Annual Report - 2012

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Project Title:	Evaluation of new bactericides for control of fire blight of apples caused by *Erwinia amylovora* and evaluation of new postharvest fungicides for pome fruit
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SUMMARY

Fire blight

1. In our 2012 survey on antibiotic resistance in *Erwinia amylovora*, twenty-one isolates from orchards in several counties were obtained. In two orchards, a high level of streptomycin-resistance was present. All isolates of the pathogen belonged to the high-resistance category, and these isolates also showed reduced sensitivity to oxytetracycline. Thus, reduced sensitivity to oxytetracycline was found in two additional orchards from the ones previously identified.
2. In a field trial on the management of fire blight on Granny Smith apple with 23 treatments, Kasumin (two formulations) continued to perform very well. The 2L formulation numerically resulted in the second lowest disease incidence among the single-active ingredient treatments. Among the mixture treatments evaluated, Kasumin-Firewall, Kasumin-Fireline, Kasumin-Prophyt, and Kasumin-Manzate had the lowest incidence of disease. The biocontrol Blossom Protect (*Aureobasidium pullulans*) was also very effective in 2012, and Actinovate (*Streptomyces lydicus)* showed intermediate efficacy.
3. Kasugamycin (Kasumin) registration in the United States is pursued on pome fruit with a California registration expected in 2013.
4. In studies on the molecular mechanism of streptomycin resistance in *E. amylovora,* a new mode of resistance for moderately resistant isolates was confirmed where *strA*-*strB* resistance genes on transposon Tn*5393* are located on plasmid pEU30. This plasmid is also present in highly resistant isolates, but here it does not carry *strA*-*strB* genes. For these strains, resistance is due to a point mutation in a chromosomal gene.

Postharvest decay control

1. Postharvest experimental packingline studies using in-line drench applications were conducted to determine the efficacy of the new DMI fungicide difenoconazole and best usage rates of a new formulation of the difenoconazole-Scholar pre-mixture to ultimately provide a highly efficacious and cost-effective pre-mixture.
2. Difenoconazole showed efficacy against gray mold and was highly effective against blue mold, similar to Scholar or Penbotec. It was also highly effective against bull's eye rot. The pre-mixture at all rates tested was highly effective against the three decays and thus, there was no negative interaction between the active ingredients.
3. Fruit temperature in relation to treatment-solution temperature is an important parameter for fungicide residues on fruit. Lower fruit temperature than the treatment solution temperature reduced the amount of fludioxonil residue of Granny Smith and Fuji apple, as well as on pear.
4. Polyoxin-D that recently obtained an exempt status was similarly effective to Penbotec in reducing the incidence of gray mold, but it was not effective against blue mold. This compound is also known to be highly effective against *Alternaria* species. Thus, it has the potential to be the most effective organic treatment ever available.
5. High in vitro sensitivities of mycelial growth of *Alternaria* spp. to difenoconazole and fludioxonil indicated that these fungicides can be very effective in reducing postharvest Alternaria rot. In

agreement with the low efficacy of difenoconazole in managing postharvest gray mold, sensitivity of nine *Botrytis cinerea* isolates against this fungicide was low.

6. Resistance potential studies using the SGD method difenoconazole, fludioxonil, and pyrimethanil indicated that difenconazole has the lowest resistance potential of the three postharvest fungicides for selecting resistant isolates of the pathogen *Penicillium expansum*.

INTRODUCTION

Epidemiology and management of fire blight. Fire blight, caused by the bacterium *Erwinia amylovora,* is a very destructive disease of pome fruit trees worldwide. In addition to cankers, the pathogen overwinters in flower buds, diseased fruit, small twigs, and branches left on the ground after pruning. In the spring, blossoms are infected through natural openings in nectaries and pistils. After infecting the blossoms, the bacteria grow into the peduncles and spurs. During warm and humid weather, ooze droplets consisting of new inoculum, are exuded from the peduncles. Young fruitlets often become infected, and they also turn black, dry, shrivel, but usually remain attached to the tree. The disease spreads rapidly. After invading blossoms, the bacterial pathogen can invade adjacent leaves through stomata, trichomes, hydathodes, and through wounds caused by hail or wind whipping. Succulent twigs, suckers, sprouts, and shoots are the next tissues infected. Secondary infections may occur throughout the growing season. Inoculum is spread by wind, rain, insects, birds, or by man, e.g. by means of contaminated pruning tools. Primary and secondary infections may develop into the branch. At this time the infection, if walled off, produces a canker or it penetrates further into the branch and then into the trunk. From here the bacteria may move into other branches and finally the trunk. Trunk cankers will eventually girdle the tree and the whole tree will die. The disease can be very severe in some years, causing repeated infections during warm and wet weather.

Fire blight is one of the most difficult diseases to manage. The infection period is long, and moreover, very few effective chemicals are available. Integrated programs that combine sanitation and orchard management with chemical and biological controls are the best approaches. If the disease is in its early stage and only a few twigs are blighted, it often can be eliminated by pruning. Thus, aggressive and regular scheduled pruning of diseased tissue is essential for keeping inoculum levels low in an orchard.

Current chemical control programs for fire blight control are based on protective schedules, because available compounds are contact treatments and are not systemic. Control with copper compounds is only satisfactory when disease severity is low to moderate. These treatments are only used during dormant and bloom periods because phytotoxic effects commonly occur on fruit as russeting. Still, new formulations of copper are being developed with low metallic copper equivalent (MCE) that might not cause phytoxicity at low application rates. Antibiotics for blight control include streptomycin and the less effective oxytetracycline that both target sites in the protein biosynthesis pathway of the pathogen. Pathogen resistance against streptomycin is widespread in California.

