EFFECTS OF GROUP, CULTIVAR, AND CLIMATE ON THE ESTABLISHMENT AND PERSISTENCE OF *XYLELLA FASTIDIOSA* INFECTIONS CAUSING ALMOND LEAF SCORCH

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ABSTRACT
Almonds are one of the most widely-grown crops infected by *Xylella fastidiosa (Xf)*. To get a better idea of the conditions that lead to almond leaf scorch outbreaks, and to determine the risks that *Xf* infections may pose to adjacent vineyards, three factors were assessed that may influence the establishment of *Xf* infections and almond leaf scorch development: almond cultivar, *Xf* genetic group, and winter severity. Experimental plots of 100 trees each were planted at two field sites, Armstrong Farm at UC Davis (UCD) and Intermountain Research and Extension Center at Tulelake (IRC). In field plots, equal numbers of highly susceptible 'Peerless' and less-susceptible 'Butte' almond trees were inoculated with grape and two almond *Xf* isolates. Because *Xf* infections must survive multiple winters in an almond tree cause almond leaf scorch, field sites were selected with moderate (UCD) and severe (IRC) winter temperatures. To better understand the role of cold temperatures in overwintering *Xf* infections, a controlled dormancy test was also done. Potted almond trees were inoculated with almond-type *Xf*, and infected trees held ind dormancy outside, or in cold rooms at 1.7ºC or 7ºC. Ten trees from each treatment were brought back into the greenhouse to break bud after 1, 2, or 4 months.

INTRODUCTION
Because almonds are one of the most widely-grown crops that can host *Xylella fastidiosa (Xf)* in the Central Valley, they might serve as a source of *Xf* infections in grapes, although for unknown reasons *Xf* dispersal between almond orchards and vineyards is uncommon (A. Purcell – *unpublished data*). Almond leaf scorch (ALS) is caused when *Xf* multiplies extensively within the xylem of infected trees, eventually severely limiting nut production (Davis et al. 1980). The disease was first formally described in 1974, and outbreaks occurred in Los Angeles and Contra Costa counties in the 1950's (Moller et al. 1974). Symptoms of ALS are similar to Pierce's disease in grapes and include leaves with marginal necrosis and chlorosis and poor terminal growth. Initial infections spread slowly and often occur only on one branch, but after a few years are visible on the entire tree (Almeida and Purcell 2003c), reducing almond productivity (Mircetich et al. 1976, Moller et al. 1974). In both grapes and almonds, *Xf* multiplies to high populations (1,000,000 bacteria per gram of plant tissue) and is acquired and transmitted by insect vectors (Almeida and Purcell 2003a, Almeida and Purcell 2003c, Purcell 1980a). In laboratory tests, *Xf* was transmitted to almonds by 5 species of xylem-feeding insects, including the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis;* formerly *H. coagulata*) (Almeida and Purcell 2003b, 2003c).

In previous studies, almond cultivars varied greatly in their susceptibility to ALS, with some developing extensive leaf scorch, and others showing little disease. *Xf* inoculations made from May through July had the best odds of surviving the following winter. (B. Kirkpatrick – *unpublished data*). We compared *Xf* infection establishment and survival in two cultivars, highly susceptible 'Peerless' and less-susceptible 'Butte'.

The genetic type of *Xf* may affect development of almond leaf scorch. Cross-inoculation studies in the greenhouse showed that the genetic type influenced the ability of the bacteria to over winter in grapes or almonds, as almond types died in grapes and grape types died in almonds (Almeida and Purcell 2003c). For this reason, we also used different genetic types of *Xf* in our field trials. Three genetic types of *Xf* have been identified from almond trees. One type was identical to *Xf* from Pierce's disease infected grapevines. The other two genetic types were unique to almonds (Hendson et al. 2001). The three types were distinguished by growth on selective media and DNA digestion with restriction enzymes (Almeida and Purcell 2003c).

