Differential Susceptibility of Prunus Germplasm (Subgenus Amygdalus) to a California Isolate of Xylella fastidiosa

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Additional index words. almond, hybrid, peach, rootstock, titer

Abstract. Seeding peach (Prunus persica Batsch) and clonal peach–almond hybrids are popular rootstock choices for commercial almond growers in California. In this study, clonal replicates of peach and almond (P. dulcis [Mill.] D.A. Webb) rootstock germplasm and a first-generation peach–almond hybrid created from them were challenged with Xylella fastidiosa isolate M23. Clonal replicates were needle-inoculated with M23 and maintained in a greenhouse environment for a growing season. Typical almond leaf scorch disease symptoms began to develop on M23-inoculated almonds 11 weeks after inoculation. No leaf scorch symptoms were observed on M23-inoculated peach or peach–almond hybrids. Quantitative real-time polymerase chain reaction revealed consistent levels of X. fastidiosa DNA among inoculated almond replicates, whereas X. fastidiosa DNA was undetectable in replicates of peach–almond hybrids. A trace level of X. fastidiosa DNA was detected in a single peach replicate, and statistical analysis demonstrated that this level differed significantly (P < 0.001) from that detected in almond replicates. Selected almonds were further sampled sequentially along their meristematic axes to examine bacterial titer throughout the trees. Selected almond trees differed significantly (P = 0.036) in bacterial titer, but no significant differences were noted in levels of X. fastidiosa DNA from different vertical sections of the main growth axes. The results suggest that peach and peach–almond hybrid rootstock germplasm used by commercial almond tree nurseries in California are not primary inoculum sources for X. fastidiosa-induced diseases.

Among the cultivated hardy trees and shrubs of North America, five subgenera are defined for Prunus by Rehder (1940), among which are the species of Amygdalus. This subgenus includes peach (P. persica), a widely grown tree throughout North America, and almond (P. dulcis), a tree nut cultivated almost exclusively in California orchards in North America. Both species are economically important for California and have significant acreage throughout its various growing regions. In 2007, almonds were a $2 billion U.S. crop grown on nearly 250,000 ha, whereas peaches (fresh market freestones and canning clings) occupied ≈25,000 ha in California (Anon., 2008).

Xylella fastidiosa (Xf) is a genetically diverse Gram-negative bacterium with numerous hosts (Freitag, 1951), including a number of Prunus species, typically causing irregular patterns of leaf necrosis or “scorching” in infected plants (Hopkins and Purcell, 2002). In California, Xf is responsible for both Pierce’s disease (PD) of grapes and almond leaf scorch disease (ALSD) in almond. Xf biotype differences based on single nucleotide polymorphisms of 16S rDNA sequences have been determined (Chen et al., 2005) with “A-genotype” biotypes causing only ALSD and “G-genotype” biotypes capable of causing both PD and ALSD. Using rootstock transmission studies and serology, Wells et al. (1981) demonstrated biotype differences for Xf causing both phony peach disease and plum leaf scald in the southeastern United States. Healthy plums grafted with infected root segments from peach trees expressing phony peach symptoms developed leaf scald symptoms after 9 months of growth. However, similarly, healthy peach trees grafted with roots from plum trees expressing plum leaf scald remained symptomless even after 2 years of growth (Wells et al., 1983). Similar results have been noted for Xf biotypes responsible for citrus variegated chlorosis and coffee leaf scorch diseases (de Lima et al., 1998; Li et al., 2001). Several other transmission and molecular-based studies have demonstrated the complexity of Xf biotype–host interactions with regard to pathogenicity and fulfillment of Koch’s postulates (Hernandez-Martinez et al., 2006; Miretich et al., 1976; Montero-Astúa et al., 2007; Raju et al., 1982). Both random and aggregated patterns of ALSD distribution were observed in sampled almond orchards (Groves et al., 2005), indicating differences in almond cultivar susceptibility to the Xf biotypes present and raising questions relative to the original source(s) of primary inoculum. Using geostatistics to determine spatial patterns of disease development, Roberto et al. (2002) concluded that commercial citrus nurseries were the primary sources of citrus variegated chlorosis-infected orange trees in citrus orchards; however, primary inoculum sources of ALSD in almond orchards are yet undetermined. Natural vegetation in habitats bordering California commercial grape and stone fruit orchards has been shown to harbor Xf and has been implicated as primary inoculum sources for PD in adjacent vineyards (Redak et al., 2004). When these same habitats border commercial tree nurseries, Xf-infected vectors are capable of inoculating susceptible rootstocks and budded trees throughout the growing season.

