

EPIDEMIOLOGY AND MANAGEMENT OF WALNUT BLIGHT

J. E. Adaskaveg, H. Förster, K. Nguyen, D. Thompson, D. Cary, L. Wade, C. Leslie, R. Buchner, and J. Grant.

ABSTRACT

In 2016, we continued to evaluate inoculation methods for testing the susceptibility of walnut cultivars to *Xanthomonas arboricola* pv. *juglandis* (Xaj) to be used in a breeding program to select new resistant genotypes. Pre- and post-infection mechanisms (e.g., morphological defense barriers, pre-formed inhibitors, hypersensitive reactions, chemical response reactions) may determine the resistance of a host plant to pathogen infection. Inoculations of fruit and leaves in the field or laboratory were successful using water (low disease) or diluted surfactant (high disease) bacterial suspensions. A relatively good relationship between natural blight incidence in the field and severity of disease was obtained with aqueous- or surfactant-inoculated fruit in the field or using surfactant-inoculated detached fruit in the lab. Because leaf and fruit susceptibility are not clearly correlated, leaf assays on young plants to screen germplasm prior to fruit development will result in a very high chance of losing valuable germplasm for walnut blight resistance of fruit. In blight management trials, we continued to evaluate copper products, mancozeb, antibiotics, biocontrols, natural products, and enhancers of copper activity. Copper-mancozeb continued to be very effective. In evaluating new copper enhancers, effectiveness of ChampION⁺⁺ was significantly increased with the addition of the experimental thiadiazole derivative ATD, similar to Manzate at one of the commercial test sites. In future trials, thiadiazoles will be evaluated using an improved agricultural formulation (i.e., ZTD) supported by a US registrant. Another new potential enhancer of copper, SBH, was not toxic to Xaj in vitro but when mixed with copper, toxicity was greatly enhanced. In detached walnut leaf assays, the copper-SBH mixture significantly reduced disease severity as compared to copper alone. An agricultural formulation of SBH will be evaluated in 2017. The antibiotic Kasumin by itself or in mixture with mancozeb and/or the acidic, non-ionic surfactant LI-600, as well as in mixture with copper, glycerol, or the antimicrobial ϵ -poly-L-lysine was highly effective, and was sometimes numerically the best treatment among all others included in a trial. Efficacy of selected Kasumin mixtures has been very consistent over numerous years. These mixtures provide excellent control of walnut blight, are effective in resistance management programs especially when mixed with mancozeb, and reduce overall copper usage and environmental accumulation. The antibiotic oxytetracycline showed intermediate efficacy when used alone, but performed very well in mixtures with Kasumin or with mancozeb. This antibiotic was accepted into the IR-4 residue program for registration on walnut. Under low disease pressure, the biocontrols Actinovate, Botector, and the natural product EXP-1552 were effective. Biocontrols generally performed better with the addition of sucrose, a promising perspective in the use of biocontrol agents for disease control. In molecular studies, highly specific primers for Xaj were developed from a unique region of the Xaj genome sequence. These primers amplified all nine Xaj genotypes representing the diversity of strains in California as determined by Rep-PCR, but not closely related *Xanthomonas* species or other bacterial species of different genera isolated from walnut buds that form yellow colonies. qPCR was sensitive in detecting the pathogen using bacterial cells or plant extracts. The limit of detection using pure bacterial suspensions was 1 to 10 bacterial cells. When bacteria were added to plant tissue before DNA extraction and amplification, the limit of detection was 10 to 100 bacterial cells.

OBJECTIVES

- I. Epidemiology - Evaluate populations of the bacterial pathogen using molecular approaches.
 - A) Characterize bacterial species in walnut buds (commensals and pathogens) (*ongoing*).
 - B) Evaluate diversity within Xaj populations and rapid identification and quantification of the pathogen using molecular methods (e.g., qPCR) (*ongoing*).
 - C) Conduct surveys to determine the distribution of strains with reduced sensitivity to copper mancozeb (*ongoing-as samples are submitted by growers, PCAs, and farm advisors*).
- II. Natural host resistance of new walnut genotypes against walnut blight. New genotypes to be evaluated as supplied by the walnut breeding (C. Leslie) and Plant Science (A. Dandekar) programs.
 - A) Develop leaf and fruit pathogenicity assays for rapid screening of walnut genotypes (*ongoing*).
 - B) In lab, greenhouse, and field studies, evaluate leaves of potted plants and detached leaves and fruit using different inoculation methods with and without surfactants (*ongoing*).
 - C) Compare bud populations of the pathogen in walnut genotypes using qPCR (*ongoing*).
- III. New treatments - Evaluate the toxicity of alternative copper and non-copper based materials against Xaj in the laboratory and the efficacy of these materials for managing walnut blight under ambient and simulated rain conditions at Kearney Ag Research and Extension Center (KARE), UC Davis, and in commercial orchards.
 - A) Compare the efficacy of new copper bactericides such as reduced-MCE products (Kocide 3000, ChampION⁺⁺, Badge X2) and other copper products (CS-2005) (*ongoing*).
 - B) Optimize the performance of kasugamycin and oxytetracycline in mixture rotations with copper, EBDCs, and other compounds for sustained usage and stewardship of the antibiotic. Support the full-registration of Kasumin in copper, mancozeb, and other mixtures (*ongoing*).
 - C) Evaluate biocontrols (Actinovate, Nacillus Pro, Botector) with nutritional additives to enhance growth, natural bactericides (EXP-1552), and bactericides used in food preservation: Niprosin (nisin), €poly-L-lysine, and calcium propionate (*ongoing*).

SIGNIFICANT FINDINGS

Development of Xaj-specific primers for pathogen identification and epidemiological studies.

- Highly specific primers for Xaj were developed from a unique region of the published Xaj genome sequence. These primers consistently amplified all nine Xaj genotypes, but not closely related *Xanthomonas* species or other bacteria with yellow colonies isolated from walnut buds.
- qPCR was successful and sensitive using bacterial cells or plant extracts as template. The limit of detection using pure bacterial suspensions was 1 to 10 bacterial cells. When bacteria were added to plant tissue before DNA extraction and amplification, the limit of detection was 10 to 100 bacterial cells.

Development of screening methods for susceptibility testing of walnut cultivars to Xaj.

The goal of developing screening methods for susceptibility testing of walnut cultivars to Xaj is to help in the breeding of new walnut cultivars and speed the selection for blight-resistance.

- Inoculations of fruit and leaves in the field or in the laboratory were successful when bacterial suspensions were prepared in water or diluted-surfactant, and tissues were kept at high humidity.
- A relatively good relationship between natural blight incidence in the field and severity of disease was obtained with inoculated fruit using or not using a surfactant.
- Leaf assays will result in a very high chance of losing valuable germplasm for walnut blight resistance in fruit because leaf and fruit susceptibility to infection by Xaj are not clearly correlated.

Evaluation of alternative bactericides for management of walnut blight in laboratory and field studies.

The incidence of walnut blight in 2016 was generally moderate at many locations. Our trials were conducted in orchards with a history of high disease levels or trees were inoculated with the walnut blight pathogen (i.e., at KARE) and thus, efficacy data were obtained for registered and new treatments.

- **Copper-mancozeb** continued to be very effective in several trials.
- In evaluating **alternative mixture partners as potential enhancers of copper efficacy**, effectiveness of ChampION⁺⁺ was significantly increased with the addition of the experimental thiadiazole derivative **ATD**, similar to Manzate at one of the commercial trial sites. Thiadiazoles will be continued to be evaluated using an improved agricultural formulation called **ZTD** that is supported by a US registrant.
- Another new potential enhancer of copper, **SBH**, was not toxic to Xaj in vitro at 50 ppm, but when mixed with copper, toxicity was greatly enhanced. In detached walnut leaf assays, the copper-SBH mixture significantly reduced disease severity as compared to copper alone. An agricultural formulation of SBH will be evaluated in field trials in 2017. This compound and ZTD have a high potential as alternatives to mancozeb for improving copper efficacy.
- The **antibiotic Kasumin** by itself or in mixture with mancozeb and/or the acidic, non-ionic surfactant LI600, as well as in mixture with copper, glycerol, or the antimicrobial ε-poly-L-lysine was highly effective, and was sometimes numerically the best treatment among all others included in a trial. Efficacy of selected Kasumin mixtures has been very consistent over numerous years. These mixtures provide excellent control of walnut blight, are effective treatments in resistance management programs especially when mixed with mancozeb, and reduce overall copper usage and environmental accumulation.
- The **antibiotic** oxytetracycline (Fireline, Mycoshield) showed intermediate efficacy when used alone, but performed very well in mixtures with Kasumin or with mancozeb. This antibiotic was accepted into the IR-4 residue program for registration on walnut.
- Under low disease pressure, the **biocontrols** Actinovate and Botector and the **natural product** EXP-1552 were effective. Nacillus Pro showed intermediate to low efficacy. Biocontrols generally performed better with the addition of sucrose (sugar). Thus, nutrient additives are a promising perspective in the use of biocontrol agents for disease control.