After vector inoculation, *Xf* must survive multiple winters in an almond tree to reach sufficient populations for sharpshooter acquisition and economic impact disease levels. Growth chamber and field studies with grapevines showed that the degree of plant dormancy, as well as severe cold, affected the over winter survival of *Xf* (Feil and Purcell 2001, Purcell 1980b). To date, there is no information available on the effects of winter dormancy on *Xf* infections in almonds. Therefore, field sites were selected with moderate and severe winter temperatures (Armstrong Farm at UCD, and IRC at Tulelake, CA, respectively) in order to study treatment impact under different winter temperatures. A controlled dormancy severity test with potted plants and growth chambers was also started at Kearny Agricultural Center, near Parlier, California.

OBJECTIVES

1. Compare the establishment and multi-year persistence of *Xf* isolates belonging to three ALS genetic groups in almond cultivars with either low or high susceptibility to almond leaf scorch.
2. Compare effects of winter severity and the degree of plant dormancy on the infection rate, symptom severity, and titer of *Xf* in inoculated almonds.
3. Use collected data on almond leaf scorch development to determine if almond orchards may serve as a reservoir of *Xf*.

RESULTS

Field trials. One hundred bare-root almond trees, 50 of each cultivar, were planted in spring 2005 at two different field sites: Armstrong Farm at University of California, Davis, CA (UCD), and Intermountain Research and Extension Center, Tulelake, CA (IRC). Trees were planted in a complete randomized block design with a split plot (almond cultivars) in each block. There were ten replicates of each treatment combination (*Xf* isolate × almond cultivar). Trees are drip irrigated at UCD and sprinkler irrigated at IRC.

The almond trees were inoculated with different genetic types of *Xf*, either Fresno-ALS (isolated from almonds but genetically similar to *Xf* that causes Pierce's disease in grapes; PD-Xf), Dixon (ALS-*Xf* type 1) and ALS 6 (ALS-*Xf* type 2), Medeiros (from grapes), or buffer control. All isolates of *Xf* were isolated from infected plants in Solano, Fresno, or San Joaquin Counties, and were pathogenic in recent greenhouse tests. In 2005, inoculations were done in early May (UC Davis) and early July (IRC) when the young shoots were at least 6 mm in diameter. Inoculum was prepared in the field from two week old cultures of *Xf*. Each tree was inoculated with approximately 100,000 CFU of *Xf* following Hill and Purcell 1995. Inoculation sites were marked with permanent metal tags and paint. Twenty trees at UCD were re-inoculated on May 15, 2006 with ALS-6 or Dixon *Xf* strains. Leaves immediately adjacent to the inoculation sites were tested for *Xf* in fall 2005 to determine the number of successful inoculations, bacterial titer, and symptom development in field-grown almond trees.

Two trees at UCD died in winter 2006, and 64 trees (or inoculated branches) died at IRC. The infected trees remaining with living inoculated branches were evenly distributed among isolate treatments, with 7 buffer-inoculated, 5 ALS6-inoculated, 3 Dixon-inoculated, 2 Fresno-inoculated, and 6 Medeiros-inoculated trees surviving. While mortality was high, similar losses were seen in previous studies examining the over winter survival of *Xf* in grapevines (Purcell 1980b). Assessment of the number of over-wintering infections is ongoing in mid-September 2006. Trees will also be evaluated for the presence of *Xf* in 2007 and 2008. The severity of infection was rated by the number of scorched leaves on the inoculated stem. Almond petioles from each tree were cultured to determine *Xf* infection and population. Strain identification of *Xf* was done by re-streaking growing bacteria on two different artificial media, PD3 and PWG (Davis et al 1980, Hill and Purcell 1995). All types of *Xf* grow on PWG, while ALS-*Xf* type 2 and Pierce's disease types grow on PD3 as well. ALS-*Xf* type 1 does not (Almeida and Purcell 2003c). To separate ALS and PD isolates, polymerase chain reaction (PCR) was used to amplify DNA from the bacteria, and *Rsa 1,* a restriction enzyme, cut the DNA of ALS-*Xf* isolates into two pieces, but did not cut the DNA of PD-*Xf* (Almeida and Purcell 2003c).

