Annual Report - 2011

Prepared for the California Apple commission

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
Project Leader:	Dr. J. E. Adaskaveg, Department of Plant Pathology, University of California, Riverside CA 92521.
Cooperators:	L. Wade (Arysta Life Science), Dr. H. Förster, D. Felts, D. Cary, and D. Thompson

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SUMMARY

1. In the 2011 survey, the incidence of fire blight was low at most locations in 2011 due to cool springtime temperatures and low rainfall during bloom. Population studies of the pathogen indicated an intermediate incidence of streptomycin resistance with resistance present in 5 of 10 locations. Only moderately resistant strains of *E. amylovora* were found. No strains less sensitive to oxytetracycline or kasugamycin were obtained.
2. In a field trial on the management of fire blight on Granny Smith apple, the new antibiotic Kasumin continued to perform well. The new 8L formulation resulted in numerically the lowest disease incidence among the single-active ingredient treatments. Among the 13 mixture treatments evaluated, Kasumin-Firewall, Kasumin-Manzate, and Kasumin-Rezist had the lowest incidence of disease. The two biocontrols evaluated, Actinovate (*Streptomyces lydicus)* and Blossom Protect (*Aureobasidium pullulans*), only numerically reduced the disease from that of the control.
3. Kasugamycin (Kasumin) registration in the US is being pursued on pome fruit with federal registration expected in 2012.
4. Studies on the molecular mechanism of streptomycin resistance in *E. amylovora* revealed a point mutation in the chromosomal *rps*L gene in highly resistant isolates. Moderately resistant isolates contained the *strA-strB* genes on transposon Tn*5393*. Preliminary studies indicated that in California isolates the resistance genes were not located on plasmids previously shown to harbor resistance (i.e., pEa29, pEa34), but on another plasmid, pEU30 that has never been described as a plasmid carrying the streptomycin resistance genes.
5. Experimental packingline studies were conducted on the management of postharvest decays of apples. All fruit were treated using the currently most effective application method, the in-line, recirculating drench system. The DMI fungicide difenoconazole was evaluated at selected rates by itself and in mixtures with Scholar. This was done with the registrant's support to design an effective and cost-effective pre-mixture.
6. In contrast to most previous studies, difenoconazole showed efficacy against gray mold in two of the three studies where freshly harvested fruit were used for the tests. Difenoconazole was consistently highly effective against blue mold and a minimum rate of 360 ppm is recommended. The difenoconazole-Scholar mixtures reduced gray mold and blue mold to zero or very low levels.
7. A new formulation of TBZ, Alumni, was not effective against gray mold and blue mold decay caused by TBZ-resistant isolates of the respective pathogens. Decay, however, was effectively controlled when Alumni was mixed with Scholar.
8. Molecular identification of *Neofabraea perennans*, cause of bull's eye rot, was demonstrated using species-specific primers.
9. Although Scholar is active in vitro against *N. perennans*, the fungicide is not highly efficacious on fruit because it is a contact material and does not inhibit existing infections. Contrastingly, difenoconazole, pyrimethanil (Penbotec), and TBZ are locally systemic and are highly efficacious on fruit against this decay.

10. The in vitro sensitivity of mycelial growth of *N. perennans* to difenoconazole was evaluated. The baseline sensitivity range was 0.003 to 0.07 ppm. Sensitivity of isolates was normally distributed with most isolates sensitive between 0.009 and 0.02 ppm in a frequency histogram.

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. From 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 infect 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 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. Integrated programs that combine sanitation and treatments with chemical and biological controls are the best approaches available. Sanitation starts with healthy planting material and an effective quarantine regulation to prevent importation of all plant material from areas where the disease is known to occur. 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 regularly 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 (MycoShield) that both target sites in the protein biosynthesis pathway of the pathogen. Pathogen resistance against streptomycin is widespread in California.

New materials for fire blight control have to be developed in order to initiate resistance management practices. This is to ensure that resistance to oxytetracycline will not develop in the pathogen population and resistance to streptomycin does not continue to build up. An ideal material should be effective, locally systemic, not phytotoxic, should target multiple sites of action within the bacterial pathogen, have a mode of action different from currently used bactericides, and should be safe to humans and the environment. In our research on fire blight of apple and pear, we demonstrated that the antibiotic kasugamycin (Kasumin) has a similar efficacy to streptomycin and oxytetracycline l. Members of the kasugamycin chemical class are not being used in human and animal medicine, in contrast to streptomycin and other antibiotics (e.g., Starner) that we previously evaluated. Kasugamycin has a different mode of action and there is no cross-resistance to streptomycin or oxytetracycline. Through our efforts, kasugamycin is planned for registration in 2012.

A high efficacy of kasugamycin was again observed in our field trial in 2011. We tested a new formulation of the compound (i.e., 8L) that is more concentrated (easier to use on a commercial base) than the previous 2L formulation and that has better storability than the 10L formulation that we evaluated last year. Kasumin was applied by itself in 2011 and in mixtures with selected other materials, including biological treatments. We also included additional new materials in our field evaluations in 2011 to find new alternatives that can be used in rotation programs. Among these materials were the biocontrols Actinovate (*Streptomyces lydicus*) and Blossom Protect (*Aureobasidium pullulans*), the natural products Citrox and

ProAlexin, as well as the fungicides dodine (Syllit), captan, mancozeb (Manzate), and quinoxyfen (Quintec). We demonstrated activity of Captan and Syllit in fire blight trials previously and of Quintec in the management of bacterial spot of tomato. In previous years' field trials we demonstrated that kasugamycin in mixture with captan, mancozeb, dodine, or low rates of new formulations of copper with lower MCE (e.g., Badge X2) often had an increased efficacy with no phytotoxic effects. Laboratory studies were conducted in 2011 to find out if this increase in efficacy is based on synergism or additive activity.