New, more effective materials for fire blight control with a different mode of action from currently used bactericides is being developed to combat this destructive disease. These could be incorporated into a resistance management program rotations and mixtures. The most effective alternative treatment that we identified over the years with an efficacy equal to streptomycin is the antibiotic kasugamycin (Kasumin). This compound has also shown very good efficacy in controlling fire blight in other pome fruit growing areas of the country. Concerns have been expressed by regulatory agencies regarding the use of antibiotics in agriculture that are also used in human medicine, but kasugamycin is not used in human and animal medicine and has a different mode of action from streptomycin or oxytetracycline (no cross-resistance). Through our efforts, registration of Kasumin in California is expected in 2013.

Kasugamycin was again effectively used in our field trials in 2012. It was applied by itself and in mixtures with selected other materials, including biological treatments. This was done to identify effective mixture treatments that would reduce the potential for resistance development. A new material that we included in 2012 was AgriTitan, an oxidizing sanitizer for field use. Additionally, we continued to evaluate the biocontrols Actinovate (*Streptomyces lydicus*) and Blossom Protect (*Aureobasidium pullulans*), the natural products Citrox + ProAlexin, the fermentation product polyoxin-D (Ph-D), as well as the fungicide quinoxyfen (Quintec) that was shown to have antibacterial activity by us in the management of bacterial spot of tomato and by others for selected bacterial diseases. We also evaluated the reduced MCE copper compound Badge in a program with four consecutive sprays.

In another objective of our project we are investigating the molecular mechanism of streptomycin resistance in California isolates of *E. amylovora*. Several mechanisms have been described for isolates of the pathogen from various locations. The two major groups are: i) a point mutation in the chromosomal *rps*L gene; and ii) resistance genes *StrA* and *StrB* that are associated with a transposon (i.e., Tn*5393*) and that are most commonly located on one of several plasmids. Strains with a high level of streptomycin resistance are associated with the chromosomal gene; whereas, moderate resistance is associated with the *StrA* and *StrB* genes in California. We have determined that the majority of recent streptomycin-resistant isolates in California have the *StrA* and *StrB* genes. These are, however, located on a plasmid that previously has not been found to carry resistance genes. This novel mode of resistance was further investigated in 2012 in an attempt to better understand the biology of the pathogen and how it responds to selection pressures.

Management of postharvest decays. Apples, like other pome fruit, can be stored for some period of time using the correct storage environments. Still, postharvest decays caused by fungal organisms can cause serious crop losses. The major postharvest decays of apples include *Penicillium expansum, Botrytis cinerea, Alternaria alternata,* and *Mucor piriformis* causing blue mold, gray mold, black mold, and Mucor decay, respectively. Bull's eye rot caused by *Neofabraea* species can be a major problem in the apple growing areas of the Pacific Northwest, but can also cause losses in California.

New postharvest fungicides including Penbotec (pyrimethanil - 2005), Scholar (fludioxonil - 2005), and Judge (fenhexamid – 2007) were developed by us and others because Captan at the registered postharvest rate of 2 lb/200,000 lb is ineffective against blue mold and TBZ- (Mertect 340F) resistance is widespread in populations of *B. cinerea* and *P. expansum.* These new treatments are just recently being utilized in California and the Pacific Northwest (PNW) because many countries had to establish maximum residue limits (MRLs) to allow the import of fruit.

Although five fungicides (Captan, TBZ, Scholar, Penbotec, Judge) are now registered for postharvest use on apple, only two of them are highly effective against TBZ-resistant blue mold (Scholar, Penbotec). Thus, we are identifying and developing additional postharvest fungicides, and we continued our evaluation of the sterol biosynthesis inhibitor difenoconazole. Our laboratory selection studies indicated that Scholar and Penbotec have a similar high risk to develop resistance. For difenoconazole, the resistance potential has not been determined. Resistance to Penbotec in the field and in the packinghouse has already been reported in other pome fruit growing areas of the US (e.g., PNW). To prevent field resistance from developing in packinghouses, anti-resistance strategies that include the use of fungicide rotations and mixtures need to be followed. One goal is to ultimately provide a pre-mixture of fludioxonil and difenoconazole that is both highly efficacious and cost-effective. For this, we are optimizing usage rates, application methods, and we are evaluating different formulations of a pre-mixture for managing gray mold, blue mold, and bull's eye rot. Although this latter decay is only of sporadic importance in California (but very important in the Pacific Northwest), management strategies need to be known in the event of a disease outbreak. We also evaluated the effect of incubation temperature between fruit inoculation and treatment for selected fungicide applications to provide additional information on usage strategies. Temperatures during harvest and packing in late summer/fall can vary widely under California conditions, but are generally low under Pacific Northwest conditions.

In 2012, we also determined the sensitivity of *Alternaria* isolates from pome fruit to fludioxonil and difenoconazole. Both fungicides are effective against Alternaria rot. Baseline sensitivity data are used for establishing a reference point of toxicity of a fungicide to a selected population of a pathogen. This information is used to compare populations before and after introduction or registration and use of a fungicide so that changes or shifts in sensitivity can be documented. Previously, we developed baseline sensitivity data for fludioxonil and pyrimethanil against *Penicillium* and *Botrytis* spp. and for difenoconazole against populations of *P. expansum* and *Neofabraea perennans*.

None of the currently registered postharvest treatments with high efficacy is approved for organic production. Recently the bio-fungicide polyoxin-D that we have been developing for use on tree crops has obtained an exempt registration status. We previously evaluated this compound as a postharvest treatment for stone fruit and found it to be very effective on some crops (e.g., cherries), but we never evaluated it for pome fruit. With the exempt status, higher rates can now be used than recommended previously. Thus, we initiated our postharvest evaluations with polyoxin-D.