Walnut blight caused by the bacterium *Xanthomonas arboricola* pv. *juglandis* (Xaj) is a continuous problem for walnut growers worldwide. In California, the disease mainly occurs in central and northern production locations where precipitation in the springtime is generally higher than in the South. The incidence of disease varies widely between years, but crop losses can be very high under favorable environments (temperature and wetness as described by XanthoCast). The pathogen

infects catkins, female blossoms, fruit, green shoots, leaves, and buds of English walnut and survives from one year to the next in twig lesions, living and dead buds, and diseased fruit that remain on the tree. In 2016, a high incidence of dieback that expanded from the peduncles of infected fruit into the twigs was observed at some locations. These infections may provide additional survival sites of the bacterial pathogen and may be colonized by opportunistic fungi such as those in the Botryosphaeriaceae. Fruit infections occur soon after flowering and these infections account for most of the economic loss. The extended period of host susceptibility and the availability of few effective treatments are the main challenges in controlling walnut blight.

Management of walnut blight is done with bactericidal chemical treatments that have to be effective, persistent, and are properly timed based on an understanding of the biology and epidemiology of the pathogen. Management also includes cultural practices (e.g., irrigation and nutrition management), and selection of cultivars that have proven to be less susceptible in the respective growing region. Breeding for walnut blight resistance has been hampered by lack of appropriate methods for screening breeding material at an early selection stage. Previous studies conducted in California on walnut cultivar susceptibility to blight used mature trees (Woeste et al., 1992). In 2015, we initiated field, greenhouse, and laboratory experiments on inoculation methods of attached and detached leaves and fruit with the goal to determine susceptibility at an earlier plant age and assist the walnut breeding program at UC Davis. Pre- and post-infection mechanisms (e.g., morphological defense barriers, pre-formed inhibitors, hypersensitive reactions, chemical response reactions) may determine the resistance of a host plant to pathogen infection, and these may be identified using different inoculation strategies (e.g., inoculations that include a surfactant will more easily overcome morphological defense barriers). Furthermore, these mechanisms may vary between fruit and leaf tissues. Therefore, potentially more than one inoculation method will have to be used to identify these mechanisms. To speed the selection process for new blight-resistant cultivars, foliar assays on walnut seedlings would be ideal in a breeding program. In last year's studies using spray- and infiltration inoculation methods, we successfully obtained disease on leaves and fruit in the laboratory and field, and differences in disease severity among cultivars were observed. For some cultivars, there was a good correlation between natural incidence of blight on fruit on mature trees and disease after inoculation. We had concluded that spray-inoculation (without surfactant) of leaves of potted seedlings in humidity chambers or possibly blunt-syringe-tip infiltration of attached leaves in the field at the beginning of the season or of detached leaves collected at leaf emergence may possibly be effective methods for screening genotypes at an early stage in a breeding program. This would have to be followed by secondary spray-surfactant-inoculations of fruit in the field to confirm a correlation between leaf and fruit susceptibility in a cultivar before its further development in multiplication blocks. In 2016, we expanded on these inoculation studies. Only spray-inoculations were done because syringe infiltrations resulted in too much injury, and bacterial inoculum was used as aqueous or diluted-surfactant suspensions.

For the proper timing of applications in chemical disease management, we developed the forecasting model XanthoCast™ to predict infection periods based on environmental requirements of the pathogen, mostly wetness period duration and temperature. The website www.agtelemetry.com provides the basic XanthoCast™ information with XanthoCast indices for weather stations in the northern portion of the California walnut production region where environments are most favorable for the disease. Our timing studies indicated that early female flower emergence applications followed by treatments based on XanthoCast provide the most

consistent disease control. A catkin expansion, bactericide treatment is only needed when high precipitation occurs at catkin flowering in an orchard with a history of high disease levels.

For the management of walnut blight since 1994 we evaluated numerous non-copper and copper compounds. Copper formulations included copper hydroxide, cuprous oxide, and copper oxychloride, basic copper sulfate, copper octanoate, as well as reduced-copper products (e.g., Kocide 3000, Badge, ChampION⁺⁺). Our program has assisted in the development of copper-EBDC treatments. We showed that mancozeb (Manzate, Dithane, Penncozeb, etc.) is effective in mixtures with copper or the antibiotic Kasumin to control disease caused by copper-resistant populations of Xaj. An important outcome of this was the full registration of mancozeb (i.e., Manzate in 2013 and Dithane in 2014 for 2015 season) for use on walnuts.

Copper-mancozeb applications are currently the most effective treatment for walnut blight. In recent years, some growers suffered high disease losses although copper-mancozeb was applied. In 2013 - 2015, we confirmed the presence of Xaj strains that were less sensitive to copper-mancozeb. With full registration of mancozeb postponed for so many years and with continued safety concerns with this compound, we started evaluating alternatives for mancozeb in laboratory and field studies. These include Tanos, Stout, the thiazazole derivatives terrazole and ATD, and in 2016 we initiated the evaluation of another new potential enhancer of copper, SBH.

Among possible alternatives to copper-EBDC treatments that we evaluated, kasugamycin (Kasumin, Arysta Life Science) has immediate potential in walnut blight management with registration pending at the US-EPA. This antibiotic is used in agriculture in other countries but not in human or animal medicine. Kasumin by itself is moderately effective, but in mixtures with EBDC or copper, efficacy is consistently very high and similar to or higher than that of copper-mancozeb. In 2016, Kasumin was used in mixture with acidifying agents (lactic acid or the non-ionic surfactant LI-700), with glycerol, or with ϵ -poly-L-lysine, and efficacy was mostly increased significantly. Kasumin was federally registered on pome fruit for management of fire blight in 2014. Once registered federally on walnut, we would like to have statewide, research-authorized demonstrations. The other antibiotic we are currently working on is oxytetracycline (Fireline, Mycoshield). With an expanded risk cup, the registrant is requesting input for bacterial diseases on additional crops for labeling. This antibiotic is highly active against Xaj but has a short residual life. Research is needed to improve the residual activity and performance consistency. No phytotoxicity has been observed with either of these antibiotics. The short residual life should ensure non-detectable residues at the time of harvest. In 2016, IR-4 accepted oxytetracycline into the residue program for registration on walnut.

In 2016 we continued evaluations on other possible alternatives including the experimental natural product EXP-1552, the biocontrols Actinovate, Botector, and Nacillus Pro (that was highly effective in 2015), and the antimicrobial ϵ -poly-L-lysine that is used commercially as a food preservative in the United States and some other countries (e.g., Japan, Korea). Some of these compounds demonstrated promising results in our trials previously. Thus, Actinovate was registered for use in 2011 based on our efforts and in 2016, we obtained improved efficacy when the biocontrol was mixed with sugar (likely providing a nutrient source for the biocontrol agent). Our overall goal for management of walnut blight is to ultimately have multiple treatments available that provide equal or improved efficacy to that obtained with traditional copper-containing treatments (e.g., copper-

mancozeb). New treatments will need to be effective against copper-sensitive and -resistant populations of the walnut blight pathogen. This strategy will result in a lower usage and decreased environmental impact of any one material and a lower potential for selecting resistant populations of the pathogen.

PROCEDURES

Genetic diversity among Xaj strains and development of specific primers for pathogen identification. The genetic diversity among Xaj strains was evaluated by Rep-PCR using BOX, ERIC, and REP primers following published protocols. The published Xaj genome sequence was used to select and evaluate putative unique genomic regions for primer design. To test the specificity of the primers, DNA from nine strains of Xaj (representing the intraspecies diversity based on Rep-PCR), closely related *Xanthomonas* species including *X. campestris* pv. *vesicatoria* from pepper, *X. fragariae* from strawberry, and *X. arboricola* pv. *pruni* from almond, as well as 15 previously molecularly characterized bacterial strains from walnut buds with yellow colonies were used as amplicons in PCR reactions. Additionally, DNA was extracted from walnut buds and infected tissues using the DNeasy Plant Mini kit. Amplifications were performed by conventional PCR or by qPCR using a CFX-96 Real-Time System with cycling conditions as follows: 95°C for 2 min; 40 cycles of 95°C for 20 s; and 57°C for 30 s. The limit of detection in qPCR was determined using serial dilutions of bacterial suspensions or by adding a specific number of bacterial cells to walnut tissue before extraction and amplification. PCR products were separated in agarose gels or amplifications were visualized as Ct diagrams and Ct values were determined.

Development of screening methods for susceptibility testing of walnut cultivars to Xaj.

1. Natural incidence of blight of selected walnut cultivars in the walnut nursery block at UC Davis. The natural incidence of blighted fruit of cultivars Cheinovo, Chandler, 76-80, Manregian, Sinensis 5, Payne, Ashley, Vina, PI159568, and Serr was recorded on 5-25-16 at the UC Davis Plant Science Department (Pomology Division) nursery and germplasm orchard locations where we also conducted field inoculations. There were two trees of each cultivar in each orchard. For natural incidence, percent blighted fruit was determined based on evaluation of 50 random fruit for each tree.