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. For California isolates, the main mechanism reported to date is a point mutation in the chromosomal *rps*L gene that confers a high level of resistance. For three isolates with moderate levels of resistance, the streptomycin resistance genes *StrA* and *StrB* were found to be located on plasmid pEa8.7 that closely resembles the broad-host-range plasmid RSF1010 (Palmer et al., Appl. Envir. Microbiol. 63:4604-4607, 1997). Among streptomycin-resistant isolates collected in Michigan, the chromosomal point mutation was rare and for the majority of isolates, *StrA* and *StrB* were found to be on transposon Tn*5393* that is integrated into either plasmid pEa34, or more recently, into plasmid pEa29 (the ubiquitous non-conjugative plasmid). Less commonly, the genes were found to be integrated in the bacterial chromosome. In 2011, we continued to analyze streptomycin resistance mechanisms in California isolates of the pathogen. This will help us to better understand the biology of the pathogen, how it responds to selection pressures, and this may lead to improved management strategies.

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. Historically, thiabendazole (TBZ) has been the main postharvest fungicide available for pome fruit. With extensive usage, resistant populations of *Penicillium* and *Botrytis* spp. have developed and are commonly found in packinghouse storage rooms. The risk of resistance development in the postharvest apple pathogens to fungicides is high because fruit are stored for extended periods of time and often receive more than one postharvest treatment, leading to an increased selection pressure in the pathogen populations. Additionally, the pathogens produce abundant spores, favoring the selection of resistant individuals.

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). Our laboratory selection studies indicated that the latter two fungicides have a similar high risk to develop resistance. 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 becoming established in packinghouses, anti-resistance strategies with use of fungicide rotations and mixtures need to be followed. For this, we are identifying additional potential postharvest fungicides, and we continued our evaluation of the sterol biosynthesis inhibitor difenoconazole. We are working in close collaboration with the registrant of Scholar and difenoconazole, Syngenta Crop Protection, who is very supportive of these studies. The goal is to ultimately provide a pre-mixture of these fungicides that is both highly efficacious and cost-effective and for this; we are optimizing usage rates and application methods.

An additional goal of our studies in 2011 was to evaluate the efficacy of treatments against bull's eye rot. Although this 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. For the

development of a baseline sensitivity range for difenoconazole, we obtained over 70 isolates of *Neofabraea perennans* that were identified using species-specific primers.

OBJECTIVES FOR 2011

1. Evaluate the efficacy of treatments for the management of fire blight in small-scale and air-blast field trials in cooperation with UCCE.
   A. Evaluate the antimicrobial kasugamycin (Kasumin) as compared to the antibiotics oxytetracycline or streptomycin
   B. Evaluate the efficacy of fungicidal compounds (e.g., Captan, Dithane, Syllit)
   C. Evaluate the efficacy of new biocontrol agents (i.e., Actinovate, Blossom Protect) and natural products (e.g., Cerebrocide)
   D. Evaluate the efficacy of selected mixtures and rotations.
2. Evaluate new postharvest fungicides for managing apple decays in storage
   A. Evaluate the efficacy of *new* formulations of difenoconazole alone and in mixtures with fludioxonil, TBZ, or pyrimethanil using low- and high-volume spray applications and in-line drench applications.
   B. Optimize postharvest fungicide usage by evaluating different rates of *pre-mixture* treatments and adjuvants with the goal to develop treatments that minimize the potential for selecting resistant populations of decay pathogens.
   C. Evaluate organic fungicides – CX 10440 and Ph-D for pome fruit (first active ingredient registered in EPA biopesticide program).
3. Evaluate sanitation treatments – sodium hypochlorite and peroxyacetic acid in combination with surfactants and fungicides (e.g., captan).

MATERIALS AND METHODS

Isolation of* E. amylovora*, bacterial culturing, and verification of species identity. Plant samples with fire blight symptoms were obtained in the spring and early summer of 2011 from orchards in Sacramento Co. A total of 47 isolates of *E. amylovora* from ten orchard locations were obtained. 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.

***Laboratory studies on the toxicity of bactericides against* E. amylovora.** Kasugamycin (Kasumin 2L, Arysta Life Sciences), streptomycin, and oxytetracycline 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), and ii) the minimal concentration that inhibited growth by >95% (MIC). 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.

To investigate the interaction between kasugamycin and captan, mancozeb, or dodine, a microtiter plate assay was used. *E. amylovora* was grown in nutrient broth in the absence (control) or presence of kasugamycin or any of the three fungicides, or in mixtures of kasugamycin with the fungicides. Rates were chosen where growth of *E. amylovora* was only partially inhibited to be able to

see interaction effects. Plates were shaken for 20 h, and the optical density at 600 nm was determined as a measurement of growth. Reduction of growth by the treatments was compared to the untreated control.

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 the Kearney AgCenter, two treatments were applied using an air-blast sprayer (100 gal/A) at 90% and 100% bloom. One day after the second treatment, trees were inoculated with an antibiotic-sensitive strain of *E. amylovora* using an air-blast sprayer. 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. 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 genomic region of the *rps*L gene from highly and moderately resistant as well as from sensitive isolates of *E. amylovora* was amplified and sequences were compared (Chiou and Jones, Phytopathology 85:324-328, 1995). The presence of *strA-strB* and of transposon Tn*5393* was evaluated using published primers (McGhee et al., Phytopathology 101:182-191, 2011). For representative isolates containing *strA-strB* and Tn*5393,* amplifications were done using primers derived from the transposon and from sequences of selected plasmids. A positive amplification would thus indicate the location of the resistance genes on a particular plasmid. Additional sequence analysis was done to confirm the results. This work was done in collaboration with Dr. G. Sundin at Michigan State University.