Susceptibility of almond to Xf biotypes causing PD and ALSD is well documented. Although accessions of peach are susceptible to phony peach Xf biotypes, several studies indicate that they resist infection by Xf biotypes responsible for PD and ALSD (Miretich et al., 1976; Wells et al., 1983). Seed-propagated peaches are used extensively in California as rootstocks for almonds and other stone fruit species with rootstock cultivars such as ‘Nemaguard’ being favored by growers because of resistance to root knot nematodes (Meloidogyne incognita and M. javanica). In cases in which more scion vigor is desirable, peach × almond (PEAL) hybrids are being used, predominantly in newer almond plantings. The susceptibility of PEAL hybrids to Xf isolates causing ALSD is currently unknown. In this study, we examined disease development and bacterial titer within parental peach and almond genotypes together with an interspecific hybrid created from them over the course of a growing season.

Materials and Methods

Plant materials. On-site trees of peach (P248-139), almond (Y113-20-99), and PEAL hybrid (062002) derived from them were used as clonal sources in the study. The trees are part of the Prunus breeding effort at the San Joaquin Valley Agricultural Sciences Center in Parlier, CA. P248-139 is an F₁ hybrid of ‘Harrow Blood’ × ‘Okinawa’ parentage created for the generation of vegetative diversity in the development of Prunus rootstocks. Accession Y113-20-99 (‘Tuono’ × 74-26) has been selected as a self-compatible hard-shell almond, but also demonstrates good performance as an almond seedling rootstock, having good field emergence rates under climatic conditions in the central San Joaquin Valley. PEAL hybrid 062002 was created as a prospective vigorous clonal rootstock selection for almond trees, and we therefore desired to examine its reaction to an aggressive Xf isolate endemic to central California. Clonal materials of these three

Received for publication 28 July 2009. Accepted for publication 3 Sept. 2009.

We thank Dr. Jianchi Chen for supplying the primer sequences. We gratefully acknowledge the technical assistance of Mark Schreiber and Sherry Peterson in the laboratory and greenhouse, respectively. Thanks are also given to Dr. Drake Stenger for helpful comments in the editorial review of the manuscript.

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accessions were dormant-grafted on 1-year-old ‘Nemaguard’ peach seedling rootstocks.

One-year-old ‘Nemaguard’ peach seedlings (Reisner Nursery, Visalia, CA) were used as rootstocks for the dormant scions. The dormant rootstocks were planted in 15.6-L pots filled with #4 Sunshine Mix (SunGro, Seba Beach, Alberta, Canada) providing sufficient volume for root development during the growing period. Three weeks after planting, rootstocks were whip-grafted during mid-Feb. 2008 and allowed to develop and break bud under ambient environmental conditions. Trees demonstrating vegetative bud swell on the grafted scion were then moved to a climate-controlled greenhouse in mid-March. Trees were maintained in a greenhouse throughout the growing season, and cultural practices, including irrigation, fertilization, and insecticides, were administered as needed to maintain an optimal environment for plant growth. Vegetative buds pushed quickly in response to the warmer bud swell on the grafted scion were then averaged to provide a single titer value for each tree. PCR was performed on each tree at each of two locations spaced 1 to 2 cm apart. Six trees of both inoculated and control peach and almond trees, with one peach tree, were sampled from each section of the three trees chosen for analysis. A single DNA extraction was performed from each petiole pair, and triplicate PCRs were performed.