In 2006, the onset of almond leaf scorch symptoms was delayed in trees at UCD. Despite sampling two weeks later than in 2005, *Xf* was recovered from only one tree inoculated in 2005 and one inoculated in 2006. The *Xf* populations in those two trees in 2006 were 10-fold lower than populations recovered from similarly infected trees one year previously. Almond leaf scorch symptoms were only visible on the trees that tested positive via culture. Re assessment of trees for disease symptoms and *Xf* presence is planned in late September.

In 2005, almond leaf scorch symptoms were much more severe at UCD, especially in 'Peerless' trees, with an average of 4.6 scorched leaves per tree, compared to 0.8 in 'Butte'. Both cultivars at IRC had no scorched leaves, an average of 0.2 and 0.1 leaf per tree for 'Butte' and 'Peerless', respectively. However, there was no difference in the proportion of infected trees at UCD (32 of 78 infected at UCD, 41 of 96 infected at IRC; Chi-square $P > 0.05$), nor in the median populations of *Xf* present in inoculated trees at UCD (6.2×10^6 CFU/g) or IRC (1.3×10^7; $\log_{10}$-transformed; $P = 0.26$). The difference in symptoms may have two explanations: i) trees at UCD were tested for *Xf* 3.5 months after inoculation and had longer to develop symptoms, compared to trees at IRC, which were tested 2 months after inoculation; or ii) the infected trees were under more moisture stress at UCD, which led to the development of disease symptoms.

While it is too soon to tell how infections overwintered in 2006, in 2005 there were not large differences between infection percentage (41% of 'Butte, 38% of 'Peerless'; Chi-Square $P>0.05$), or *Xf* population (2×10^6CFU/g for 'Peerless' and 9×10^6CFU/g for 'Butte; $\log_{10}$-transformed; $P = 0.11$) for the two cultivars. 'Peerless' had much fewer scorched leaves than 'Butte' at UCD, but not at IRC, as discussed in the previous paragraph. Also in 2005, grape strain *Xf* was more frequently recovered from inoculated trees than either almond strain. Fresno and Medeiros were recovered from 64 and 77% of trees, respectively, whereas ALS6 and Dixon were recovered from 27 and 28% of trees. Leaf scorch symptoms were more severe in trees inoculated with grape-type isolates Fresno and Medeiros (an average of 2.8 and 3.2 scorched leaves/tree), compared to almond isolates Dixon and ALS6 (0.3 and 0.9 scorched leaves/ tree), and background leaf scorch in buffer-inoculated trees (0.1/ tree).

In 2005, bacterial populations in trees infected with grape and almond isolates were similar, even though infection percentage and symptom severity was greater in grape isolates of *Xf*. Median populations of *Xf* in infected trees were: 6.2 x 10^6 CFU/g (ALS6), 2.8 x 10^6 CFU/g (Dixon), 5.5 x 10^6 CFU/g (Fresno), 2.4 x 10^7 CFU/g (Medeiros), and 0 CFU/g (buffer). Bacterial populations were high even in only a few trees in the treatment were infected with *Xf*, as in ALS6 inoculated plants at UCD. In future analyses, ANOVA will be used where applicable to detect differences in infection percentage and bacterial populations between cultivars and bacterial isolates. Temperature data collected from on-site weather stations will be compared for both sites as well, to determine the number of hours with temperatures outside the growth range of *Xf*.