Efficacy of postharvest treatments using single fungicides and mixtures. The efficacy of difenoconazole (formulation A8574D), Scholar 230SC, as well as mixtures of these two fungicides were evaluated using different rates and were compared to treatments with Penbotec, Alumni (TBZ), Scholar+Alumni or Ph-D. Granny Smith apples were wound-inoculated with TBZ-resistant isolates of *B. cinerea* or *P. expansum* (10^5 conidia/ml for *B. cinerea,* 5 x 10^5 conidia/ml for *P. expansum*), incubated for 18-19 h at 20C, and then treated. For studies on bull's eye rot, apples were inoculated with *N. perennans* and *N.* sp. *nova* (10^6 conidia/ml). Before fungicide treatment, fruit were 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 a carnauba-based fruit coating (Decco 231). After treatment, fruit were stored at 20 C, 95% RH for 6 to 8 days and then evaluated for decay. Data were analyzed using analysis of variance and least significant difference mean separation procedures of SAS 9.1.

Molecular identification of species of* Neofabraea*, the causal agents of bull's eye rot. Species of this genus are highly variable in cultural morphology, conidia are not always produced, and thus, isolates are difficult to identify. We used a multiplex PCR method (Gariepy et al., Can. J. Plant Pathol. 27:118-124, 2005) where four species-specific primers are used in a single amplification reaction. Amplification products were separated in agarose gels. Reference isolates used for each species were previously obtained from R. Spotts (Oregon State University).

Baseline studies for sensitivity of* N. perennans *to difenoconazole. A total of 72 isolates of *N. perennans* were included in the evaluation. 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 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 47 isolates from 10 orchard locations in Sacramento Co. (2 to 8 isolates per location) were obtained and were subsequently tested for their sensitivity against antibiotics. For oxytetracycline and kasugamycin, none of the collected strains showed reduced sensitivity and all isolates were considered sensitive in the 2011 survey. For streptomycin, moderate resistance (LIC values 13.9 to 24.3 ppm, MIC values 20.8 to

37.6 ppm) was found at 5 locations with an incidence of between 42.8% and 75%. Thus, the occurrence of streptomycin resistance was moderate again in 2011. This is in agreement with our conclusion that in low-disease years when fewer antibiotic applications are made and selection pressure on the pathogen population is lower, wild-type sensitive isolates will gradually replace the resistant population that appears to be less fit as compared to sensitive isolates. This information is very useful for the implementation of resistance management strategies. It implies that at locations with resistance against streptomycin, the incidence can possibly be reduced if more rotational treatments are available, making this important management tool more effective again. This emphasizes the need for registration of new bactericides.

Laboratory studies on the interaction between kasugamycin and captan, mancozeb, or dodine. Our results from a microtiter plate growth assay demonstrate the in vitro inhibition of growth of *E. amylovora* by the fungicides captan, mancozeb, and dodine and support our previous findings that these compounds can reduce the incidence of fire blight in the field. As seen in Table 1, in the interaction between captan and kasugamycin and between mancozeb and kasugamycin, the inhibitory action of the compounds is additive and not synergistic. Concentrations of each of these chemicals were used that only partially inhibited growth of *E. amylovora*, so the additive effect could be more clearly seen.

***Field studies on fire blight using protective treatments during the growing season*.** In an air-blast spray field trial on cv. Granny Smith apple where 2 applications of each treatment were made before trees were inoculated with a streptomycin-sensitive isolate of *E. amylovora*, Kasumin continued to perform well (Fig. 1). The new 8L formulation resulted in numerically the lowest disease incidence among the single-active ingredient treatments. Among the 13 mixture treatments evaluated, Kasumin-Firewall, Kasumin-Manzate, and Kasumin-Rezist had the lowest incidence of disease. The two biocontrols, Actinovate (*Streptomyces lydicus)* and Blossom Protect (*Aureobasidium pullulans*), only numerically reduced the disease from that of the control. As in 2010, Captan was effective in reducing the incidence in blight. Overall, disease reduction by most treatments was only at a moderate level. This probably was due to the late timing of applications (90% and 100% bloom). A high disease incidence was present in this orchard in previous seasons and thus, with high inoculum levels, natural infections could have occurred before the first application was done.

In summary, our field trials in 2011 again indicate that kasugamycin is a highly effective treatment against fire blight of apple that can be used in resistance management programs with mixtures and rotations. No phytotoxicity was observed after two consecutive applications at 100 ppm. A California registration of kasugamycin for management of fire blight is expected for 2012. 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. Thus, 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 using molecular approaches. *Isolates highly resistant to streptomycin.* After sequencing the ribosomal protein S12 (*rps*L) gene from three highly resistant (MIC>50 ppm), two moderately resistant, and two sensitive isolates, sequences for codon 43 were AGA, AAA, and AAA, respectively. Thus, the amino acid change in the highly resistant isolates was from lysine to arginine as has been described for highly resistant isolates from other locations (Chio and Jones, 1995). Other mutations at this site previously reported (i.e., changes to asparagin or threonine) were not detected. This mechanism of resistance was described to be the primary mechanism for isolates from the western United States in the 1990s. In our surveys over the last years, however, highly resistant isolates of *E. amylovora* were only detected at one location and thus, these isolates have been replaced by moderately resistant strains that have different resistance mechanisms.