**Xf quantification.** Xf titer in petioles was measured by quantitative real-time PCR. Total DNA was extracted from petioles by a protocol previously reported method (Dellaporta et al., 1983) and further purified using nucleotide removal columns according to the manufacturer’s instructions (Qiagen, Inc., Valencia, CA). PCR was performed on a Step One Plus real-time thermocycler using Applied Biosystem’s Sybr Green Master Mix according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Primers FX145-60F (5′-TATCATCGGAACTCTAC-3′) and FX145-60R (5′-CGTATTACGAGTTTC-3′) that amplify portions of the 16S ribosomal RNA gene were used for PCR. Total DNA was quantified by OD260 on a NanoDrop1000 spectrophotometer (ThermoScientific, Wilmington, DE).

**Statistical analyses.** Data were compiled and analyzed in JMP stats (Version 7; SAS, Cary, NC) using the “Basic” category to obtain means and SDs of initial real-time PCR analyses. A t-test was used, assuming unequal variances, to compare Xf titer differences between peach and almond replicates. Analysis of variance (ANOVA) was used to examine effects of tree replicates and tree vertical sections on Xf titer. Before ANOVA, data were subjected to tests to ensure homogeneity of variance. If significant F-test statistics were obtained, Tukey’s honestly significant difference test was used to compare differences among means.

### Results

Xf-inoculated and control trees grew vigorously in the greenhouse for the first several months without manifesting symptoms. In mid-July, ~11 weeks after inoculation, irregular patches of chlorosis/yellowing began to appear in leaves of inoculated almond trees. Discolored leaves first appeared just below the level where trees were initially pruned after inoculation. The discoloration intensified, leading to eventual necrotic patches (scorching) near leaf margins. At the same time, symptoms progressed acropetally in the inoculated trees (Fig. 1A). New leaves appeared along the main growing axis. By mid-September, scorching leaves were evident from the inoculation site to within 30 cm of the shoot apex. Noninoculated almond trees and all trees of peach and PEAL remained symptomless throughout the growing season. Initial PCR analyses of leaf petioles harvested near inoculation sites corresponded well with symptom development in Xf-inoculated trees. Aside from a trace amount observed in one peach tree, Xf DNA was quantified only in Xf-inoculated almond trees (Fig. 1). No Xf DNA was detected in any of the Xf-inoculated PEAL trees. In these preliminary samples, levels of Xf DNA in the almond trees ranged from 2.17 × 10^(-3) ± 7.86 × 10^(-4) to 3.12 × 10^(-3) ± 3.21 × 10^(-4) ng Xf DNA/ng total DNA and averaged 6.30 × 10^(-3) ± 1.49 × 10^(-4) ng Xf DNA/ng total DNA. In contrast, the trace level observed in one of six Xf-inoculated peach trees was 1.80 × 10^(-3) ± 1.56 × 10^(-4) ng Xf DNA/ng total DNA, more than two orders of magnitude less than levels observed in almonds.

Additional samples were collected from trees testing positive for Xf DNA to determine whether significant differences in Xf titer existed between Xf-inoculated and control trees. In a comparison of Xf titer in petioles collected near inoculation sites, an unequal-variance t test (F ratio = 49.104, df = 1, 17, t ratio = 7.007) revealed highly significant (P < 0.001) differences in titer of Xf DNA between the two germplasm types. The six tested almond trees averaged 6.26 × 10^(-4) ng Xf DNA/ng total DNA, whereas 7.20 × 10^(-5) ng Xf DNA/ng total DNA was quantified in the sampled peach tree. Leaf petioles harvested from water-inoculated peach and almond controls as well as all PEAL trees were absent of Xf DNA as determined by quantitative real-time PCR.