OBJECTIVES FOR 2012

1. Evaluate the efficacy of treatments for managing fire blight and characterize antibiotic resistance.
    A. Evaluate the antimicrobial kasugamycin (Kasumin) as compared to the antibiotics oxytetracycline or streptomycin and the efficacy of fungicidal compounds (e.g., Captan, Dithane, Syllit, Ph-D, and Quintec) in selected mixtures with antimicrobials
    B. Evaluate the efficacy of new biocontrol agents (i.e., Actinovate, Blossom Protect) and natural products (e.g., Cerebrocide)
    C. Evaluate the efficacy of sanitizing agents (Deccosan) and other treatments (titanium dioxide – AgriTitan).
    E. Characterization of streptomycin- and oxytetracycline-resistant strains using molecular approaches: characterize plasmids that harbor the resistance genes and compare to *E. amylovora* populations from other parts of the country.
2. Evaluate new postharvest fungicides for managing apple decays in storage
    A. Evaluate the efficacy of *final* formulations of difenoconazole alone and in mixtures with fludioxonil, TBZ, or pyrimethanil using low- and high-volume spray applications and in-line drench applications. Temperature effects will also be evaluated.
    B. Determination of baseline sensitivities and evaluation of the resistance potential
        - *P. expansum* resistance potential to difenoconazole - Exposure of large populations to a gradient of fungicide concentrations using the SGD method.
        - Determination of baseline sensitivities of fludioxonil & difenoconazole against *Alternaria* spp.
        - Determine if higher concentrations of the organic polyoxin-D fungicide are effective against blue mold.

MATERIALS AND METHODS

***Laboratory studies on the toxicity of bactericides against* E. amylovora.** Kasugamycin (Kasumin 2L, Arysta Life Sciences, Cary NC), streptomycin (Sigma, St. Louis, MO), and oxytetracycline (Sigma) were evaluated for their in vitro toxicity using the spiral gradient dilution method. For this, a radial bactericidal concentration gradient was established in nutrient agar media in Petri dishes by spirally plating out a stock concentration of each antimicrobial using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Norwood MA). After radially streaking out suspensions of the test bacteria (10 µl of 10^8 cfu/ml as determined by measurement of optical density at 600 nm) along the concentration gradient, plates were incubated for 2 days at 25ºC. Measurements were visually taken for two inhibitory concentrations: i) the lowest inhibitory concentration (LIC; the lowest concentration where inhibition of bacterial growth was observed, i.e., where the bacterial streak became less dense visually), and ii) the minimal concentration that inhibited growth by >95% (MIC). The actual antibiotic concentrations were obtained by entering the radial distances of inhibition (measured from the center of the plate) into the Spiral Gradient Endpoint computer program (Spiral Biotech, Inc.).

Isolation of* E. amylovora, *bacterial culturing, and verification of species identity. Fruit samples with fire blight symptoms were obtained in the spring and early summer of 2012 from orchards in selected counties. Infected plant material (flowers, fruit, stems, and pedicels) was surface-disinfested for 1 min using 400 mg/L sodium hypochlorite, rinsed with sterile water, cut into small sections, and incubated in 1 ml of sterile water for 15 to 30 min to allow bacteria to stream out of the tissue. Suspensions were streaked onto yeast extract-dextrose-$CaCO_3$ agar (YDC). Single colonies were transferred and the identity of the isolates as *E. amylovora* was verified by colony morphology and by PCR using primers specific for the ubiquitous *E. amylovora* plasmid pEA29 described by Bereswill et al. (Appl. Environ. Microbiol. 58:3522-2536). The presence of a 1-kb DNA fragment after gel electrophoresis confirmed a positive identification. A total of 21isolates of *E. amylovora* from eight orchard locations were obtained in 2012.

Field studies on fire blight using protective treatments during the growing season. In a field study on apple cv. Granny Smith in an experimental orchard at KARE, four treatments were applied at 30% bloom (3-21-12), 85% bloom (3-24-12), 95% bloom (4-3-12), and 100% bloom (4-15-12) using an airblast sprayer at 100 gal/A. There were five single-tree replications for each treatment. Trees were evaluated for incidence of fire blight and for potential phytotoxic effects of the treatments in May of 2012. Data were analyzed using analysis of variance and LSD mean separation procedures of SAS 9.1.

Characterization of streptomycin-resistant strains using molecular approaches. The location in the genome of the *strA-strB* resistance genes that previously were found to be associated with transposon Tn*5393* was characterized for representative California isolates of *E. amylovora*. Additionally, the exact location was determined on plasmid pEU30 by plasmid mapping. Plasmids were also isolated from sensitive, moderately resistant and highly resistant isolates using a commercial kit and digested with *Kpn*I. Fragments were separated on agarose gels and banding patterns were analyzed visually for the presence of pEU30. This work was done in collaboration with Dr. G. Sundin at Michigan State University.

Efficacy of postharvest treatments and application methods using single fungicides and mixtures. The efficacy of difenoconazole (formulation A8574D), Scholar 230SC, mixtures and pre-mixtures (i.e., A20171A) of these two fungicides at different rates, and of Penbotec was evaluated. Granny Smith apples were wound-inoculated with TBZ-resistant isolates of *B. cinerea* (10^5 conidia/ml), *P. expansum* (10^6 conidia/ml), or with *N. perennans* (10^6 conidia/ml), incubated for 16-17 h at 20C, and then treated. Before fungicide treatment, fruit were first sprayed with chlorine at 100 ppm and then rinsed with water. Fungicides were applied on an experimental packingline at the Kearney Agricultural Center as aqueous solutions using in-line drench applications that were followed by low-volume spray applications with fruit coating (Decco 231, a carnauba-based coating). After treatment, fruit were stored at 20 C, 95% RH for 6 to 8 days and then evaluated for the incidence of decay. Data were analyzed using analysis of variance and least significant difference mean separation procedures of SAS 9.1.

To evaluate the effect of fruit temperature on fludioxonil residues, Fuji and Granny Smith apple fruit or Bosc pear fruit were equilibrated to temperatures of 1.5, 12.5, or 20 C and then dipped for 30 sec in an aqueous solution of 180 ppm fludioxonil at 10C. Fruit were then air-dried and processed for residue analysis. Two experiments were done with a total of five residue values for each temperature.