Glasshouse and Growth Chamber trial. An additional experiment examined the effect of over wintering temperature in the survival of *Xf* infections in controlled environments. One hundred and fifty-five potted two-year-old 'Peerless' almond trees were inoculated in spring 2005, 125 with ALS 6 *Xf* and 30 with buffer alone. Trees were kept in the greenhouse and tested for infection in fall 2005. Ninety trees infected with *Xf*, and 27 buffer-inoculated trees were used for the rest of the experiment. Trees went dormant in screen cages outside, and were divided equally between treatments in January 2006. One-third remained outside in the screen cage, 1/3 were kept in a cold chamber at 7°C (45°F), and 1/3 at 1.7°C (35°F). *Xf* dies at these temperatures in grapevines (Almeida and Purcell 2003c, Feil and Purcell 2001). Trees were removed from each cold treatment at intervals of 1, 2 and 4 months, and allowed to break bud in the greenhouse. These intervals were reflective of dormancy periods used in previous studies with almonds and grapevines (1 month; Almeida and Purcell 2003c, Feil and Purcell 2001), typical dormancy in the central valley (2 months; going fully dormant in December and flowering in February) and an extreme treatment for abnormally long dormancy (4 months). Plants were kept the greenhouse until they developed almond leaf scorch, in mid-August, then assessed for disease severity, and *Xf* presence and population as previously described.

Preliminary analysis of data collected in September 2006 indicated no differences in the number of symptomatic leaves between cold treatments, with trees held at 1.7ºC averaging 23.3 symptomatic leaves per tree, trees at 7ºC 24.3 symptomatic leaves per tree, and trees kept outside an average of 18.5 symptomatic leaves per tree. Trees exposed to one or two months of cold treatment had fewer symptomatic leaves than trees left outside or in the cold box for 4 months (1 month = 17.8 leaves, 2 months = 15.4, 4 months = 32.2), regardless of temperature. Initial implications suggest that almond *Xf* strains have different overwintering survival characteristics that grape *Xf* strains, as has been indicated by previous studies (Almeida and Purcell 2003c).

CONCLUSIONS

Analysis of data collected in August and September 2006 is ongoing; however some interesting preliminary results emerged. In 2005, ALS symptoms at UC Davis were more severe than at IRC, probably the result of the longer interval between inoculation and sampling (3.5 months for UCD and 2 months for IRC). The current data do not suggest an explanation why ALS group isolates infected trees more frequently at IRC than at UCD. In spring 2006, an additional 20 trees at UCD were inoculated with almond-type isolates to determine if they do not infect the trees as frequently, or if the low infection rate observed in 2005 was an anomalous result. Potted almond trees in our controlled overwintering study showed no "curing," in fact trees exposed to a 4-month dormancy had greater numbers of symptomatic leaves than trees dormant for one or two months. Not all field-grown trees have been analyzed for infection severity and *Xf* presence; complete data will allow better documentation of the fate of overwintering *Xf* infections.

Although the effect of cold on *Xf* infection survival was investigated in grapes (Feil and Purcell 2001, Purcell 1980b), there is little data on the effect of dormancy on bacterial over wintering in almonds. Previous studies suggested (Almeida and Purcell 2003) that almond-type *Xf* had better over winter survival in almonds. Since both grape and almond strains reached approximately the same titers in plants, and the grape strains in this study initially infected almond trees at a greater rate, over winter survival may be explain why almond strain *Xf* is so prevalent in naturally-occurring infections.

Table 1. Number of leaves showing almond leaf scorch symptoms on trees held under varying overwintering conditions of different temperatures and cold storage periods.

Cold Storage (in months)	Temperature (°C)		
	1.7	7.0	Ambient (Fresno Co.)
1	17.33 ± 8.22 a, A	35.14 ± 9.26 a, B	23.71 ± 8.21 a, B
2	22.67 ± 5.51 a, AB	22.22 ± 6.82 a, B	16.50 ± 6.37 a, B
4	50.00 ± 9.75 a, B	43.25 ± 7.18 a, B	30.50 ± 5.08 a, B

Different letters (small case) indicate a significant different in each row among tested temperatures under similar cold storage periods, and different letters (upper case) in each column among different cold storage periods (upper case) under similar temperatures. Tukey's test, $P < 0.05$.