2. Field inoculation of immature green fruit and leaves of selected walnut cultivars. Leaves and fruit of selected walnut genotypes at the UC Davis walnut nursery block (well established trees) and at the germplasm block (younger trees) were spray-inoculated on the upper and lower surfaces until runoff. Inoculum consisted of either Xaj (1×10^8 CFU/mL) suspended in water or in 0.5% surfactant solution (BreakThru). As controls, leaves or fruit were sprayed with water or 0.5% surfactant solution alone. Inoculated branches were bagged for 24 h. Leaves and fruit were collected and evaluated for phytotoxicity and disease symptoms after 21 days. For this, digital images were acquired and were analyzed for percent fruit or leaf area affected using the APS Assess software. To account for minor effects of phytotoxicity on leaves and fruit, dark tissue areas resulting from water or surfactant sprays were averaged for each cultivar and deducted from disease area values for each cultivar inoculated with Xaj or Xaj-surfactant. Diseased area data were arcsine-transformed before statistical analysis using mean separation procedures of SAS 9.4. To verify that lesions were caused by Xaj, re-isolations were done from a sub-set of samples. Tissues were surface-sterilized for 60 s in 10% bleach. Lesions were excised, suspended in sterile water,

and shaken for 5 min. The suspension was streaked onto Modified Tween Medium (Schaad, 2001), and plates were incubated for 4 days at 25°C.

3. *Laboratory inoculation of detached young green fruit and leaves.* The petioles of detached walnut leaflets were placed inside wire racks in a plastic container containing 20 ppm gibberellic acid solution in the bottom. Leaves were spray-inoculated on the abaxial surface with either 0.4% surfactant or Xaj (1×10^8 CFU/ml) suspended in 0.4% surfactant using an airbrush sprayer set at 20 psi. Containers were bagged to maintain high relative humidity and incubated for 5 days at 25°C under fluorescent light with a 12-h photoperiod, and evaluated after 21 days. Detached fruit were placed into plastic fruit trays (Panta Paks) in clear plastic containers that were bagged for the duration of the trial. Fruit were spray-inoculated to run-off with either 0.5% surfactant or Xaj (1×10^8 CFU/mL) suspended in 0.5% surfactant. Fruit were evaluated for disease symptoms after 21 days. Evaluation of leaves and fruit was done as described for the field inoculations above. Data were evaluated using analysis of variance or general linear model procedures and mean separation procedures of SAS 9.4.

Evaluation of alternative bactericides for the management of walnut blight in field studies.

Trials were established in experimental orchards at KARE (Fresno Co.; cvs. Chico, Vina, Chandler, Hartley) and in commercial orchards in Solano (cv. Tulare; Xaj partially copper-resistant) and Sutter-Yuba Co. (cv. Tehama - Xaj partially copper-resistant; and cv. Tulare - Xaj highly copper-resistant at 50 ppm MCE). The trials at KARE were conducted with simulated rain applied for 6-7 h on selected days as indicated in the figures of the results. The other trials were conducted under natural rainfall conditions with bactericide applications based on XanthoCast, and four treatments were done with treatment timings indicated in Figs. 4-11. Trees at KARE were inoculated with a copper-resistant strain of Xaj as indicated in the figures.

Copper compounds evaluated included the micro-encapsulated copper hydroxide Kocide 3000, copper hydroxide (ChampION++), copper octanoate (Cueva), basic copper sulfate (Cuprofix Ultra Dispers), and copper sulfate (MagnaBon CS2005). Mancozeb products (Manzate DF, Manzate Max), and the antibiotics kasugamycin (Kasumin) and oxytetracycline (Fireline, Mycoshield) were used either alone or in selected mixtures. Copper products were used together with the new potential enhancer of copper activity, the thiadiazole derivative ATD. Other additives included an agricultural oil (Gavicide), glycerol, lactic acid, the non-ionic surfactant LI-700, and the antimicrobial ϵ -poly-L-lysine. Biocontrols evaluated included Actinovate (a fermentation product of *Streptomyces lydicus*), Botector (*Aureobasidium pullulans*), Nacillus Pro (*Bacillus subtilis* and 3 other species, 5 strains total), and Serenade Opti (QST 713 strain of *Bacillus subtilis*). Sugar was added to some of the biocontrol treatments to provide a nutrient source. The experimental natural product EXP-1552 was also evaluated. Incidence of disease was based on the number of infected fruit of 50-150 fruit evaluated for each of four or five single-tree replications. Data were evaluated using analysis of variance or general linear model procedures and mean separation procedures of SAS 9.4.

Evaluation of the copper activity enhancer SBH in a detached walnut leaf assay. Young succulent walnut leaves were collected in the field, placed into wire racks in plastic boxes with a 20-ppm gibberellic acid solution in the bottom, and spray-treated to run-off. After air-drying, leaves were sprayed with 0.25% surfactant or with Xaj (1×10^8 CFU/ml) suspended in 0.25%

surfactant. Disease was evaluated after 3 weeks of incubation at 22°C using a severity rating scale from 0 to 5. Data were evaluated using analysis of variance or general linear model procedures and mean separation procedures of SAS 9.4.

RESULTS AND DISCUSSION

Genetic diversity among Xaj strains and development of specific primers for pathogen identification. Nine banding patterns representing different Xaj genotypes were differentiated using rep-PCR. Primers Xaj417f and Xaj417r that were designed from a coding region of the published Xaj genome were found to be highly specific. Amplification products were present for all Xaj genotypes and additional strains evaluated (Fig. 1A). No amplification occurred using other related species of *Xanthomonas* or 15 bacterial strains from walnut buds with yellow colonies belonging to other bacterial genera (Fig. 1B). qPCR was successful and sensitive using bacterial cells or plant extracts as template. The limit of detection using pure bacterial suspensions was 1 (Ct value = 29) to 10 (Ct value = 25) bacterial cells. When bacteria were added to plant tissue before DNA extraction and amplification, the limit of detection was 10 (Ct value = 33) to 100 (Ct value = 30) bacterial cells.

Development of screening methods for susceptibility testing of walnut cultivars to Xaj.

1. Natural incidence of blight of selected walnut cultivars in the walnut nursery block at UC Davis. The natural blight incidence was evaluated for ten cultivars that were also used in selected inoculation studies (see below). In a late-May evaluation, cvs. Serr, Vina, Ashley, Payne, Sinensis, as well as genotype PI159568 had a higher incidence of disease than cvs. Cheinovo, Chandler, and genotype 76-80 (Fig. 2). These results are mostly consistent with last year's observations, except that less disease was present on PI159568 in 2015 than in 2016. The basis of resistance for the latter cultivars is mostly unknown, and several mechanisms may be involved as indicated in the Introduction. For the late-blooming cv. Chandler, resistance has been described as an escape strategy because, due to the late bloom of this cultivar, flowers and developing fruit are less likely to be exposed to favorable environmental conditions (e.g., rain) for pathogen dissemination and infection that commonly occur in early spring in California. In fact, in years with late-spring rains, disease on cv. Chandler can be very high.

2. Field and laboratory inoculation of immature green fruit and leaves of selected walnut cultivars.

The blunt-syringe-tip infiltration procedure that we described in 2015 was not used in 2016 because this method resulted in a high amount of mechanical damage that made disease evaluation very difficult. Instead, we repeated field spray-inoculation studies similar to 2015 and we were again successful in obtaining disease on leaves and fruit. Inoculations containing a surfactant resulted in some minor phytotoxicity on leaves and fruit. Because phytotoxicity symptoms could not be easily differentiated from disease symptoms, ratings of affected tissue area were subtracted from those after inoculation, resulting in a more accurate measurement of actual disease. Although plant material was not available from all cultivars in the two orchard locations to compare the two spray-inoculation methods (inoculum prepared with and without a surfactant), results can be summarized as follows:

Fruit inoculations. Results of spray inoculations of fruit in the field were similar for the two orchards for each of the two inoculation methods (i.e., sprays of aqueous vs. diluted surfactant

suspensions of Xaj). There was no interaction between orchard location and cultivar and, therefore data were combined for the two orchards for each of the two inoculation methods (note: 2 of 11 cultivars that were not evaluated in both orchards were not included in the statistical analysis for the surfactant inoculation). There were significant differences in cultivar/genotype susceptibility ranking between the inoculation methods (Fig. 2A). Overall, disease severity was mostly much lower in the no-surfactant inoculations. Inoculations of detached fruit were statistically analyzed separately. Inoculations with surfactant suspensions in the field and of detached fruit in the laboratory had similar results for ranking the cultivar susceptibility to blight. Representatives of surfactant-inoculated fruit of several cultivars and genotypes are shown in Fig. 3.

Overall, inoculations with bacterial suspensions with and without surfactant mostly related to the natural disease incidence. For example, cvs. Cheinovo and Manregian, as well as genotype 76-80 developed very low to low levels of disease; whereas cvs. Vina and genotype PI159568 had higher levels of disease in natural incidence evaluations and after inoculation with and without surfactant. Although natural blight incidence on cv. Ivanhoe could not be assessed, this cultivar has a known history of high blight susceptibility and developed disease in our inoculations with surfactant. Natural incidence of genotype PI159568, however, was low in 2015 and mostly very low over 6 years in our variety block at Kearney Agricultural Research and Extension Center (KARE). This may indicate that PI159568 is basically susceptible, but escapes infection in most years because susceptible tissues are not present at times when environmental conditions are favorable for the pathogen or that the cultivar does not support an overwintering population of the pathogen. These possibilities can be tested with inoculations next spring and by testing of buds for populations of the pathogen during tree dormancy. The first possibility is also evident in the late-blooming cv. Chandler that blooms when environmental conditions are generally not favorable for disease under California conditions and thus, escapes disease. In seasons with late spring rains, however, the cultivar may be devastated by blight. High disease development of cv. Chandler in our fruit inoculations supports that the cultivar is susceptible to Xaj infection.