Isolates with moderate resistance to streptomycin. StrA and *strB* gene, as well as transposon Tn*5393* sequences were amplified from representative isolates with moderate resistance but not from sensitive or highly resistant isolates (Fig. 2). Amplifications for plasmid pEa8.7 that was described for a few isolates from central California in 1997 were negative. Additionally, *strA-strB* genes could not be located on plasmid pEa34 that often harbors the resistance gene in isolates from Michigan. This indicated

that a different mechanism of resistance is present in the current California pathogen population with moderate resistance. Sequence analysis of DNA regions flanking the transposon indicated that it was located on plasmid pEU30. This plasmid was first described from isolates from the western United States in 2004, but is not known to date to carry resistance genes. 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. Further characterization of the genetic base of resistance of these isolates is ongoing, but data indicate that the pathogen is highly adaptive in changing its defense strategies against streptomycin and will continue to challenge the use of this antibiotic. A diagram summarizing current knowledge on mechanisms of streptomycin resistance in *E. amylovora* is presented in Fig. 3.

Efficacy of postharvest treatments using single-fungicides and mixtures. Experimental packingline studies were conduced to evaluate single-fungicide and mixture treatments to optimize efficacy of new fungicides. Aspects evaluated included: comparison of efficacy of rates of difenoconazole used by itself or in mixtures with fludioxonil, efficacy of Alumni-Scholar mixtures to control decay caused by TBZ-resistant isolates, and efficacy of Alumni and Ph-D (an organic fungicide) against gray and blue mold decays.

Rates of difenoconazole used by itself or in mixtures with fludioxonil. In contrast to most previous studies, difenoconazole showed efficacy against gray mold in two of the three studies (Figs. 4,5). This could be because fruit were used for the tests soon after harvest. Thus, they were not senescent, and the gray mold pathogen was less aggressive in the invasion of the host tissue. Difenoconazole was consistently highly effective against blue mold, similar to Penbotec (Figs. 4-6). Rates of 360 ppm used in in-line drench applications reduced the incidence of blue mold to very low levels as compared to the untreated control, lower rates were less effective. The difenoconazole-Scholar mixtures reduced gray mold and blue mold to zero or very low levels (Figs. 4-6). Mixture rates that resulted in a high efficacy were 180 ppm for Scholar and 270 ppm for difenoconazole. These results will help in the development of a new pre-mixture by the registrant of these two fungicides (i.e., Syngenta Crop Protection) that is both highly efficacious and cost-effective.

Treatment efficacy was generally very high using these low fungicide rates even when applied 18-19 h after inoculation and incubation at 20C. This can be attributed to the use of the in-line re-circulating drench application method that we previously identified as being significantly more effective as compared to a low-volume spray application.

Efficacy of Alumni (TBZ) against gray mold and blue mold. Alumni is a new formulation of TBZ with safer inert ingredients that is replacing the Mertect formulation. When used by itself, Alumni was not effective against gray mold and blue mold caused by TBZ-resistant isolates of the respective pathogens (Figs. 4,5). In other studies not presented here, fruit were inoculated with lower conidial concentrations of *P. expansum* resistant to TBZ, and decay was significantly reduced from the control. This indicates that TBZ can still have partial efficacy when disease pressure is low. Scholar mixed with Alumni resulted in high efficacy against both green mold and blue mold, indicating no negative interaction between the two fungicides. Additionally, in 2011 as in 2010, we showed that Ph-D is effective in CDA and drench treatments against gray mold but not blue mold of apple (*data not shown*). Registration of organic Ph-D would be similar to the registration of Judge with the efficacy limited to gray mold.

Efficacy of treatments against bull's eye rot. Although Scholar has in vitro activity against *N. perennans*, the fungicide is not highly efficacious on fruit because it is a contact material and does not inhibit existing infections. In our studies, difenoconazole at rates as low as 270 ppm, mixtures of Scholar with difenoconazole, Alumni, and mixtures of Alumni with Scholar were highly effective in managing bull's eye rot that has a sporadic occurrence in California (Figs. 5,6). Penbotec at 500 ppm was similarly effective (Fig. 6) although treatment failures using this fungicide have been reported from the Pacific Northwest. It remains to be investigated if inappropriate treatment methods or resistance of the pathogen is responsible for the lack of efficacy of Penbotec treatments. The active ingredient of Penbotec is pyrimethanil and formulations of this compound are also registered (e.g., Scala) for preharvest use. Thus, multiple applications with the same active ingredient on the same fruit in preharvest and postharvest treatments is

potentially a very high-risk practice in the selection of resistant sub-populations within pathogen populations.

Summary of new postharvest fungicide treatments. In our postharvest studies we found that mixtures of Scholar with difenoconazole were highly effective in managing gray and blue mold, as well as bull's eye rot of apple. Difenoconazole is not very effective against gray mold and generally does not provide an additive effect in blue mold control when used in mixtures with Scholar as compared to using Scholar alone. Still, registration of a pre-mixture will be an important tool to decrease the risk of fungicide resistance development in populations of *Penicillium* spp. Additionally, because difenoconazole is very effective against bull's eye rot, this pre-mixture will increase the spectrum of activity for postharvest decay control. These results support our plans to support a difenoconazole registration for postharvest use on pome fruit through the IR-4 program. Because Syngenta Crop Protection is the registrant for both active ingredients, the marketing of this pre-mixture will be feasible. This is the strategy that we are developing with other crops (e.g., stone fruit – Scholar and Mentor; citrus – Graduate, Mentor, and Diploma).