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EVALUATING THE ROLES OF PILI IN TWITCHING AND LONG DISTANCE MOVEMENT OF *XYLELLA FASTIDIOSA* IN GRAPE XYLEM AND IN THE COLONIZATION OF SHARPSHOOTER FOREGUT

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ABSTRACT
Our investigations have focused on intra-plant movement and colonization by *Xylella fastidiosa* (*Xf*). This study is particularly directed toward elucidating how *Xf*, once introduced into xylem vessels, moves in these elements farther upstream (down shoots and canes). In addition, it reports on the influence of the physical and chemical environments of the xylem as they relate to *Xf* attachment, colony development, and biofilm formation in both plant tissues as well as microfludic chambers fabricated to mimic xylem elements. Toward these goals, we have made extensive use of mutants deficient for various traits of pili and fimbriae.

INTRODUCTION
How *Xylella fastidiosa* (*Xf*) moves in xylem elements upstream against the flow of the transpiration stream and into petioles from the leaf or down shoots and canes has long been a particularly puzzling and important question, the answer of which could provide clues about better disease control practices through knowledgeable timing of pruning or roguing. Certainly, it would provide significant advances in the biology of the pathogen. Our studies have been directed toward elucidating movement of *Xf in planta* and *in vitro*, as well as how these bacteria colonize and establish biofilms. Toward this, we have made use of a number of *Xf* mutants deficient in traits important for cell attachment, movement, and colonization. Previously, we reported that migration of individual *Xf* bacteria occurred both *in vitro* and *in planta*, and that such migration occurred against a strong current of flowing media (Meng et al., 2005; Hoch, 2005). Such movement is characteristic of twitching motility that occurs in some gram-negative bacterial species. It is mediated by type IV pili (Mattick, 2002). There are several important implications of this observation: this was not only the first observation of twitching movement by a non-flagellated plant pathogenic bacterium (albeit, *Ralstonia solanacearum*, that sometimes has flagella, has been shown to exhibit colony features characteristic of twitching (Liu et al., 2001)), it was also the first time that such movement by *Xf* was observed. Such motile behavior is important in explaining, in part, the query posed above about how the bacteria spread in the grapevine from an inoculation point to upstream locations.

Type IV pili are long (1-5.8 μm in length) filamentous appendages (a.k.a, fimbriae) located at either one or both poles, depending on the species (Bradley, 1980), are generally 5-7 nm in diameter, and may be up to several micrometers in length. They are assembled primarily from single structural protein subunits, pilin (PilA) (Mattick, 2002). Twitching movements are generated as the pili are retracted and dissembled. Because the pili tips are attached to the substratum, the cell moves toward that point of contact as the pili shorten (Mattick, 2002; Skerker and Berg 2001). Genomic analysis of *Xf* indicates that there are at least 26 genes related to pili synthesis and function (Simpson et al., 2000). In addition to the type IV pili, *Xf* has shorter (0.4-1.0 μm in length) type I pili, also positioned on the same cell pole. The unique dual-pili composition of the wild type *Xf* presents an opportunity to study the two types of pili comparatively in the same experimental setting. Biofilm deficient mutants (e.g., 6E11), the result of a disruption of the *fimA* gene (lacking type I pili), were previously shown to continue to migrate since they still possess the type IV pili; whereas, mutants deficient in genes that code for type IV pili (e.g., 1A2) are migration deficient and develop robust biofilms (Meng et al., 2005). Attachment of *Xylella* cells at their polar ends is well documented in the precibarium region of the sharpshooter foregut. At this point, however, little is known about how they attach in this orientation (other than the conjecture that the pili may be involved) to this preferred region, as opposed to other foregut regions. Additionally, little is known about how they detach from this region.

OBJECTIVES
Our goal is to understand how *Xf* colonizes plants and insects. One aim is to identify factors that contribute to attachment (and detachment) and migration of *Xf* cells on surfaces. Using wild-type and mutants of *X. fastidiosa*, we have proposed to