***Evaluation of the resistance potential to difenoconazole in populations of* P. expansum.** In laboratory studies, selection plates with a continuous concentration gradient for difenoconazole were prepared using a spiral plater. Conidia of *P. expansum* (10^8/plate) of single-spored sensitive isolates were plated onto these selection plates, and plates were incubated for up to 7 days. Fungal colonies growing inside the EC_{95} concentration ranges were sub-cultured and evaluated for their fungicide sensitivity. Resistance frequencies were calculated based on the number of resistant isolates obtained per plate of the total number of spores plated out.

***In vitro fungicide sensitivity studies for* Alternaria *spp. and* Botrytis cinerea.** A total of 34 isolates of *Alternaria* sp. from decayed pome fruit were evaluated for their sensitivity to fludioxonil and difenoconazole, and nine isolates of *B. cinerea* were evaluated for their sensitivity against difenoconazole. Fungicide sensitivity was determined using the spiral gradient dilution method. A conidial suspension of the fungus was streaked along the radial fungicide gradient in the agar Petri dish and the 50% inhibitory concentrations for mycelial growth were determined as described previously.

RESULTS AND DISCUSSION

***Survey of antibiotic sensitivity among* E. amylovora *strains collected in California*.** Isolates of *E. amylovora* were confirmed for species identity by PCR amplification of a 1-kb DNA fragment using specific primers for plasmid pEa29 that is ubiquitously found in this bacterium. A total of only 21 isolates from 8 orchard locations (1 to 5 isolates per location) were obtained and tested for their sensitivity against streptomycin, oxytetracycline, and kasugamycin. Still, this limited survey produced some interesting results.

In some orchards all isolates were found to be sensitive against the three antibiotics (Table 1). In other orchards (Sacramento and Fresno Co.), however, high levels of resistance against streptomycin were present. In 2012, a high level of streptomycin-resistance was present in two orchards. All isolates of the pathogen belonged to the high-resistance category, and minimum concentrations to completely inhibit growth of the bacterium exceeded 50 ppm. Furthermore, these resistant isolates all also had a reduced sensitivity to oxytetracycline. MICs of isolates sensitive to oxytetracycline were 0.09 - 0.38 ppm; whereas those for isolates with reduced sensitivity were 1.25 to 1.88 ppm. Thus, this is very similar to what we observed at a few orchard locations in 2007 and 2009: isolates with high resistance to streptomycin had a reduced sensitivity to oxytetracycline. In contrast, other isolates with high-streptomycin resistance that we found in our previous surveys were sensitive to oxytetracycline. High-streptomycin resistance that is due to a chromosomal mutation was the first type of streptomycin resistance described in West coast growing areas (Chio and Jones, 1995). Based on our surveys over the years, his type of resistance has been mostly replaced by moderate resistance where *strA-strB* resistance genes are located on plasmids. All isolates collected in 2012 were sensitive to kasugamycin.

Field studies on fire blight using protective treatments during the growing season. In a field trial to control natural incidence fire blight on Granny Smith apple, 23 treatments were evaluated using four applications each. Kasumin (two formulations) continued to perform very well (Fig. 1). The 2L formulation (that ultimately will be marketed) numerically resulted in the second lowest (after Firewall) disease incidence among the single-active ingredient treatments. Among the mixture treatments evaluated, Kasumin-Firewall, Kasumin-Fireline, Kasumin-Prophyt, and Kasumin-Manzate had the lowest incidence of disease. Mixture partners for kasugamycin and the registered antibiotics need continued evaluation to maximize the efficacy of treatments and as part of a resistance management program. A California registration of kasugamycin for pome fruit is expected for 2013. The biocontrol Blossom Protect (*Aureobasidium pullulans*) was also very effective in 2012, and Actinovate (*Streptomyces lydicus)* showed intermediate efficacy, similar to copper. In 2011, these biocontrols only numerically reduced the disease from that of the control. No new effective treatments were identified. The sanitizer AgriTitan and the biofungicide polyoxin-D were not effective at the rates evaluated. In summary, in our program identification of integrated fire blight programs with copper, fungicides, antibiotics, and biocontrols, as well as optimum application conditions (e.g., water pH) is successfully being pursued for the California pome fruit industries.

Characterization of streptomycin-resistant strains of* E. amylovora *using molecular approaches. High-resistance to streptomycin in California isolates was previously found to be correlated with a mutation in the ribosomal protein S12 (*rps*L) gene located on the bacterial chromosome, similar as was described for West coast isolates by Chio and Jones in 1995. We continued to investigate the molecular mechanism of moderate streptomycin resistance that is based on acquisition of *strA-strB* resistance genes. Based on our surveys over the past seven years, this type of resistance is currently much more common than the high-resistance based on a chromosomal mutation. We previously had confirmed the presence of *strA-strB* and transposon Tn*5393* in California isolates. It was found to be located on plasmid pEU30 that has been described from isolates from the western United States in 2004, but not on plasmid pEa34 or pEa29 as in isolates from Michigan. Thus, California isolates show a unique mode of resistance. PCR amplifications confirmed the association of *strA-strB* with pEa30 in all evaluated moderately resistant isolates that were collected between 2006 and 2011 from various locations in California (no moderately resistant isolates were found in 2012 due to limited sampling). Based on restriction enzyme analysis, plasmid pEU30 is also present in highly resistant isolates, but does not carry *strA-strB*. We continued to molecularly analyze this new mode of resistance, and in collaboration with G. Sundin, were able to determine the insertion site of the resistance genes in the plasmid. This information, together with our streptomycin resistance survey data, is currently being prepared for publication.

Efficacy of postharvest treatments using single-fungicides and mixtures. Experimental packingline studies were conduced to evaluate single-fungicide, mixture, and pre-mixture treatments (Figs. 2,3). Efficacy of most treatments against blue mold was lower in the second study (Fig. 3), most likely because fruit were more ripe and therefore more susceptible to decay. Scholar at 180 ppm in in-line drench applications effectively reduced blue mold and gray mold, but not bull's eye rot (Figs. 1,2). Difenoconazole was highly effective against blue mold and also bull's eye rot, but also against gray mold in these studies. Previously, this fungicide did not show good efficacy against gray mold, indicating that the physiology of the fruit and its susceptibility to decay determines the success of gray mold control using difenoconazole. Overall, difenoconazole should be regarded as a weak treatment for the management of gray mold; it is mostly effective against blue mold and bull's eye rot.