Lower relative disease levels occurred on cvs. Sinensis and Serr using the non-surfactant inoculation as compared to the surfactant inoculation. These cultivars had moderate or high levels of natural disease incidence, respectively. The difference in disease between the inoculation methods could reflect different mechanisms for resistance for these cultivars. The surfactant likely allows the bacteria to enter stomata, and low disease in the non-surfactant inoculation may indicate a mechanism that prevents the bacterium from entering the fruit. Higher amounts of epicuticular wax may prevent the spreading out of inoculum and differences in stoma morphology (or function – closed stomata) may prevent outside water from gaining entry into the leaf. These are possible mechanisms of preformed, morphological mechanisms of host resistance. Disease levels after inoculation with surfactant on cvs. Ashley and Payne were similar to natural incidence and this was expected because both are considered to have the same genetic background.

Leaf inoculations. In spray inoculations of leaves, field and laboratory-detached-leaf inoculations with Xaj suspensions containing a surfactant resulted in a similar ranking of the cultivars with the exception of Manregian having higher disease in the field (Fig. 2B). There were differences in disease levels and ranking for some cultivars when using aqueous, non-surfactant inoculation suspensions. In comparison with natural incidence of disease on fruit, only a few cultivars/genotypes showed similar results in sensitivity. These were Chandler and PI159568, both

with a high level of disease and Manregian with a low level of disease. In contrast to fruit rankings, Cheinovo and genotype 76-80 had high levels of leaf infection (Fig. 2B). Therefore, if a breeding program was based on leaf inoculations to select for resistance, only three of the ten cultivars would have been correctly rated. These results further support previous data that walnut leaf and fruit susceptibility to infection by Xaj are not clearly correlated.

Summary and conclusions. In 2015, when we initiated these inoculation studies, we were successful in obtaining disease in the field and laboratory, and in 2016, we further optimized these leaf and fruit inoculations. Leaf assays for sensitivity screening of germplasm looked promising in 2015, however, with more detailed comparisons done in 2016, we conclude that leaf assays will result in a very high chance of losing valuable germplasm for walnut blight resistance in fruit. This may not be acceptable in a resistance breeding program considering that there may be a low probability of germplasm that has both, leaf and fruit resistance. This year, we obtained a relatively good relationship between natural blight incidence in the field and severity of disease on inoculated fruit using or not using surfactant. Therefore, the first crop of fruit of breeding lines should be inoculated in the field and rated. Relatively few fruit are needed for this inoculation screening, and this still can be done at a relatively early stage of propagation when other characteristics of the genotypes such as tree growth, bloom date, and kernel characteristics are also evaluated. Fruit inoculation methods may be further improved for use in walnut genotype screening. Ultimately, molecular mechanisms of resistance and genetic markers need to be identified to accelerate breeding for blight resistance.

Walnut blight incidence in California in 2016. Although the winter to early spring of 2015/16 was an El Niño season, the incidence of walnut blight was moderate at most locations and the disease was generally successfully managed with properly timed bactericide applications. There were no new reports on failure of copper-mancozeb treatments. One few samples were received for Xaj verification and resistance testing that resulted in continued verification of high copper resistance with either no or very slight changes in mancozeb sensitivity. Our trials were conducted in orchards with a history of high disease levels or trees were inoculated with the walnut blight pathogen (i.e., orchards at KARE) and thus, efficacy data were obtained for registered and new treatments.

Evaluation of alternative bactericides for management of walnut blight in field studies. Data on new treatments for managing walnut blight were obtained from commercial and experimental orchards. **Copper-mancozeb** continued to be very effective in several trials (Figs. 4, 5, 6, 7, 8). Still, in several studies, the **antibiotic Kasumin** by itself (Figs. 7,8) or in mixture with mancozeb (Figs. 4,5) and/or the acidic, non-ionic surfactant LI-700 (Figs. 5, 8), as well as in mixture with copper, glycerol or the antimicrobial ϵ -poly-L-lysine (Fig. 5) resulted in a significantly or numerically lower disease incidence, and sometimes was numerically the best treatment among all others included in a trial [Figs. 4, 5 (Kasumin-Manzate and ChampION++ both had no disease), 7, 8]. Some of these treatments were also highly effective under high disease pressure with almost 50% blight incidence in the control (Fig. 4). In a commercial orchard with pathogen populations highly resistant to copper (50 ppm MCE), a Kasumin-Manzate mixture significantly reduce the disease (Fig. 9). Lactic acid and ϵ -poly-L-lysine were used as mixture partners because they have anti-microbial action. Lactic acid also lowers the pH, and glycerol reduces water activity, both making the micro-environment less favorable for bacterial growth. Thus, Kasumin continues to show high efficacy, especially in selected

mixtures, and over years of trials has demonstrated consistency over a range of environmental conditions. These mixtures provide excellent control of walnut blight, are excellent treatments in resistance management programs especially when mixed with mancozeb, and reduce overall copper usage and environmental accumulation. As indicated above, Kasumin was federally registered on pome fruit for management of fire blight in 2014. Residue data for kasugamycin on walnut developed by IR-4 were submitted to EPA, and full registration of kasugamycin is pending. The Walnut Commission of California has encouraged CDFA and EPA to register Kasumin in a timely manner.

In comparing **different copper formulations**, the CS-5000 MagnaBon product was similarly effective to Kocide 3000, but in mixture with mancozeb, the efficacy of CS-5000 was not improved (Fig. 6). CS-5000 is a new registration in California and some marginal leaf injury was observed when the product was used at and above 27 fl oz/A. More trials are needed to ensure the product's tree safety under a variety of conditions and cultivars. Cueva was used in a study that compared various biological treatments and it performed well under low disease pressure (Fig. 12). Addition of the agricultural oil Gavicide did not improve performance of Kasumin-ChampION++ (Fig. 4) or of Manzate-ChampION++ (Fig. 5).

In evaluating **alternative mixture partners as potential enhancers of copper efficacy**, effectiveness of ChampION++ was significantly increased with the addition of **ATD** (similar to Manzate) at one of the commercial sites (Fig. 4), but not in another one (Fig. 5). This compound is an analog to a compound registered in China and represents a new mode of action of bactericides called thiadiazoles. The thiadiazoles will continue to be evaluated using an improved agricultural formulation (i.e., zinc thiadiazole or **ZTD**) from China. A potential US registrant for ZTD has been identified. Another new potential enhancer of copper that became available late in the season was evaluated in in vitro laboratory sensitivity and detached leaf studies. This material, **SBH**, was not toxic to Xaj in vitro at 50 ppm, but when mixed with copper, toxicity was greatly enhanced, indicating a synergistic effect. In detached walnut leaf assays, the copper-SBH mixture significantly reduced disease severity as compared to copper alone (Fig. 10A,B). The surfactant used in the Xaj inoculation suspension caused some phytotoxicity, but the affected leaf area (browning) was much increased after inoculation with the pathogen (Fig. 10B). SBH in an improved formulation will be evaluated in field trials in 2017. These compounds have a high potential as an alternative to mancozeb for improving copper efficacy, and ZTD by itself also as a new bactericide.

The **antibiotic oxytetracycline** (Fireline, Mycoshield) was used by itself or in selected mixtures. Used by itself, it showed very good (Fig. 7 - moderate disease pressure) or intermediate (Fig. 4 - high disease pressure) efficacy and performed very well in mixture with Kasumin or copper (Figs. 4, 6, 8), or with Manzate (Fig. 7). The **antimicrobial ε-poly-L-lysine** was also very effective used by itself under low disease pressure (Fig. 5). This compound is an antimicrobial food preservative and has potential for exempt-from-tolerance registration. ε-poly-L-lysine is adsorbed electrostatically to the bacterial cell surface, followed by stripping of the outer membrane. This eventually leads to cell damage and bacterial cell death.

Several 'biological' treatments were again also evaluated in 2016. The experimental **natural product EXP-1552** showed intermediate performance and reduced disease from the control by over 50% (Fig. 4). The most effective **biocontrol** treatment in 2016 was **Botector**, especially when mixed with

sugar as a nutrient source for the biocontrol agent. This treatment was similar effective to Kocide-Manzate in one study under moderate disease pressure (Fig. 11), and numerically resulted in the lowest disease incidence in a comparison of biological treatments in another study with low disease pressure (Fig. 12). **Actinovate** was also effective in two studies where it was included (Figs. 11,12). Actinovate and Botector had shown efficacy in previous years and should be rotational options for organic growers especially if copper-resistant pathogen populations occur in their orchard. The biocontrol **Nacillus Pro** that was highly effective when mixed with sugar in some studies in 2015, showed lower efficacy in 2016, but still significantly reduced the incidence of blight from the control in one of the two studies where it was included (Fig. 4). Unfortunately, 2015 product had to be used in 2016 applications, there is currently no registrant for this biocontrol.

