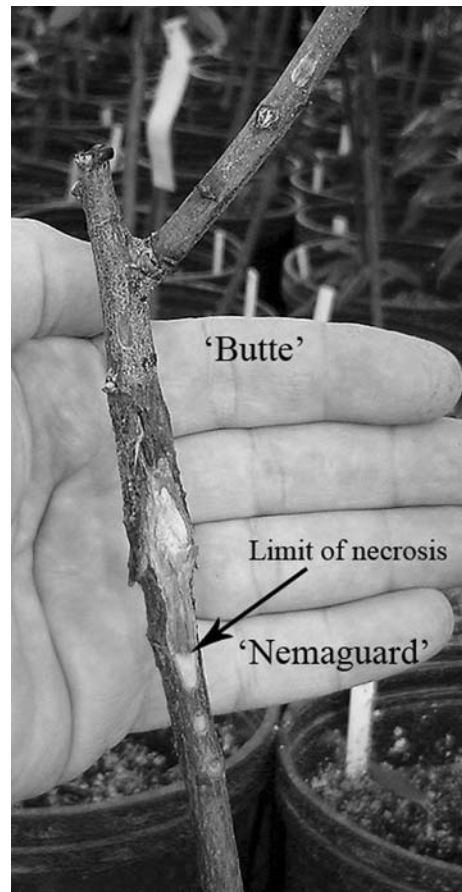


Fig. 1 Graft union region of ‘Butte’/‘Nemaguard’ tree near the end of its first season growth. Primary axis dieback proceeded to and terminated at the graft union, as evidenced by the limit of necrotic vascular tissues

Table 1 Symptomatic leaves, primary axis dieback and *X. fastidiosa* diagnoses as determined by PCR 10 weeks post inoculation in three almond accessions

	Symptomatic leaves (%)	Primary axis dieback (%) ^a	Positive PCR signals (% of population)		
			Petiole ^b	Stem ^c	
Butte	$n = 52$	9.8 ± 3.0 c ^d	23.1 ± 2.4 a	96.2	94.1
<i>P. webbia</i>	$n = 53$	54.4 ± 3.0 a	12.0 ± 2.4 b	96.2	83.0
P 63-61	$n = 62$	39.1 ± 2.8 b	0.1 ± 2.2 c	91.9	62.7

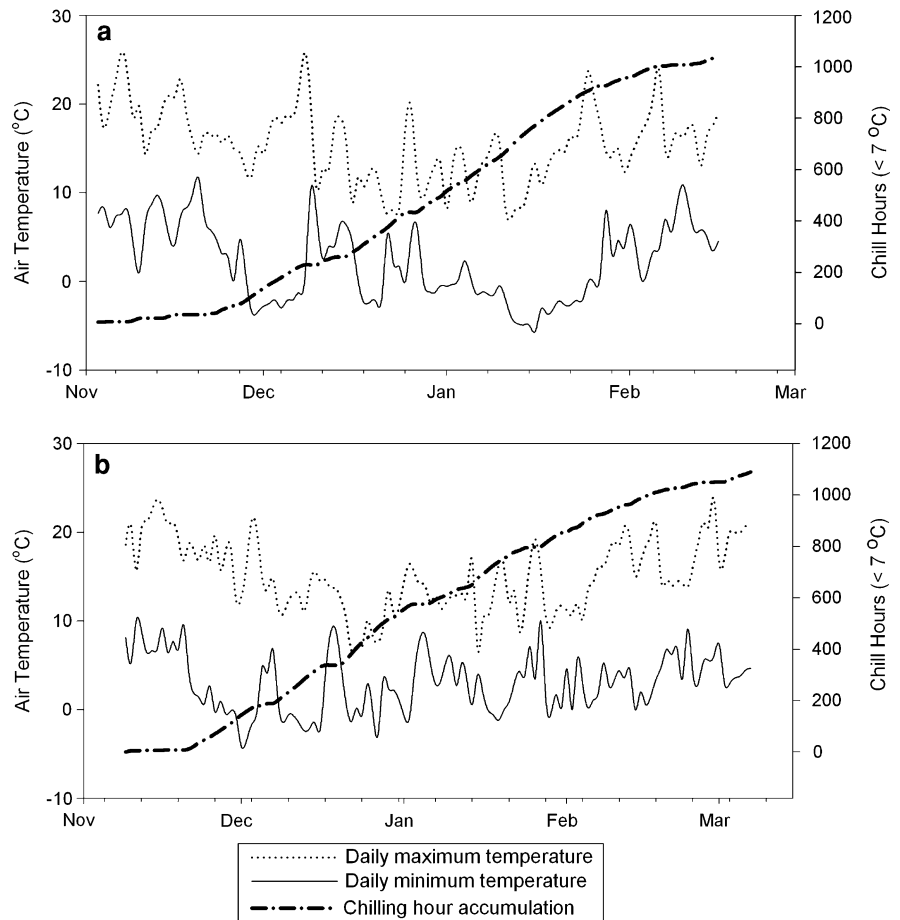
^a Primary axis dieback was scored in early November, prior to the beginning of winter chill accumulation