These additional samples also allowed analysis of Xf DNA in the orchard nearby the inoculation site in the six Xf-inoculated almond trees. At 21 weeks postinoculation, Xf titer at the inoculation site differed significantly (F ratio = 4.07, df = 5, 17, P = 0.021) among almond trees. Titer of tree five averaged 1.24 × 10^(-4) ng Xf DNA/ng total DNA, which differed significantly (P < 0.05) from trees two and six, averaging 3.21 × 10^(-4) and 4.76 × 10^(-4) ng Xf DNA/ng total DNA, respectively (Table 1). Although differences in Xf titer of these trees were statistically significant, ALSD differences incurred by almond trees infected with such variable levels of Xf may not be. The six inoculated almond trees infected with these variable levels of Xf expressed only minor differences in tree vigor and ALSD symptoms during the course of the study.

At 24 weeks postinoculation, multiple petiole samples along the meristematic axis were harvested from three Xf-inoculated almond trees to examine whether differences existed in Xf titer between trees and between vertical sections within trees. Xf titer was influenced significantly by almond tree (F ratio = 3.71, df = 2, 35, P = 0.036), and no significant differences in Xf titer were noted between tree vertical sections within sampled trees (F ratio = 2.01, df = 3, 35, P = 0.134). Averaged across tree vertical sections, Xf titer throughout the scion of almond tree five was significantly (P < 0.05) higher than that of trees one and four (Fig. 2A), reinforcing our previous observations of significant Xf titer differences near the inoculation site. Comparing tree vertical sections across the three almond trees, Xf titer was numerically

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HORTSCIENCE VOL. 44(7) DECEMBER 2009 1929

**PLANT PATHOLOGY**

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the inoculation site. Extractions from petiole samples collected near

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2 b
1
6.64
× 10
2 ab

1 6.64
× 10
2 b
2 3.21
× 10
2 a
3 5.32
× 10
2 b
4 5.27
× 10
2 ab
5 1.24
× 10
1 a
6 4.76
× 10
2 b

Table 1. Titer of Xylella fastidiosa in needle-inoculated almond trees 21 weeks post-inoculation as determined by quantitative real-time polymerase chain reaction. Bars represent SEs.

Almond tree & Titer (ng Xf DNA/ng total DNA)
\hline
1 & 6.64 \times 10^2 \text{ab} \\
2 & 3.21 \times 10^2 \text{b} \\
3 & 5.32 \times 10^2 \text{b} \\
4 & 5.27 \times 10^2 \text{ab} \\
5 & 1.24 \times 10^1 \text{a} \\
6 & 4.76 \times 10^2 \text{b} \\
\hline

\text{*Xf* titer based on three independent DNA extractions from petiole samples collected near the inoculation site. Values followed by the same letter within a column are not significantly (P < 0.05) different according to Tukey’s honestly significant difference test.

Fig. 1. Initial Xylella fastidiosa titer in petioles harvested near the inoculation site of mechanically inoculated peach (PE), peach-almond hybrid (PEAL), and almond (AL) trees 20 weeks post-inoculation as determined by quantitative real-time polymerase chain reaction. Bars represent SEs.

| Individual Xf inoculated tree | PE-6 | PE-5 | PE-4 | PE-3 | PE-2 | PE-1 | AL-6 | AL-5 | AL-4 | AL-3 | AL-2 | AL-1 |
|-----------------------------|------|------|------|------|------|------|------|------|------|------|------|------|
| ng Xf DNA / ng total DNA    | 0.0001 | 0.001 | 0.01 | 0.1 |

Nicotiana tabacum compared with other *Xf* isolates causing ALSD (Francis et al., 2008). In other concurrent greenhouse studies with M23 and almonds, we have achieved nearly 100% inoculation success and ALSD disease initiation/symptom development using the same inoculation procedures (Ledbetter et al., 2009).