Summary of biological and conventional antimicrobial treatments for blight management. In conclusion, over many years of testing potential new bactericides as copper-mancozeb alternatives for the management of walnut blight, few effective compounds have been identified. These include antibiotics and biocontrols, as well as several additives. Selected additives improve efficacy of copper, antibiotics, or biocontrol agents and may also help to reduce the risk of resistance development. Additionally, some of the additives such as ϵ -poly-L-lysine, glycerol, and lactic acid are produced naturally by fermentation processes and may be important in mixtures with copper to overcome copper-resistance in organic production. For the evaluated biocontrols, we identified nutrient additives that significantly increased their efficacy against walnut blight, but also against other bacterial diseases of crops grown in California. This again is a significant find for organic tree crop production where limited effective alternatives to copper are available.

For conventional treatments, in addition to the pending kasugamycin registration on walnuts, we identified two new chemical groups, thiadiazoles and hydrazones, that have specific active ingredients with bactericidal activity at selected rates. Two US registrants currently are developing these materials with us for registration in the United States. ATD was used as a surrogate analog to ZTD while a US registrant formed an agreement with the producer and registrant in China. ZTD is a commercial bactericide for agricultural crops in China. SBH will be used as a mix partner with copper at lower rates than mancozeb to greatly enhance the activity of copper. These materials should not be considered replacements but rather complementary rotational treatments in the development of bactericidal management programs. Ideally, new modes of action with activity against a pathogen should be mixed or rotated from the onset of registration to delay resistance developing to anyone mode of action over an extended period of time or almost indefinitely. The goal is to use any single mode of action only once per growing season. Copper-mancozeb mixtures have an outstanding twenty-five-year history of proven consistent performance for managing walnut blight under low to highly favorable conditions for disease. Unfortunately, on other crops where the mixture has been registered longer than on walnuts, such as tomato, this mixture has now failed in the eastern United States. We have demonstrated that the selection for higher copper resistance is ongoing. Once resistance to a mode of action develops in a pathogen population, the addition of a second compound only slows the continued selection for higher resistance levels to that compound. Considering shifts in copper-mancozeb sensitivity in sub-populations of the pathogen, alternatives to copper should be registered immediately to reduce the total number of applications in a given season and to ensure the continued activity of mancozeb for years to come.

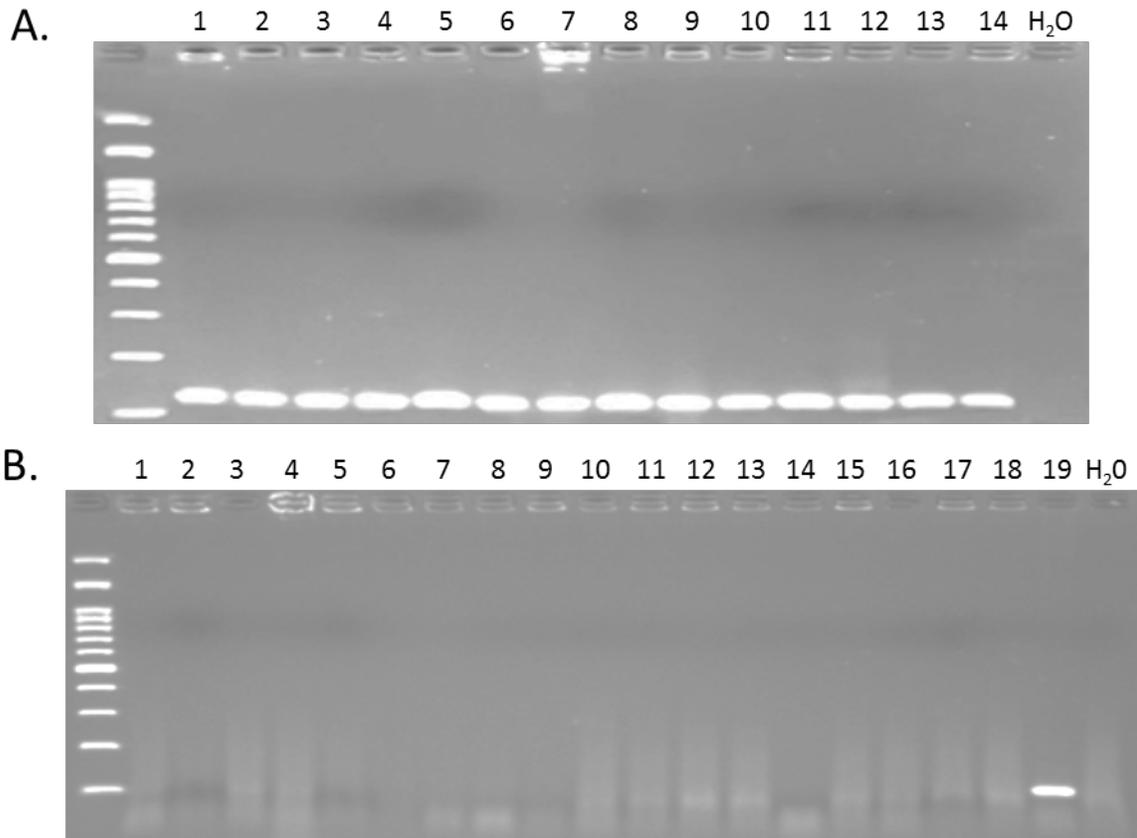
ACKNOWLEDGMENTS

Special thanks to the growers in Butte, Solano, Yolo, Sutter/Yuba, San Joaquin, and Tehama Co. who allowed us to conduct our research in their orchards, and to the chemical industry representatives and PCAs who assisted in this research.