The registrant of difenoconazole and fludioxonil (Syngenta Crop Protection) is finalizing the formulation of a pre-mixture, and thus, we evaluated its effectiveness. At all rates tested, the pre-mixture treatments reduced the incidence of the three decays to low levels (Figs. 2,3). Thus, this pre-mixture broadens the spectrum of activity of the single fungicides with managing blue mold, gray mold, and bull's eye rot. Note that the rates used are based on both active ingredients combined and are less than 500 ppm. These three decays are also controlled by Penbotec. Resistance against pyrimethanil, however, has developed in some populations of the three decay fungi at some locations and thus, this fungicide has to be rotated with different modes of action. Although difenoconazole is not effective against gray mold, and generally did not provide an additive effect in blue mold control when used in mixtures with Scholar as compared to using Scholar alone, registration of a pre-mixture will be an important tool to decrease the risk of fungicide resistance to develop in populations of *Penicillium* spp. These results support our plans to support a difenoconazole registration for postharvest use on pears and apples through the IR-4 program.

In a study using Granny Smith apple, polyoxin-D (Ph-D) at all rates tested was similarly effective to Penbotec in controlling gray mold (Fig. 4). Blue mold, however, was not reduced as compared to the control. In preliminary studies, Ph-D was also effective against bull's eye rot. Because this material is currently one of the most effective treatments for managing Alternaria diseases of several crops (including almond where we helped to get this treatment registered), it likely will also be effective against postharvest Alternaria decays of pome fruit. Polyoxin-D recently received an exempt registration status and can be used for organic fruit production. Our data indicate that it has the potential to be the most effective organic compound we ever evaluated. Thus, we will continue our studies with Ph-D in the coming season.

Fruit temperature at treatment time affected the amount of fludioxonil residue of Bosc pear, as well as two apple cultivars. A fruit temperature of 7.5C resulted in lower residues (average of 0.26 ppm on Bosc pear) than temperatures of 12.5 or 20C (averages of 0.45 and 0.46 ppm on Bosc pear, respectively) when temperature of the treatment solution was 10C (Fig. 5). Residue levels were similar for Bosc pear and Granny Smith apple and lower than on Fuji apple. Thus, fruit temperature in relation to treatment-solution temperature is an important parameter for fungicide uptake and additional fruit temperature-treatment temperature combinations could be evaluated.

***Evaluation of the resistance potential to difenoconazole in populations of* P. expansum.** Isolates of *P. expansum* with reduced sensitivity against fludioxonil and pyrimethanil were readily obtained in previous studies when large numbers of conidia were plated on selection plates. Resistance frequencies ranged from $1x10^{-8}$ to 3.6×10^{-5} for fludioxonil and from $1.2x10^{-8}$ to 1.8×10^{-6} for pyrimethanil. For fludioxonil, isolates were either moderately resistant (EC_{50} 0.77 to 3.5 mg/L; sensitive isolates: <0.02 mg/L) or highly resistant (EC_{50} >40 mg/L), whereas for pyrimethanil a range of sensitivities (EC_{50} 1.8 to >75 mg/L; sensitive isolates: <0.70 mg/L) was observed. Isolates insensitive to both fungicides were recovered at very low frequency in some tests and always displayed a lower level of resistance. Most resistant isolates were stable in culture and were pathogenic in apple fruit inoculations. Using the same protocol in several experiments in last year's studies, no isolates with reduced sensitivity to difenoconazole were obtained (Fig. 6). Our data indicate that the risk of resistance development against new postharvest fungicides for pome fruit varies and may be high for some fungicides, and that resistance management is crucial.

***In vitro fungicide sensitivity studies for* Alternaria *spp. and* Botrytis cinerea.** Sensitivities of 34 *Alternaria* spp. isolates from pome fruit against fludioxonil and difenoconazole were within a narrow range (0.011 to 0.025 ppm for fludioxonil, 0.010 to 0.040 ppm for difenoconazole) and all isolates were highly sensitive to the two fungicides (Fig. 7A,B). This is an indication that Alternaria decays will be effectively managed by postharvest treatments with the two fungicides. Additional isolates of *Alternaria* spp. will be collected in the future to obtain a full baseline range. Supporting the low efficacy of difenoconazole in controlling gray mold, the range of EC_{50} values for nine isolates of *Botrytis cinerea* was high at 0.162 to 0.884 ppm (Fig. 8). Residue values less than 1 ppm are expected on fruit with maximum applications rates of 300 ppm difenoconazole similar to fludioxonil and thus, are insufficient to be highly effective against gray mold.

Registration status of postharvest fungicides evaluated. Scholar (fludioxonil), Penbotec (pyrimethanil), and Judge (fenhexamid) have US-EPA postharvest registration. Table 2 shows maximum residue limits (MRLs) for several fungicides in selected countries. Scholar has received MRLs and Codex tolerances in most countries of the world. Additionally, a food additive tolerance (FAT) has been obtained in Japan. The FAT for pyrimethanil in Japan is pending and MRLs are being established worldwide. Difenoconazole registration is going through the IR-4 program with federal registration pending in 2014 (MRLs have already been established for preharvest use as shown in Table 2). As indicated above, polyoxin-D is currently registered with exempt status in the United States but international exemption or MRLs need to be established in other countries.