^b Petiole sample taken near site of inoculation, within region of symptomatic leaves

^c Stem sample taken within 15 cm of apical meristem, a region without symptomatic leaves

^d Values represent mean \pm standard error. Means followed by the same letter within the column do not differ significantly ($P \leq 0.05$) according to a Tukey’s Honestly Significant Difference test

Fig. 2 Winter minimum and maximum daily air temperatures ($^{\circ}\text{C}$) and chill hour accumulation ($<7^{\circ}\text{C}$) for 2006–2007 (a) and 2007–2008 (b) winters in Parlier, CA



Second season disease development

A single *P. webbii* tree scored previously with 98% primary shoot dieback failed to break bud, and it was assumed that tree death was caused by *Xf*-induced ALSD. All other scions began to grow rapidly after being brought back into the greenhouse environment. ALSD symptoms developed more slowly during the second growing season, with visible symptoms first appearing in early June on 'Butte' scions. By July, 27/51 'Butte' scions exhibited strong and obvious ALSD symptoms, whereas the majority of *P. webbii* and P 63-61 scions remained asymptomatic. ALSD symptoms continued to intensify on 'Butte' scions throughout the summer months, with 30/51 scions being scored as symptomatic at the September diagnostic period (Table 2). Four of the 52 *P. webbii* scions and 2/61 P 63-61 scions were putatively scored with ALSD symptoms in June, but no scions of these two accessions were evaluated with ALSD symptoms in

September. Three of the 52 *P. webbii* scions did exhibit 5–50% primary axis dieback although these affected scions did continue vigorous growth from lower portions of the scions. No scions of either *P. webbii* or P 63-61 were lost during the second growing season, whereas seven of the 51 'Butte' scions (13.7%) collapsed, with necrosis developing from the primary shoot apex, and spreading basipetally to the graft union.

Scions were sampled during the second growing season in June and again in September for the presence of *Xf* through plating on PW-G medium and through PCR-based analysis. In the June analysis, PCR detected similar percentages of *Xf* infected scions in 'Butte' and *P. webbii* (39/50 vs. 37/52, respectively), but failed to detect any infections in P 63-61 scions. Petiolar sap from both *P. webbii* and P 63-61 produced no *Xf* colonies, whereas 35/50 (70%) samples of 'Butte' petioles produced growing colonies of *Xf*. By the September sampling period, *Xf* was still

Table 2 Diagnostic analyses for *X. fastidiosa* in scions of three almond accessions during second (2007) and third (2008) growing seasons after exposures to ambient winter conditions in Parlier, CA

Accession	Year	June diagnoses			September diagnoses		
		Symptomatic trees	In vitro culture	PCR	Symptomatic trees	In vitro culture	PCR
	2007						
Butte		27/51	35/50	39/50	30/51	25/42	32/42
<i>P. webbii</i>		4/52	0/52	37/52	0/52	0/52	17/52
P 63-61		2/61	0/61	0/61	0/61	0/61	11/61
	2008						
Butte		28/41	31/41	16/41	31/40	28/40	31/40
<i>P. webbii</i>		3/40	0/40	1/40	0/40	0/40	1/40
P 63-61		0/11	0/11	0/11	0/11	0/11	0/11

uncultivable from *P. webbii* and P 63-61, whereas ‘Butte’ yielded 25/42 (60%) petioles with culturable levels of *Xf* present. Approximately equal proportions of PCR positives (39/50 = 78% vs. 32/42 = 76%) were obtained for ‘Butte’ during the June and September harvest periods, taking into account the seven scion deaths occurring between the sampling periods. Between the June and September sampling dates, PCR analyses revealed a 2-fold reduction in the number of positives in *P. webbii* scions (37/52 vs. 17/52, respectively). Detection of *Xf* in P 63-61 scions was observed in only 11 of the 62 scions (18%) during the September sampling period (Table 2).

Considering the results of the June and September analyses, only a portion of the trees were prepared for subsequent overwintering at the beginning of November. Specifically, trees were saved for the next season’s analyses only if *Xf* had been detected in one or both of the second season sampling periods, through either culturing or through PCR analysis. Hence, 43, 40 and 11 trees of ‘Butte,’ *P. webbii* and P 63-61, respectively, began the overwintering period in ambient temperatures after the second growing season. After two full seasons of growth in a greenhouse environment (single cycle of ambient winter exposure), percentages of inoculated trees from ‘Butte,’ *P. webbii* and P 63-61 that winter cured from *Xf* infections were 1.9%, 24.5% and 82.2%, respectively.