Molecular identification of species of* Neofabraea*, the causal agents of bull's eye rot. Bulls eye rot can be caused by several species of *Neofabraea*. *N. perennans* and *N. alba* have been more commonly isolated than *N. malicorticis* or *N.* sp. *nova* (now classified as *Cryptosporiopsis kienholzii*). All species are highly variable in cultural morphology, conidia are not always produced, and thus isolates are difficult to identify. Using a multiplex PCR method, 72 of the 74 isolates were identified as *N. perennans*; two isolates were assigned to *C. kienholzii*. PCR products from reference and newly collected isolates after agarose gel separation are shown in Fig. 7.

Baseline studies for sensitivity of* N. perennans *to difenoconazole. The in vitro sensitivity of mycelial growth for 72 isolates of *N. perennans*, the dominant species identified in our collections, to difenoconazole was determined. The sensitivity range was 0.003 to 0.07 ppm (Fig. 8A). Sensitivity of isolates was normally distributed and skewed with most isolates sensitive between 0.009 and 0.02 ppm in a frequency histogram (Fig. 8B). Thus, difenoconazole has a high in vitro activity against this pathogen and the sensitivity range can be used in the monitoring of resistance development.

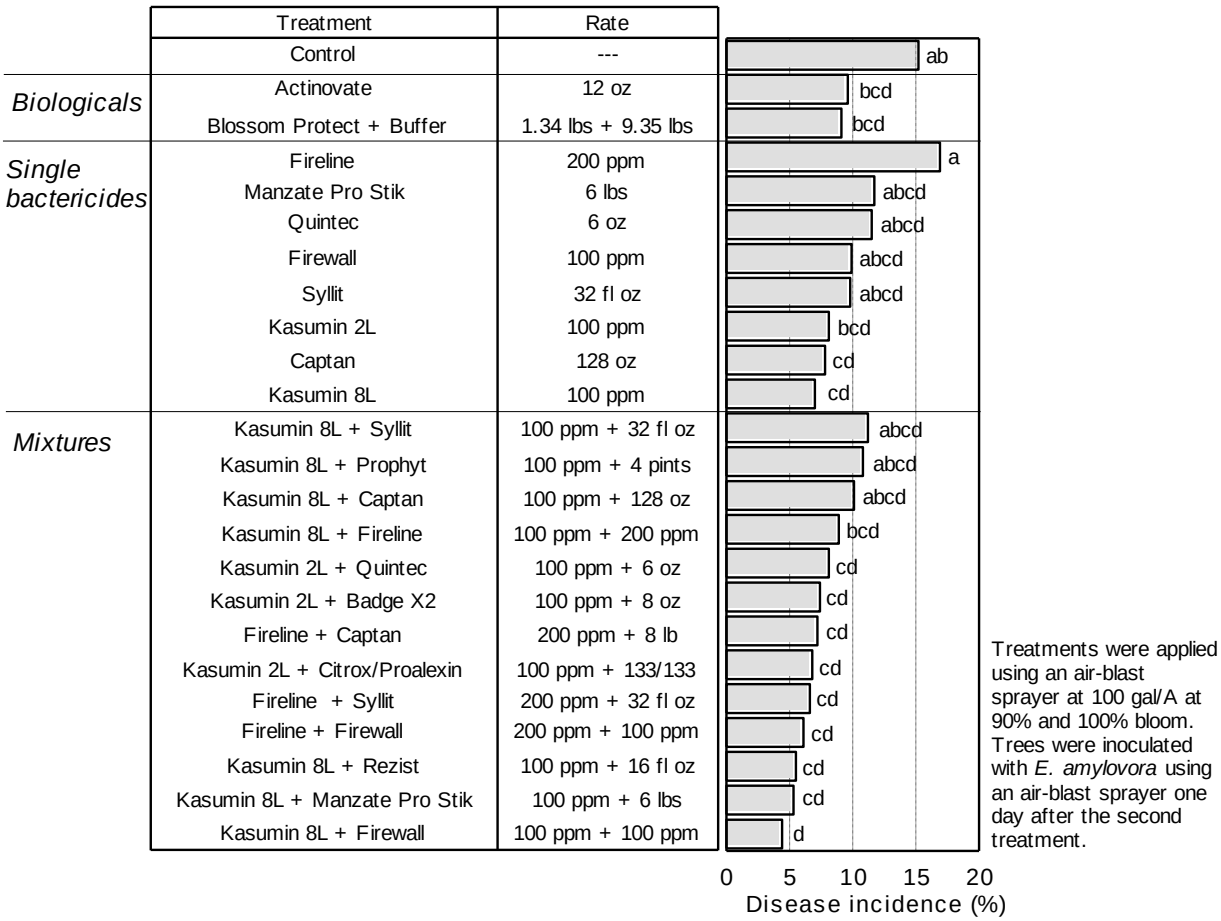
Table 1. Evaluation of the additive activity of kasugamycin and captan, mancozeb, or dodine in inhibiting growth of *Erwinia amylovora*

Treatment*	% inhibition of growth**
Kasugamycin 1 ppm	43.8
Captan 5 ppm	53.3
Kasugamycin 1 ppm + Captan 5 ppm	89.7
Mancozeb 10 ppm	37.3
Kasugamycin 1 ppm + Mancozeb 10 ppm	78.9
Dodine 0.5 ppm	99.2
Kasugamycin 1 ppm + dodine 0.5 ppm	100

*- *E. amylovora* was grown in microtiter plates in nutrient broth without or with the addition of test substances. Growth was measured by optical density readings at 600 nm.