REFERENCES

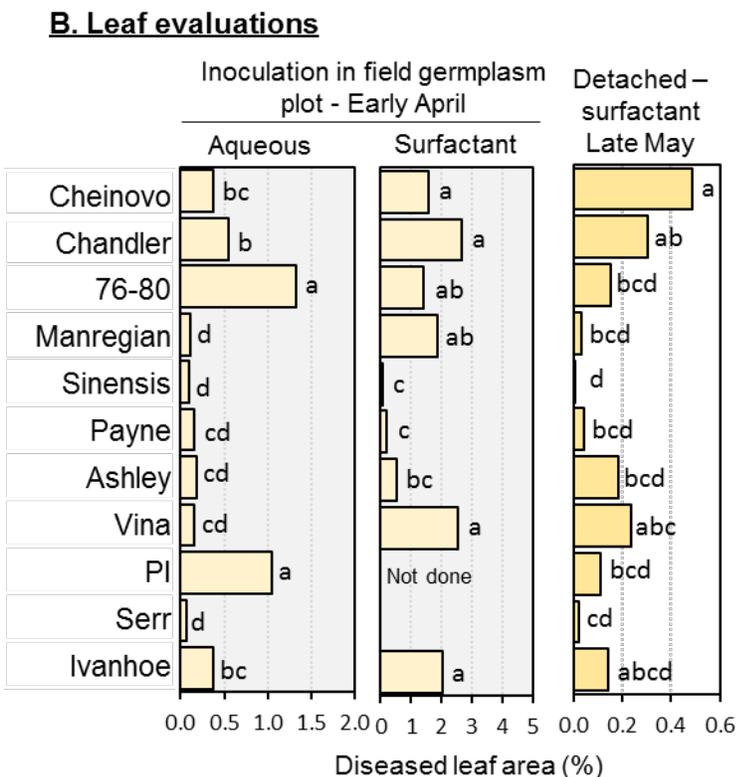
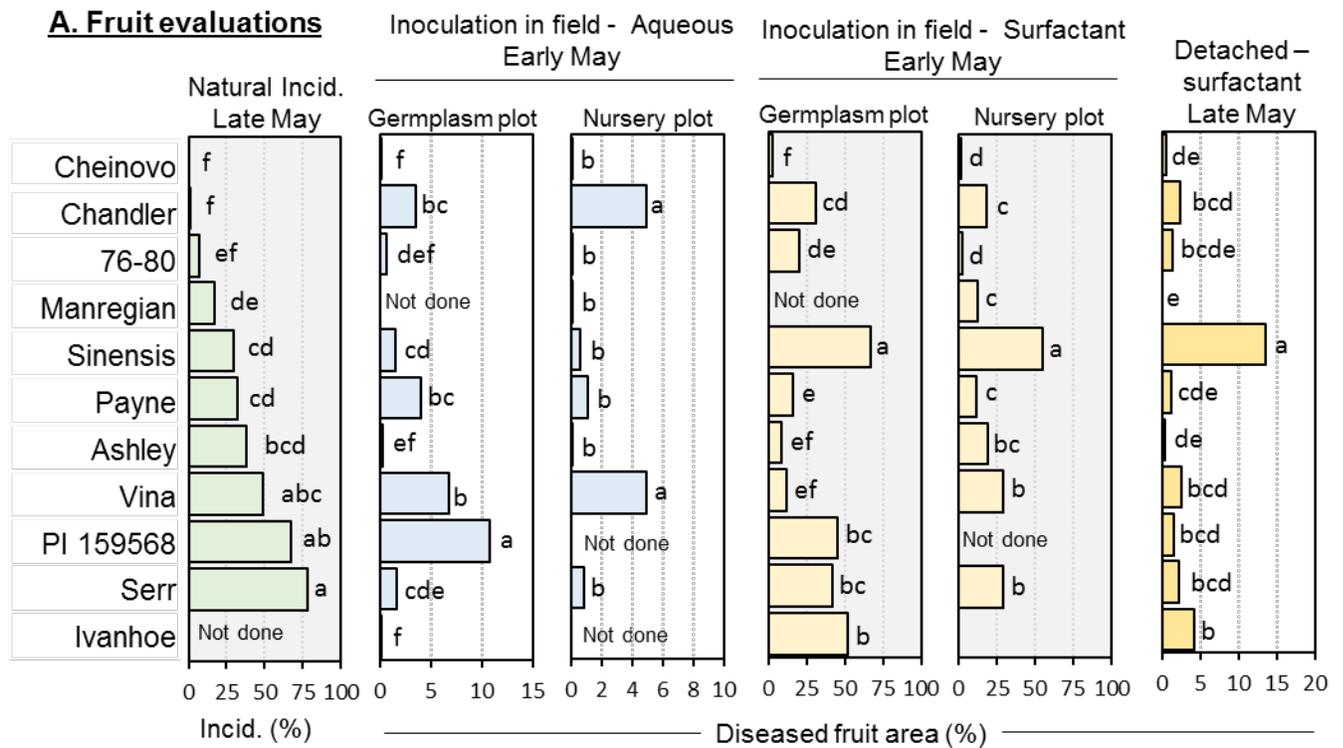
1. Adaskaveg, J. E., et al. 1998-2014. Annual Walnut Reports – 1998-2015. Walnut Marketing Board of California. Sacramento, CA.
2. Aletà, N., Ninot, A., Moragrega, C., Llorente, I., and Montesinos, E. 2001. Blight sensitivity of Spanish selections of *Juglans regia*. Proc. IV Int. Walnut Symp. Eds. E. Germain and D. Calvi. Acta Hort. 544:353-362.
3. Christofferson, C., McGranahan, G., Mills, N., and Leslie, C. 1999. Annual Walnut Reports – 1999. Walnut Marketing Board of California. Sacramento, CA.
4. Conover, R. A., and Grenhold, N. R. 1981. Mixtures of copper and maneb or mancozeb for control of bacterial spot of tomato (*Xanthomonas campestris* pv. *vesicatoria*) and their compatibility for control of fungus diseases. Proc. of Fla. State Hortic. Soc. 94: 154-156.
5. Lee, Y.-A., Schroth, M. N., Hendson, M., Lindow, S. E., Wang, X. -L., Olson, B., Buchner, R. P., and Teviotdale, B. 1993. Phytopathology 83: 1460-1465.
6. Lee, Y.-A., Sung, A.-N., Liu, T.-F., and Lee, Y.-S. 2009. Appl. Environm. Microbiol. 75:6831–6838.
7. Lindow, S., Olson, W., and Buchner, R. 2014. Colonization of dormant walnut buds by *Xanthomonas arboricola* pv. *juglandis* is predictive of subsequent disease. Phytopathology 104: 1163-1174.
8. Louws, F. J., Fulbright, D. W., Taylor Stevens, C., and de Bruijn, F. J. 1994. Appl. Environ. Microbiol. 60:2286-2295.
9. Lyman, W.R., and Lacoste, R. J. 1975. New developments in the chemistry and fate of ethylenebis (dithiocarbamate) fungicides. Environ. Qual. Saf., suppl 3, 67-74.
10. Miller, P. W., and W. B. Bollen. 1946. Oregon Agric. Exp. Stn. Tech. Bull. 9. 107 pp.
11. Schaad, N. W., Jones, J. B., and Chun, W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. Third Edition. APS Press, St. Paul, MN.
12. Scortichini, M., Marchesi, U., and Di Prospero, P. 2001. Genetic diversity of *Xanthomonas arboricola* pv. *juglandis* (synonyms: *X. campestris* pv. *juglandis*; *X. juglandis* pv. *juglandis*) strains from different geographical areas shown by repetitive polymerase chain reaction genomic fingerprinting. J. Phytopathol. 149:325-332.
13. Teviotdale, B. L., Schroth, M. N., and Mulrean, E. N. 1985. In: Walnut Orchard Management. Ed. by E. Ramos. Univ. of Calif. Coop. Ext. Publ. 21410.
14. Woeste, K. E., McGranahan G. H., and Schroth, M. N. 1992. Variation among Persian walnuts in response to inoculation with *Xanthomonas campestris* pv. *juglandis*. J. Amer. Soc. Hort. Sci. 117:527-531.
15. Schaad, N. W., Jones, J.B., and Chun, W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. Third Edition. APS Press, St. Paul, MN.
16. Young, J. M., Park, D.-C., Shearman, H. M., and Fargier, E. 2008. Syst. Appl. Microbiol. 31:366-377.

Fig. 1. Amplification of molecularly diverse strains of the walnut blight pathogen, other *Xanthomonas* spp., and walnut-bud contaminants that are phenotypically similar to Xaj using newly designed Xaj-specific primers in PCR reactions



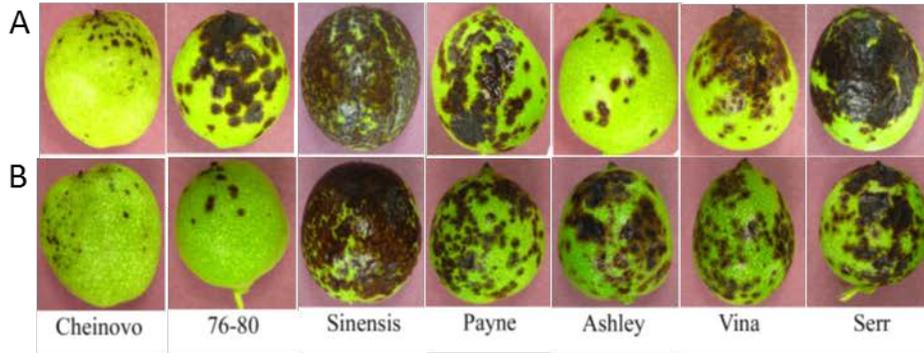
A. Amplification of 14 molecularly diverse strains of Xaj (as determined by Rep-PCR). **B.** Amplification of Xaj and other bacteria. Lanes 1-16: Yellow-colony-forming bacteria isolated from walnut buds representing 9 genera of bacteria based on sequencing of the 16S rRNA; 16: *X. fragariae*; 17: *X. a. pv. pruni*; 18: *X. campestris pv. vesicatoria*; and 19: *X. a. pv. juglandis*. The first lane of each gel is a 100-bp ladder.

Fig. 2. Evaluation of inoculation methods of walnut fruit and leaves with *X. arboricola* pv. *juglandis* to test cultivars for their susceptibility to walnut blight



The natural incidence of walnut blight was evaluated for two trees of each cultivar in the nursery block at UC Davis on 05/25/16. In inoculations, fruit and leaves were sprayed in the field with Xaj (1×10^8 CFU/ml) as aqueous suspensions or in 0.5% surfactant. Inoculated branches were bagged for 24 h, and disease was evaluated after 3 weeks. Detached fruit were spray-inoculated with Xaj in 0.5% surfactant, incubated in plastic boxes, and evaluated after 3 weeks. Detached leaves were inoculated by dipping in a suspension of Xaj containing 0.4% surfactant, placed into wire racks in plastic boxes with water in the bottom, and were evaluated after 5 days.

Fig. 3. Evaluation of inoculation methods of walnut fruit with *X. arboricola* pv. *juglandis* to test cultivars for their susceptibility to walnut blight



Fruit were sprayed in the field with Xaj (1×10^8 CFU/ml) as an aqueous suspension with 0.5% surfactant. Inoculated branches were bagged for 24 h, and disease was evaluated after 3 weeks. A, Fruit from the germplasm block; B, Fruit from the nursery block.