Third season disease development

Surviving *Xf* infected trees were exposed to ambient winter temperatures from 9 November 2007 through 7 March 2008. During this period, trees experienced a

temperature minimum of -4.2°C on 1 December, and a total chill accumulation of 1,089 h. Temperature maximums during the winter period were 23.8°C on 15 November and 23.9°C on 29 February (Fig. 2b). Two ‘Butte’ trees collapsed during the 2007–2008 winter period, leaving 41 ‘Butte’ trees to begin the third growth season. As observed in past ‘Butte’ losses, the primary axes of these trees began to darken, shrivel and die back, with necrosis spreading basipetally to the graft union. No trees of *P. webbii* or P 63-61 were lost during the second winter period.

Many of the ‘Butte’ scions developed ALSD symptoms by the end of May, approximately 12 weeks after being brought into the greenhouse environment. Possible ALSD symptoms were also noted on leaves of three *P. webbii* scions during the June 2008 sampling period (Table 2). Diagnoses of symptomatic leaves, culturing of petiolar sap and petiole PCR analyses all yielded numerically different results for ‘Butte’ during the June and September sampling periods. Apart from the ‘Butte’ scions, a single scion of *P. webbii* was diagnosed positive, based on PCR analyses, in both the June and September analyses. These positive detections occurred in different *P. webbii* scions in the June and September diagnostic periods. Moreover, neither of these was among the three symptomatic scions noted during the June diagnosis.

Overall tree survival throughout the study, and percentages of the initial populations that successfully winter cured, are listed in Table 3. Tree losses were significant in ‘Butte,’ with only 73.1% of the population surviving at the end of the study. Approximately equal proportions of ‘Butte’ trees failed to survive

Table 3 Tree survival and winter curing success from *X. fastidiosa* infections for ‘Butte,’ *P. webbii* and P 63-61 over two ambient winter periods in Parlier, CA

	Initial tree pop	After 1st ambient winter		After 2nd ambient winter		Final	
		Surviving trees	Winter cured	Surviving trees	Winter cured	Surviving pop. (%)	Winter cured (%)
Butte	52	44	1	38	10	38/52 (73.1)	11/52 (21.2)
<i>P. webbii</i>	53	52	13	52	37	52/53 (98.1)	50/53 (94.3)
P 63-61	61	61	50	61	11	61/61 (100)	61/61 (100)

during each winter period. Only a single tree was lost in the *P. webbii* population, and no trees were lost for P 63-61. Trees were classified as winter cured only when no ALSD symptoms were observed during the subsequent growing season, and neither a positive PCR signal nor a successful bacterial isolation was realized from that tree. With these criteria in mind, the entire population of P 63-61 trees was successfully winter cured, as were all but three of the 53 *P. webbii* trees. In contrast, only 21.2% of the ‘Butte’ population winter cured successfully after two ambient winter cycles.

Several inconsistencies were observed between ALSD symptoms, ability to isolate *Xf* in culture and positive PCR reactions. In particular, positive PCR reactions were frequently noted in trees that were devoid of ALSD symptoms and could not be successfully cultured. There were other cases where positive results were obtained from culturing but negative results from PCR analysis of the same scion. Pooled data of all three accessions from summertime harvest periods after the first and second winters were evaluated to establish correlations between visual ALSD symptoms, successful *Xf* isolations and PCR positive reactions. The highest correlation (0.753) noted occurred between ALSD leaf symptoms and successful *Xf* isolations, whereas the lowest correlation (0.465) was observed between ALSD symptoms and positive PCR reactions (Table 4). Among the individual accessions in this study, diagnostic methods exhibited significant correlation coefficients in ‘Butte’ only, while essentially no correlations existed between the three diagnostic methods in accessions *P. webbii* and P 63-61 during summertime rating periods.

Discussion

Initial diagnostic results from the first growing season demonstrate clearly that ‘Butte,’ *P. webbii* and P 63-61

Table 4 Pearson correlation coefficients between ALSD leaf symptoms, PCR reactions and *X. fastidiosa* isolations for ‘Butte,’ *P. webbii* and P 63-61 during the second and third growing seasons

Accession	ALSD symptoms	<i>Xf</i> isolations	PCR positives
Butte (<i>n</i> = 166)			
ALSD symptoms	1.000		
<i>Xf</i> isolations	0.547**	1.000	
PCR positives	0.356**	0.406**	1.000
<i>P. webbii</i> (<i>n</i> = 184)			
ALSD symptoms	1.000		
<i>Xf</i> isolations	0.000	1.000	
PCR positives	0.012	0.000	1.000
P 63-61 (<i>n</i> = 148)			
ALSD symptoms	1.000		
<i>Xf</i> isolations	0.000	1.000	
PCR positives	0.033	0.000	1.000
Accessions combined (<i>n</i> = 498)			
ALSD symptoms	1.000		
<i>Xf</i> isolations	0.753**	1.000	
PCR positives	0.465**	0.524**	1.000