Table 1. Incidence of resistance against streptomycin, oxytetetracycline, or kasugamycin in isolates of *Erwinia amylovora* collected in surveys of 8 California orchards in 2012

Orchard No.*	No. of isolates	Incidence streptomycin resistance (%)**	Incidence reduced oxytetracycline sensitivity (%)***	Incidence kasugamycin resistance (%)****
1	2	0	0	0
2	5	80**	80	0
7	3	0	0	0
3	4	100**	100	0
4	1	0	0	0
5	2	0	0	0
6	1	0	0	0
8	3	0	0	0
Total	21			

* - Note that some orchards had several blocks

** - Inhibitory concentrations were determined on nutrient agar using the SGD method. Minimum inhibitory concentrations (MIC, >95% inhibition) of isolates sensitive to streptomycin were 1.0 - 2.7 ppm; whereas those of isolates resistant to streptomycin were >50 ppm.

***- MICs of isolates sensitive to oxytetracycline were 0.09 - 0.38 ppm; whereas those for isolates with reduced sensitivity were 1.25 to 1.88 ppm.

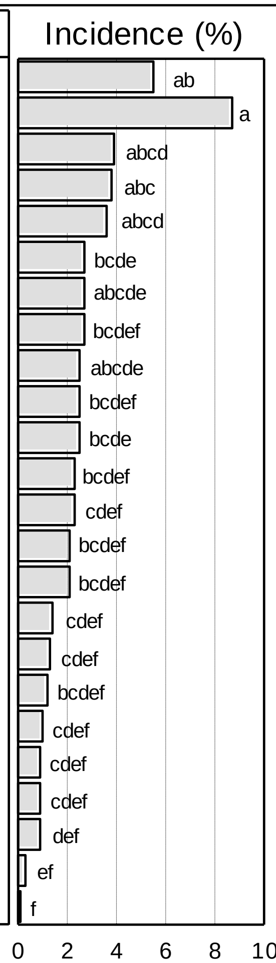
****-MICs for kasugamycin were 3.8 to 16.1 ppm.

Table 2. Maximum residue limits (MRLs) for four fungicides in the United States (US), Codex (Cod), the European Union (EU), Hong Kong (HG), Japan (Jpn), Korea (Kor), and Taiwan (Tai).

Crop	Fungicide	US *1*	Cod *2*	EU *3*	HK *4*	Jpn *5*	Kor *6*	Tai *7*
Apple	Difenoconazole	1 PPM	{0.5} PPM	{0.5} PPM	{0.5} PPM Cod	1 PPM	1 PPM	{0.5} PPM
Apple	Fludioxonil	5 PPM	5 PPM	5 PPM	5 PPM Cod	5 PPM	{1} PPM	5 PPM
Apple	Pyrimethanil	14 PPM	{7} PPM	{5} PPM	{7} PPM Cod	{5} PPM	{2} PPM	{7} PPM

Fig. 1. Efficacy of bactericides for fire blight management on Granny Smith apple in a field trial at Kearney Ag Center 2012

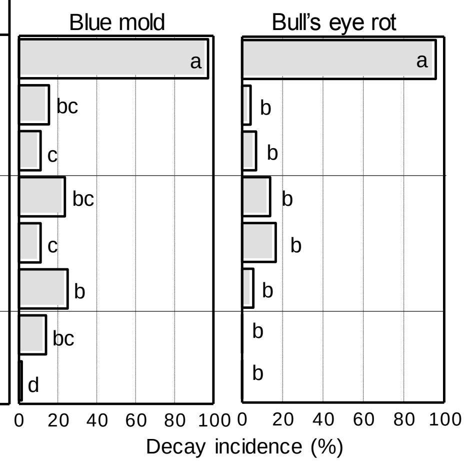
No.	Treatment*	Rate/A
1	Control	---
2	Ph-D	6.2 oz
3	Blossom Protect + Buffer + Actinovate	1.34 lbs + 9.35 lbs + 12 oz
4	Deccosan 315 foll'd by Blossom Protect + Buffer	1:50 + 1.34 lb + 9.35 lb + 8 fl oz
5	AgriTitan	1:25
6	Kasumin 2L + Captan 50WP	100 ppm + 128 oz
7	Badge X2	8 oz
8	Fireline	200 ppm
9	Kasugamycin 2L + Ph-D	100 ppm + 6.2 oz
10	Fireline + Firewall	200 ppm + 100 ppm
11	Kasugamycin 2L + Actinovate	100 ppm + 12 oz
12	Kasumin 2L + Quintec	100 ppm + 6 fl oz
13	Kasumin 2L + Syllit 65WP	100 ppm + 32 oz
14	Actinovate	12 oz
15	Deccosan 315 foll'd by Actinovate + Breakthru	1:50 + 12 oz + 8 fl oz
16	Blossom Protect + Buffer + Citrox + ProAlexin	1.34 + 9.35 lbs + 133 + 133 ml
17	Kasumin 8L	100 ppm
18	Kasumin 2L + Manzate ProStik	100 ppm + 2 lb
19	Blossom Protect + Buffer	1.34 lbs + 9.35 lbs
20	Kasumin 2L + Prophyt	100 ppm + 64 fl oz
21	Kasumin 2L	100 ppm
22	Kasumin 2L + Fireline	100 ppm + 200 ppm
23	Kasumin 2L + Firewall	100 ppm + 100 ppm
24	Firewall	100 ppm



Treatments were applied at 30% bloom (3-21-12), 85% bloom (3-24-12), 95% bloom (4-3-12), and 100% bloom (4-15-12) using an airblast sprayer at 100 gal/A. Disease was evaluated in May 2012 and the incidence was based on the number of diseased spurs of the total numberof spurs evaluated.