** - % inhibition as compared to the non-amended control

Fig. 1. Efficacy of bactericides for fire blight management on Granny Smith apple in a field trial at Kearney Ag Center 2011



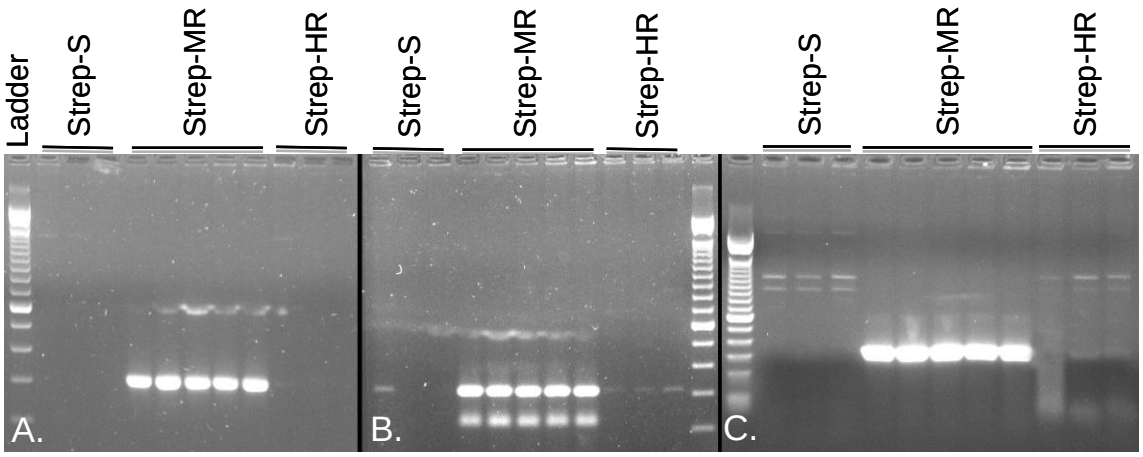


Fig. 2. PCR amplification of streptomycin resistance genes A) *StrA* and B) *StrB,* as well as C) transposon Tn*5393* in isolates of *Erwinia amylovora* sensitive (Strep-S), moderately resistant (Strep-MR), or highly resistant (Strep-HR) to streptomycin.

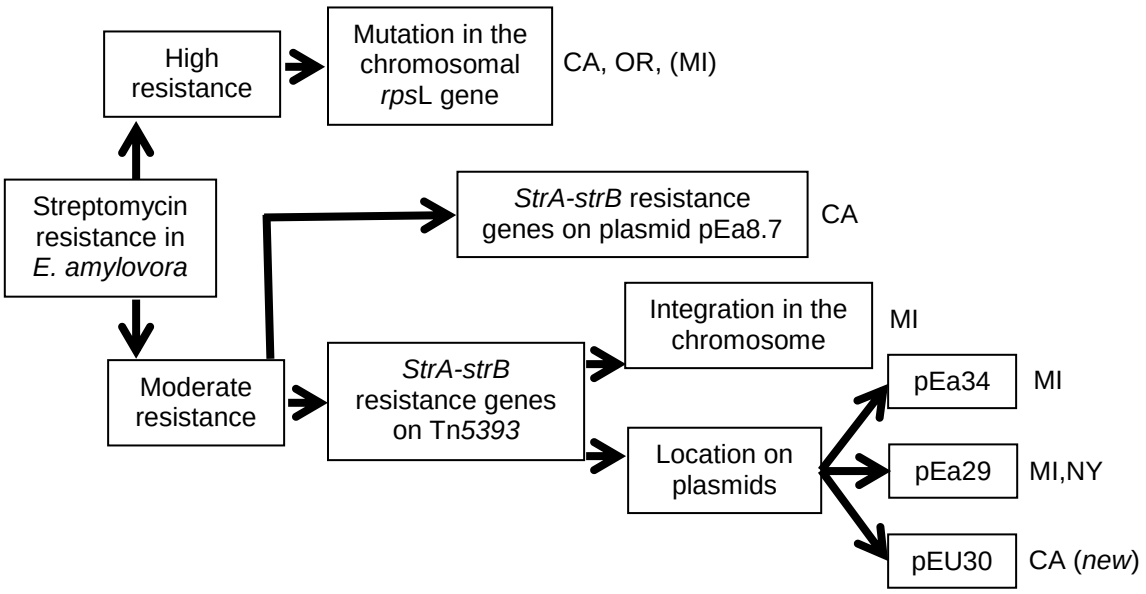
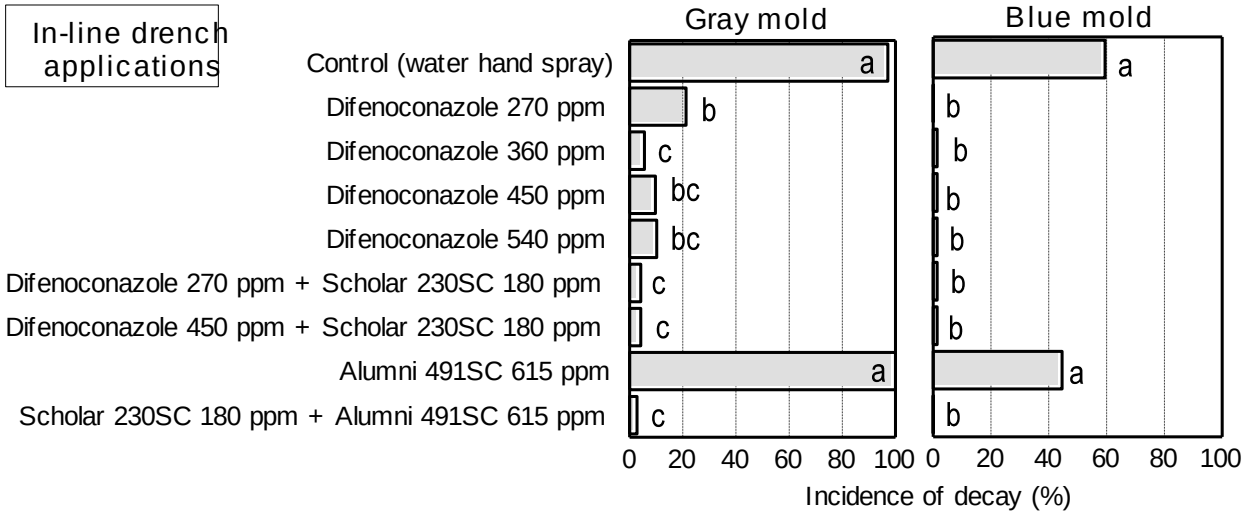