** Indicates significance at the $P \leq 0.01$ level for Pearson product-moment correlations

are all susceptible to *Xf*. Inoculation success was very good, with more than 90% of inoculated trees yielding classic ALSD symptoms on primary leaves and PCR positive reactions from petioles collected near the inoculation site. While ‘Butte’ is said to be an almond cultivar ‘less-susceptible’ to *Xf* as compared with other California adapted almonds (Daane and Purcell 2005), it is certainly of equal susceptibility to artificially inoculated *Xf* as compared with *P. webbii* and P 63-61, based on inoculation success and initial leaf symptom diagnoses. PCR diagnoses from stem tissues near the meristematic apex were more variable, ranging from 94.1% (‘Butte’) to 62.7% (P 63-61). In all cases, stem tissues were sampled approximately 60 cm from the point of

inoculation, and in no case were symptomatic leaves present near the region where stems were sampled. These results contradict those of Hopkins and Thompson (1984) who did not observe *Xf* in actively growing juvenile tissues of *Xf*-infected Muscadine grapes.

Over the course of three growing seasons, clear differences became evident in the three accessions' abilities to winter cure *Xf* infections. Specific climatic conditions during the winter likely affect the extent of this winter curing phenomenon. Duration and intensity of cold temperatures have been shown to affect *Xf* survival in both laboratory and field studies (Feil and Purcell 2001; Purcell 1980). Conversely, hot water treatments have effectively eliminated *Xf* from pecan scion wood, and thereby reduced pathogen transmission during propagation (Sanderlin and Melanson 2008). These results demonstrate the critical temperature dependency of *Xf* population survival in plants, and detached plant parts.

Winter temperature minimums and the extent of chilling accumulation during the study period were considered average for the central San Joaquin Valley during the study period. The minimum temperatures experienced by trees in this study during the first winter (-5.7°C) and second winter (-4.2°C) were well above record minimum temperatures registered during the last 120 years. Temperatures of -6.7°C or lower have occurred 16 times during that period, with an absolute minimum being -8.3°C . In the last 15 years, chill hours have ranged from approximately 700–1,400 h during the winters, averaging 982 h for the period. Thus, the degrees of wintercuring realized in this study occurred during winters that were not unseasonably cold nor exceptionally long.

Movement of *Xf* across a graft union has been clearly demonstrated (Hutchins et al. 1953; Sanderlin and Melanson 2006), and root systems have been suggested as an overwintering location for *Xf*, as an insulated location where the bacteria may escape the negative effects of low air temperatures (Henneberger et al. 2004). However, root systems must also be susceptible to *Xf*, and at least one study indicates that peach root systems resist infection of *Xf* biotypes responsible for ALSD (Mircetich et al. 1976). Given that the current study utilized potted trees placed on the bare soil surface exposed to ambient winter conditions, their insulating ability may have been somewhat less as compared with orchard-rooted trees. Further, the necrotic front of primary axis dieback

stopped abruptly at the graft unions of our almond scions with the 'Nemaguard' peach seedling rootstocks (Fig. 1). Since graft unions in this study were several cm above the soil surface, our estimates of *Xf* winter curing rates for these three accessions might therefore be higher than that which would be otherwise experienced in a commercial orchard.

In this study, winter curing of *Xf* infections was much more successful in *P. webbii* and P 63-61 as compared with 'Butte.' These results are encouraging, and suggest a possibility of developing adapted almond varieties that are capable of clearing *Xf* infections through the winter curing phenomenon. It has previously been hypothesized that *P. webbii* was a wild species progenitor responsible for the introduction of self-compatibility into edible almonds (Browicz and Zohary 1996). Our current study further demonstrates the utility of this undomesticated almond species. While not resistant to *Xf* infections, the screening of this Old World plant introduction against *Xf* has demonstrated that *P. webbii* is clearly more capable of pathogen elimination through the winter curing process compared with 'Butte,' a cultivar said to be of moderate *Xf* susceptibility. Furthermore, the F₁ hybrid P 63-61 is capable of winter curing from *Xf* infections at least as well as *P. webbii*, and has improved vegetative and carpological characters. Backcross seedlings (P 63-61 × Butte, P 63-61 × *P. webbii*) have now been secured, and will be developed to provide sufficient clonal materials for *Xf* screening. Concurrent horticultural evaluations and pathological screening of this germplasm will be necessary to identify those elite genotypes with superior nut characteristics and the enhanced ability to recover from *Xf* infections.

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