Fig. 2. Efficacy of bactericides for fire blight management on Granny Smith apple in a field trial at Kearney Ag Center 2012

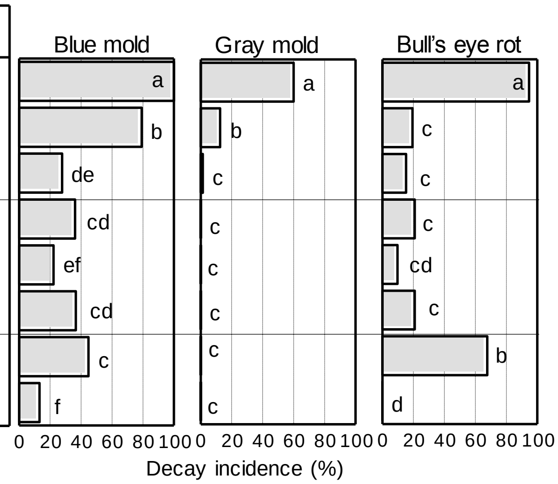
	Fungicide	Active ingredients	rate
	Control (water)	---	---
	A8574D 360SC	difenoconazole	300 ppm
	Scholar 230SC + A8574D	fludiox. + difenoc.	180 ppm + 300 ppm
New pre-mixture	A20171A 384SC	fludiox. + difenoc.	135 ppm + 225 ppm
New pre-mixture	A20171A 384SC	fludiox. + difenoc.	157 ppm + 262 ppm
New pre-mixture	A20171A 384SC	fludiox. + difenoc.	180 ppm + 300 ppm
	Scholar 230SC	fludioxonil	180 ppm
	Penbotec	pyrimethanil	383 ppm



Fruit were inoculated with conidia of a TBZ-resistant isolate of *Penicillium expansum* or with *Neofabraea perennans* (10^6 conidia/ml), incubated for 16-17h at 20C, and treated. In-line, aqueous, re-circulating drench applications were followed by a CDA application with carnauba fruit coating (Decco 231). Fruit were then incubated at 20 C for 6 days. A20171A is a new pre-mixture of fludioxonil and difenoconazole.

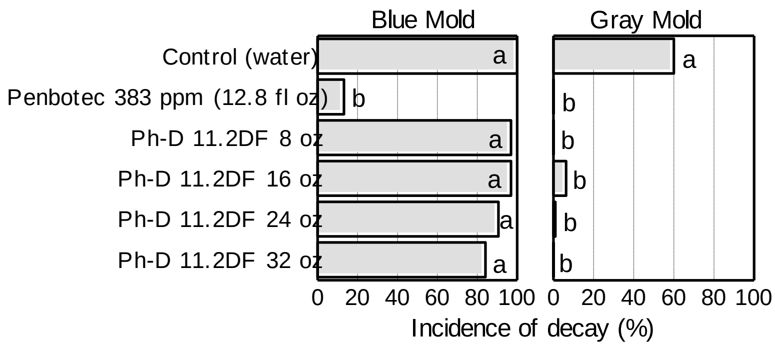
Fig. 3. Efficacy of bactericides for fire blight management on Granny Smith apple in a field trial at Kearney Ag Center 2012

	Fungicide	Active ingredients	rate
	Control (water)	---	---
	A8574D 360SC	difenoconazole	300 ppm
	Scholar 230SC + A8574D	fludiox. + difenoc.	180 ppm + 300 ppm
New pre-mixture	A20171A 384SC	fludiox. + difenoc.	135 ppm + 225 ppm
New pre-mixture	A20171A 384SC	fludiox. + difenoc.	157 ppm + 262 ppm
New pre-mixture	A20171A 384SC	fludiox. + difenoc.	180 ppm + 300 ppm
	Scholar 230SC	fludioxonil	180 ppm
	Penbotec	pyrimethanil	383 ppm



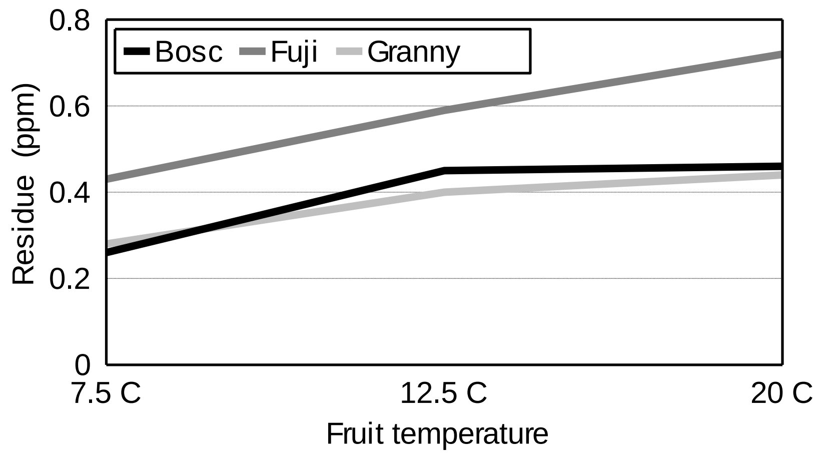
Fruit were inoculated with conidia of TBZ-resistant isolates of *Penicillium expansum* (10^6 conidia/ml) and *Botrytis cinerea* (10^5 conidia/ml), or with *Neofabraea perennans* (10^6 conidia/ml), incubated for 16-17h at 20C, and treated. In-line, aqueous, re-circulating drench applications were followed by a CDA application with carnauba fruit coating (Decco 231). Fruit were then incubated at 20 C for 6 days. A20171A is a new pre-mixture of fludioxonil and difenoconazole.