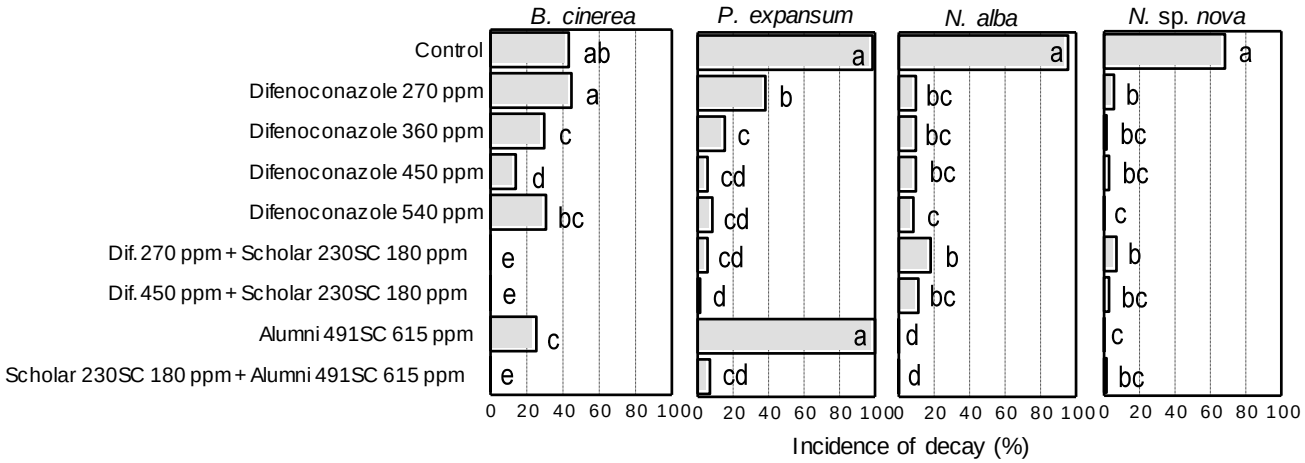
Fig. 3. Genetic mechanisms of streptomycin resistance in *Erwinia amylovora.* US States abbreviations indicate where each mechanism has been reported. Tn*5393* is a transposon.

Fig. 4. Evaluation of new postharvest fungicides for management of decay of Granny Smith apples in experimental packingline studies



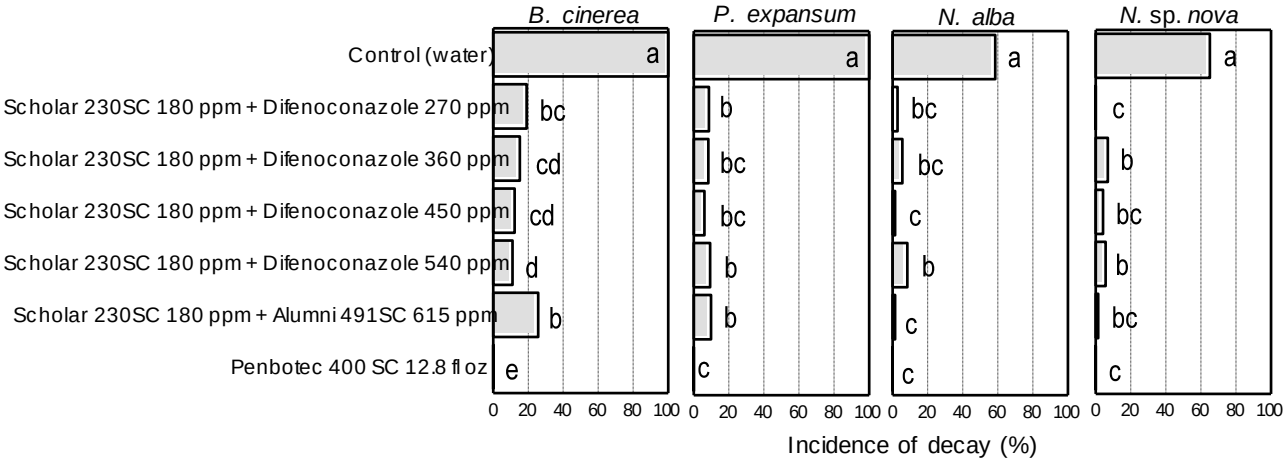
Fruit were inoculated with conidia of TBZ-resistant isolates of *Botrytis cinerea* (10^5 conidia/ml) or *Penicillium expansum* ($5x10^5$ conidia/ml), incubated for 18-19 h at 20C, and treated. In-line re-circulating drench applications were done with aqueous fungicide solutions that were followed by a CDA application with carnauba fruit coating (Decco 231). Fruit were then incubated at 20 C for 6 days. For difenoconazole, the A8574D formulation was used and for Alumni the A10466G formulation was used.