Fig. 4. Efficacy of new copper- and non-copper-based treatments for managing walnut blight on cv. Tehama under natural rainfall conditions – Sutter/Yuba Co. 2016
- Populations of *X. arboricola* pv. *juglandis* partially resistant to copper -

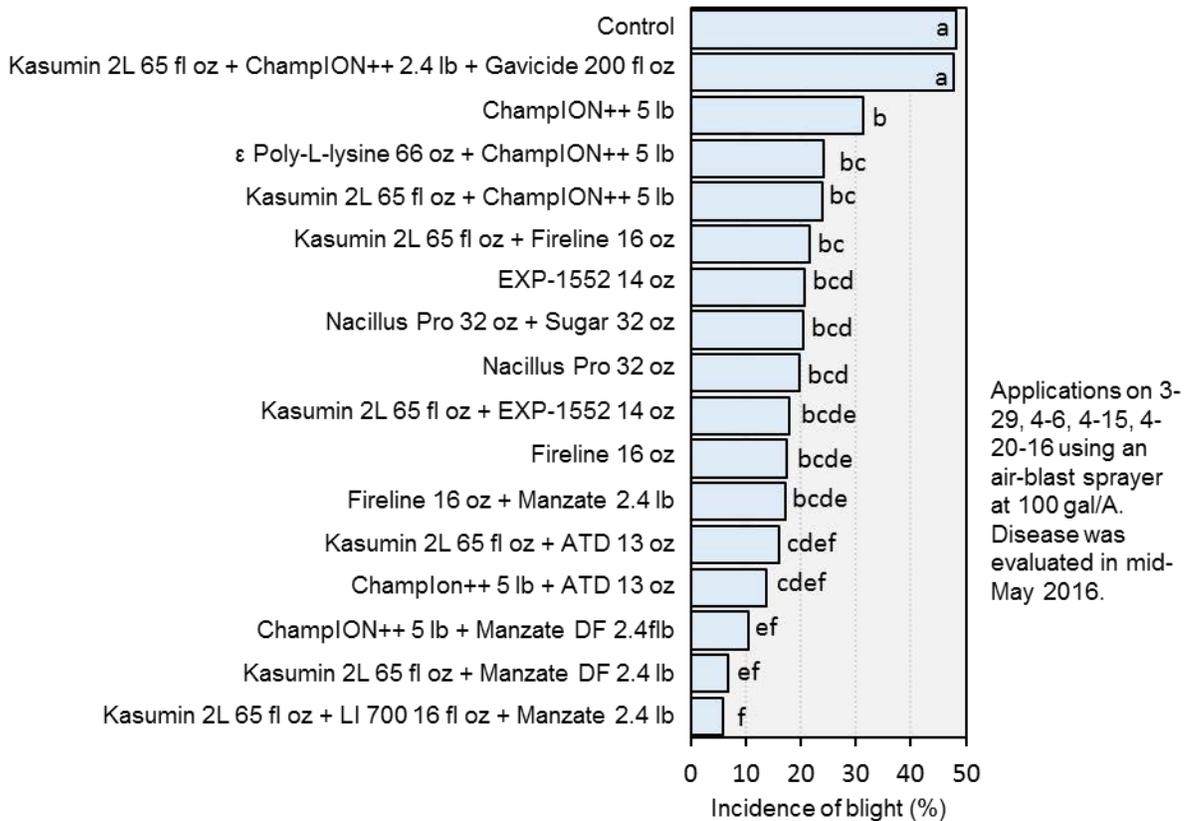


Fig. 5. Efficacy of new copper- and non-copper-based treatments for managing walnut blight on cv. Tulare under natural rainfall conditions - Solano Co. 2016
 - Populations of *X. arboricola* pv. *juglandis* partially resistant to copper -

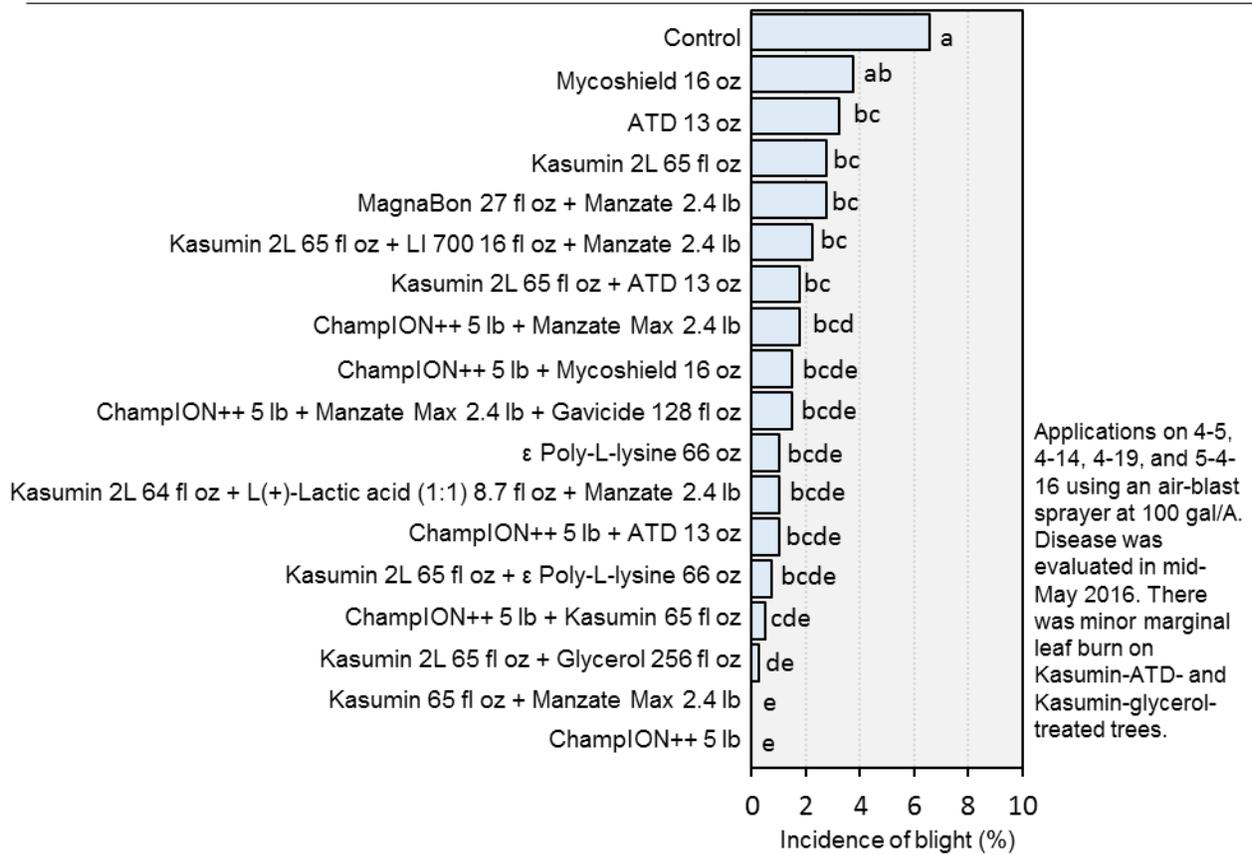


Fig. 6. Evaluation of new copper products for management of walnut blight on cv. Vina - Kearney Ag Center, Fresno Co. 2016
 - Inoculation with a copper-resistant strain of *X. arboricola* pv. *juglandis* -

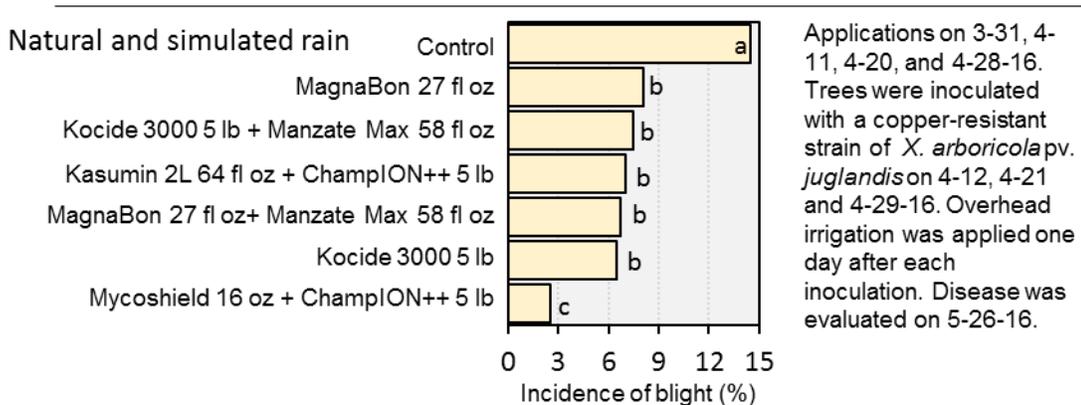


Fig. 7. Efficacy of bactericide treatments for management of walnut blight on cv. Hartley-Kearney Ag Center, Fresno Co. 2016
- Inoculation with a copper-resistant strain of *X. arboricola* pv. *juglandis* -

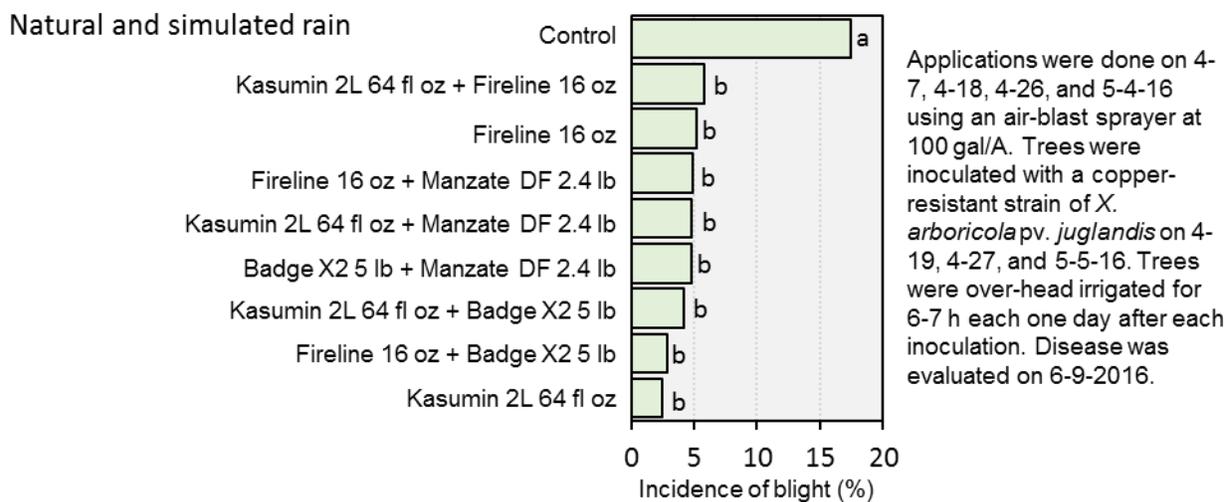


Fig. 8. Efficacy of bactericide treatments for management of walnut blight on cv. Chico - Kearney Ag Center, Fresno Co. 2016
- Inoculation with a copper-resistant strain of *X. arboricola* pv. *juglandis* -