Fig. 4. Evaluation of postharvest in-line drench applications with Ph-D (polyoxin-D) for managemer blue mold and gray mold decay of Granny Smith in experimental packingline studies



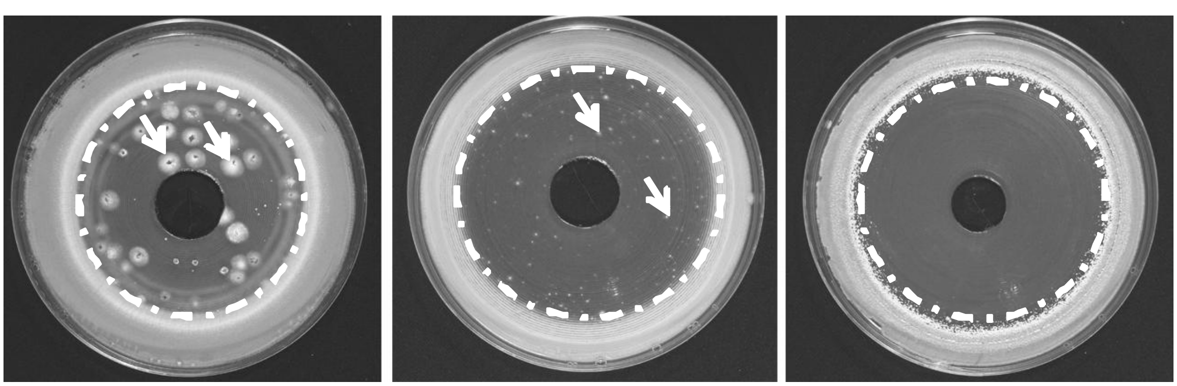
Fruit were inoculated with conidia of *Penicillium expansum* (10^6 conidia/ml) or *B. cinerea* (10^5 conidia/ml) and were incubated for 16-17 h at 20C. Treatments with aqueous fungicide solutions were done by in-line re-circulating drench applications that were followed by a CDA application with carnauba fruit coating (Decco 231). Fruit were then incubated at 20 C for 6 days.

Fig. 5. Effect of fruit temperature on fludioxonil residue after dip treatments



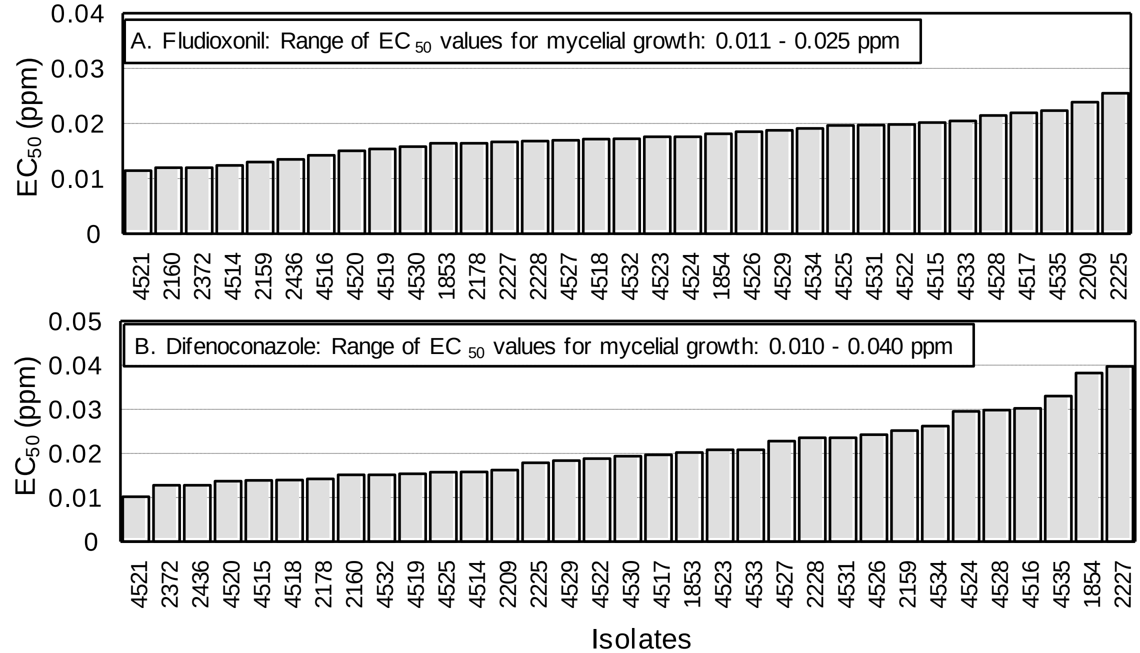
Bosc pear fruit or Fuji and Granny Smith apple fruit were equilibrated to temperatures of 1.5, 12.5, or 20 C and then dipped for 30 sec in an aqueous solution of 180 ppm fludioxonil at 10C. Fruit were then air dried and processed for residue analysis.

Fig. 6. Fungicide resistance potential assay for *Penicillium expansum* using the SGD method



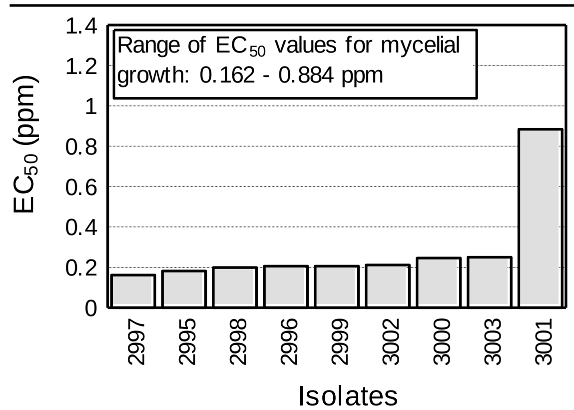
The assay was done for A. Fludioxonil on PDA; B. Pyrimethanil on AP agar; and C. Difenoconazole on PDA. High concentrations of the fungicides are near the center; whereas lower concentrations are near the perimeter of each plate. EC_{95} concentrations are shown as a dotted circle. Arrows indicate resistant isolates. Colonies are small on the pyrimethanil plates because AP media was used. No resistant isolates were detected for difenoconazole.

Fig. 7. Baseline sensitivity to fludioxonil and difenoconazole for 34 isolates of *Alternaria* spp. from pome fruit



Isolates of *Alternaria* spp. were collected from decayed fruit in packinghouses. Fungicide sensitivities for mycelial growth were determined using the spiral gradient dilution method.

Fig. 8. In vitro sensitivity to difenoconazole for 9 isolates of *Botrytis cinerea* from pome fruit



Isolates of *Botrytis* spp. were collected from decayed fruit in packinghouses. Fungicide sensitivities for mycelial growth were determined using the spiral gradient dilution method.