Fig. 5. Evaluation of new postharvest fungicides for management of decay of Granny Smith apples in experimental packingline studies



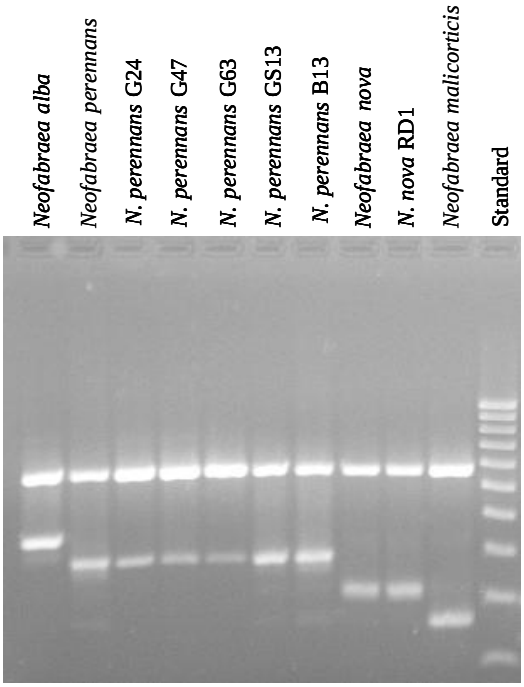
Fruit were inoculated with conidia of TBZ-resistant isolates of *Botrytis cinerea* (10^5 conidia/ml) or *Penicillium expansum* ($5x10^5$ conidia/ml), or with Neofabraea alba and N. sp. nov. (10^6 spores/ml), incubated for 18-19 h at 20C, and treated. In-line re-circulating drench applications were done with aqueous fungicide solutions that were followed by a CDA application with carnauba fruit coating (Decco 231). Fruit were then incubated at 20 C for 6 days. For difenoconazole, the A8574D formulation was used and for Alumni the A10466G formulation was used.

Fig. 6. Evaluation of new postharvest fungicides for management of decay of Granny Smith apples in experimental packingline studies



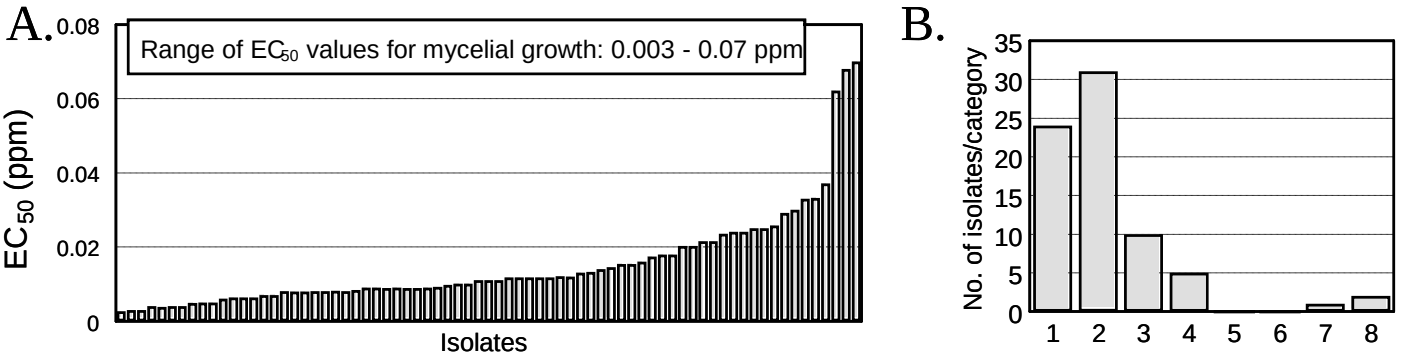
Fruit were inoculated with conidia of TBZ-resistant isolates of *Botrytis cinerea* (10^5 conidia/ml) or *Penicillium expansum* ($5x10^5$ conidia/ml), or with Neofabraea alba and N. sp. nov. (10^6 spores/ml), incubated for 18-19 h at 20C, and treated. In-line re-circulating drench applications were done with aqueous fungicide solutions that were followed by a CDA application with carnauba fruit coating (Decco 231). Fruit were then incubated at 20 C for 6 days. For difenoconazole, the A8574D formulation was used and for Alumni the A10466G formulation was used.

Fig. 7. Molecular identification of *Neofabraea* species using species-specific primers



Reference isolates are the specific taxonomic names; whereas isolates from our collections are followed by accession numbers.

Fig. 8. Baseline sensitivity to difenoconazole for 73 isolates of *Neofabraea perennans* from pome fruit



Isolates of *N. perennans* were collected from decayed fruit in packinghouses. Fungicide sensitivities were determined using the spiral gradient dilution method. For the Frequency histogram, groups are designated as follows: 1= 0-0.009, 2=>0.009-0.018, 3=>0.018-0.027, 4=>0.027-0.036, 5=>0.036-0.045, 6=>0.045-0.054, 7=>0.054-0.063, and 8=>0.063-0.072 ppm.