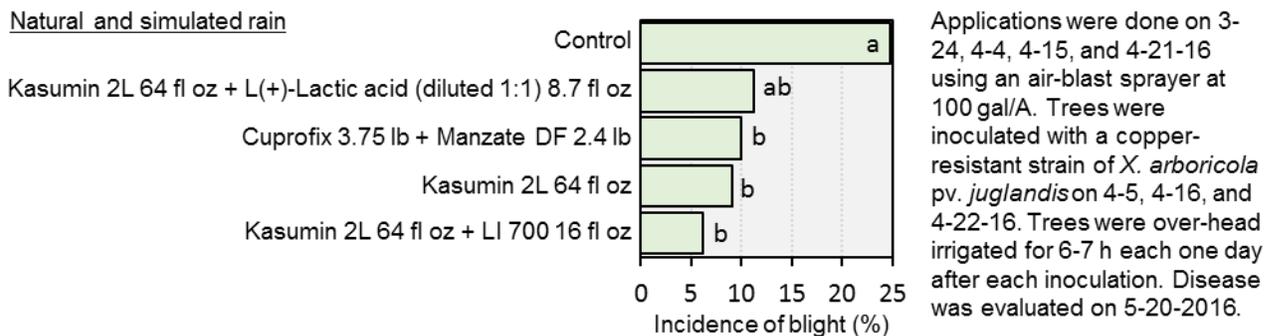


Fig. 9. Efficacy of bactericides for managing walnut blight on cv. Tulare under natural rainfall conditions – Sutter/Yuba Co. 2016
- Populations of *X. arboricola* pv. *juglandis* highly resistant to copper -

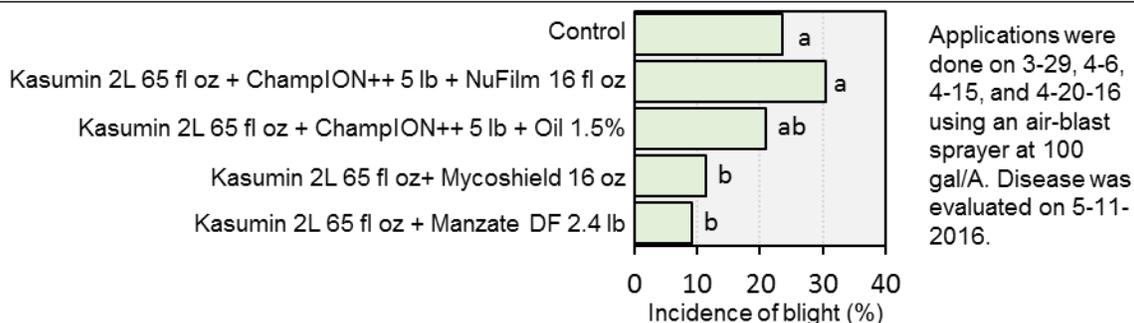
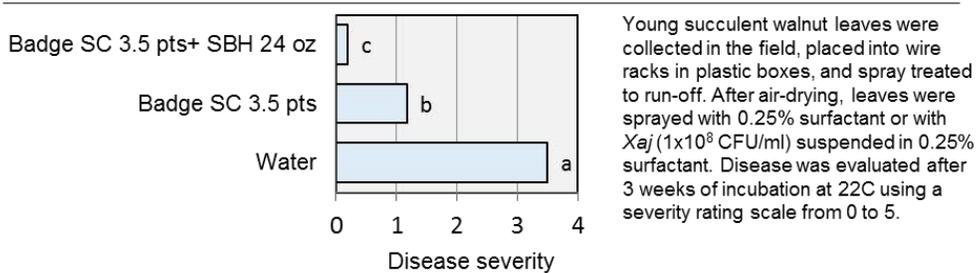


Figure 10. Efficacy of SBH and copper against walnut blight in a detached walnut leaf assay



Young succulent walnut leaves were collected in the field, placed into wire racks in plastic boxes, and spray treated to run-off. After air-drying, leaves were sprayed with 0.25% surfactant or with *Xaj* (1×10^8 CFU/ml) suspended in 0.25% surfactant. Disease was evaluated after 3 weeks of incubation at 22C using a severity rating scale from 0 to 5.

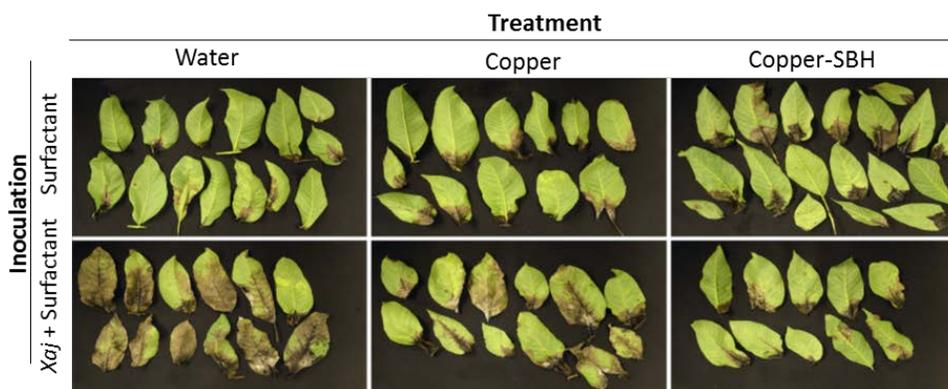
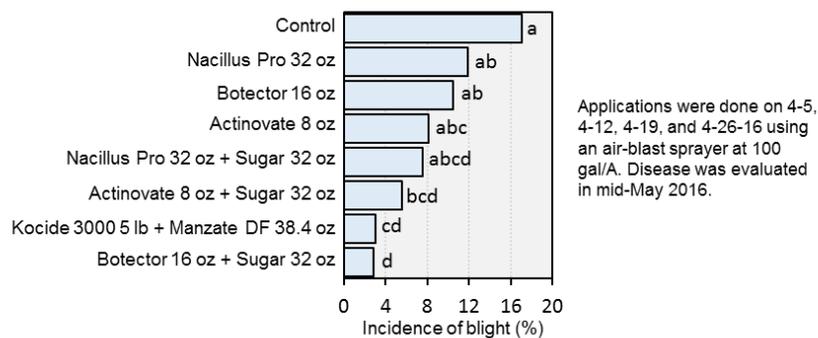
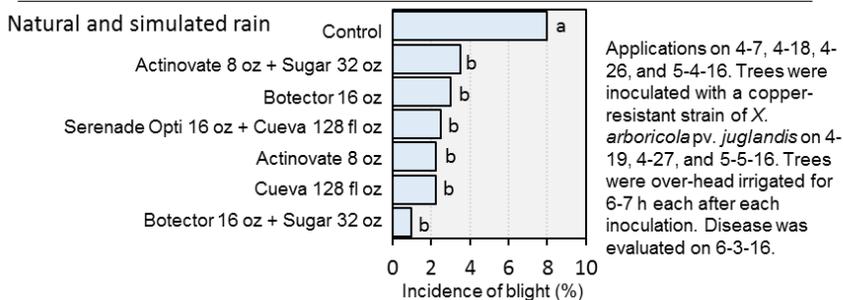


Fig. 11. Efficacy of new biological treatments for managing walnut blight on cv. Tulare under natural rainfall conditions - Solano Co. 2016 - Populations of *X. arboricola* pv. *juglandis* partially resistant to copper -



Applications were done on 4-5, 4-12, 4-19, and 4-26-16 using an air-blast sprayer at 100 gal/A. Disease was evaluated in mid-May 2016.

Fig. 12. Efficacy of new antimicrobial and biological products for management of walnut blight on cv. Chandler - Kearney Ag Center 2016 - Inoculation with a copper-resistant strain of *X. arboricola* pv. *juglandis* -



Applications on 4-7, 4-18, 4-26, and 5-4-16. Trees were inoculated with a copper-resistant strain of *X. arboricola* pv. *juglandis* on 4-19, 4-27, and 5-5-16. Trees were over-head irrigated for 6-7 h each after each inoculation. Disease was evaluated on 6-3-16.