Our results demonstrate the clear lack of host suitability of peach rootstock accession P248-139 relative to *Xf* isolate M23. Trace levels of *Xf* were quantified in the inoculation zone basal leaves of one of the six inoculated trees, albeit 100-fold lower than levels detected in any almond tree. Additionally, no leaf scorching was ever observed in that particular peach tree nor in the other five *Xf*-inoculated P248-139 replicates. No visual growth reductions were evident comparing *Xf*-inoculated P248-139 trees with water-inoculated controls. As a result of sensitivity of the diagnostic technique, it is possible that quantified *Xf* DNA from this tree was from the inoculation such that bacteria did not multiply in peach xylem vessels. The PCR-based quantitation method used here differs from the colony-forming units method used in previous reports. This method allows detection of small quantities of DNA that might not be associated with living, replicating bacterial cells. Quantitation of bacterial titer by measuring colony-forming units detects only living bacterial cells. For comparison, the mean almond titer of 6.26 \times 10^2 ng *Xf* DNA/ng almond DNA reported here is roughly equivalent to 1 \times 10^2 colony-forming units/g and is in good agreement with previous reports (Almeida and Purcell, 2003a, 2003b).

The absence of detectable *Xf* DNA in inoculated PEAL trees is an important observation for commercial almond tree nurseries. PEAL hybrids have become increasingly popular rootstock choices for California almond growers to enhance root anchorage and increase scion vigor. Increased demand for PEAL rootstocks by almond growers translates to a higher percentage of commercial nursery acreage being planted with these interspecific hybrids. If PEAL hybrids were susceptible to the *Xf* isolates endemic to regions where commercial almond nursery stock was grown, insect vectors would have a larger window of opportunity to infect planting stock and thereby disseminate *Xf*-infected trees to new orchard plantings. Because no *Xf*-resistant almond cultivars have yet been detected and *Citrus*-interspecific hybrids created from *Xf*-susceptible \times *Xf*-resistant parents have all shown to carry detectable levels of *Xf* (Coletta-Filho et al., 2007), the *Xf* host suitability status of PEAL hybrids was in question. The *Xf* susceptibility of almond accession Y113-20-99 is without question as is the *Xf* resistance demonstrated by P248-139 peach trees. Although no broad generalizations can be stated relative to *Xf* host suitability of all PEAL hybrids from the current study’s results, the data are encouraging and suggest that these interspecific hybrids are not a primary inoculum source of ALSD in orchards where PEAL hybrids have been used as rootstocks.

With the overwhelming majority of rootstock grown for commercial almond plantings being either seedling peach or clonal PEAL hybrids, our results suggest that rootstocks from California’s commercial tree nurseries are not primary inoculum sources for ALSD trees found in commercial almond plantings. Almond seedlings are used as rootstocks for almonds in other growing regions (Psallidas, 1989), but they are not offered to commercial growers as rootstock choices for almond in California. Transmission of *Xf* from infected rootstocks to scions propagated to them has been demonstrated previously in apricots (Latham et al., 1980), and this would be a concern in California almond tree nurseries if almond seedlings were used as common rootstocks. June-budding of almonds to vigorously growing rootstocks generally begins by the middle of May in San Joaquin Valley nurseries, whereas almond bud leaf development begins 20 to 30 d after propagation. Hence, although the common rootstocks used for almond may not be suitable hosts for *Xf*, almond scions propagated to them would generally be growing and available for *Xf* transmission from infected insect vectors during the final days of June. Although successful transmission of *Xf* from infected vectors to the succulent scions may not be statistically common, it may account for the low levels of ALSD-affected trees observed in commercial almond plantings without other obvious sources of primary inoculum adjacent to them.

Backcrossing of PEAL hybrid 062002 to both Y113-20-99 almond and P248-139

Discussion

We examined potential host suitability of prospective almond rootstock germplasm in this study using a virulent *Xf* isolate obtained from an ALSD-affected tree in the San Joaquin Valley. Although numerous *ALSD*-causing *Xf* isolates exist, M23 was chosen as a representative as a result of its discovery in the region where commercial tree nurseries provide planting stock to almond growers and its apparently greater virulence at lower titers in

| 0.0001 | 0.001 | 0.1 |
peach will provide further opportunities to understand host suitability of Prunus genotypes associated with almond leaf scorch disease on the same location in California. Phytopathology 95:708–714.

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