Annual Report - 2011

Prepared for the California Pear Board

Project Title:	Evaluation of Postharvest Treatments for Management of Gray Mold, Blue Mold, and other Decays of Stored Pears in California
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MAIN ACHIEVEMENTS IN 2011 RESEARCH

- Experimental packingline studies were conducted to determine best usage rates of a new postharvest formulation of the new DMI fungicide difenoconazole alone or in mixtures with Scholar. The efficacy of Scholar-Alumni (TBZ) mixtures was also evaluated in the management of postharvest decays of pears. This was done to ultimately provide a pre-mixture of these fungicides that is both highly efficacious and cost-effective for the manufacturer and end-user.
- Difenoconazole was not effective against gray mold at any of the rates tested, but was highly effective against blue mold of Bartlett and Bosc pear, similar to Scholar or Penbotec. Rates of 200 ppm used in in-line drench applications reduced the incidence of blue mold to very low levels as compared to the untreated control.
- Rates of fludioxonil-difenoconazole mixtures were evaluated. Blue mold and gray mold were reduced to very low levels using Scholar at 180 ppm and difenoconazole as low as 180 ppm for drench treatments. A new pre-mixture of the two fungicides will be developed by the registrant.
- The fungicides tested were similarly highly effective against blue mold when fruit were incubated between inoculation and treatment (16 to 17 h) at 13C (55F) or at 20C (68F). This indicates that there is some flexibility in the timing of postharvest fungicide treatments.
- Molecular identification of *Neofabraea perennans*, cause of bull's eye rot, was demonstrated using species-specific primers.
- 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 and TBZ are locally systemic and are highly efficacious on fruit against this decay.
- 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

Gray mold, caused by *Botrytis cinerea*, and blue mold, caused by *Penicillium expansum* and some additional less common species of *Penicillium*, are the most important storage diseases of pears in California. Other decays that may cause significant losses include Alternaria, Phomopsis, Rhizopus and Mucor rots, as well as occasionally bull's eye rot. Gray mold infections generally start at the stem end that is cut at harvest and becomes contaminated by the omnipresent spores of the pathogen. On Bartlett pears, calyx end-rot caused by *B. cinerea* is common that starts from infections during bloom. Additional entry points for all pathogens are wounds that are caused by abiotic or biotic agents before or during harvest. While some postharvest decay fungi like *Rhizopus* species are suppressed at storage temperatures of 0°C (32°F), *B. cinerea* and *P. expansum*, as well as *Mucor* and *Neofabraea* spp. will still grow, although slowly. Thus, additional chemical treatments are needed. Preharvest treatments with fungicides (e.g., Ziram, Captan, Pristine, Elevate) to manage postharvest decays have been inconsistent and generally unsatisfactory in their efficacy when fruit are sanitized and washed immediately after harvest. These treatments, however, can reduce the incidence of postharvest gray mold when field bins of fruit are not washed and placed directly into cold storage. Still, they are not as effective as when used as postharvest

treatments (i.e., Elevate vs. Judge). 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 resistance against TBZ (Mertect 340F) is widespread in populations of *B. cinerea* and *P. expansum.* These new treatments are just recently being utilized in California because many countries also had to establish maximum residue limits (MRLs) to allow marketing of fruit with our trade partners.

The risk of resistance development in the postharvest pear pathogens to fungicides is high because most registered materials have a single-site mode of action and because fruit are stored for extended periods of time. Furthermore, when fruit receive more than one postharvest treatment, than a repeated selection pressure allows the survivors to become the dominant pathogen population. Although five fungicides (Captan, TBZ, Scholar, Penbotec, Judge) are now registered for postharvest use on pears, 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. To prevent field resistance from developing in packinghouses, anti-resistance strategies that include the 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 have been working in close collaboration with the registrant of Scholar and difenoconazole, Syngenta Crop Protection, who is very supportive of these studies. One goal is to ultimately provide a pre-mixture of these fungicides that is both highly efficacious and cost-effective for the manufacturer and user and for this, we are optimizing usage rates and application methods. 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.

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 perennens* that were identified using species-specific primers.

Objectives

1) Comparative evaluation of postharvest fungicides (fludioxonil - Scholar, pyrimethanil – Penbotec, difenoconazole, as well as difenoconazole-fludioxonil and TBZ-fludioxonil pre-mixtures) for postharvest management of gray mold and blue mold. TBZ-sensitive, and -resistant isolates of the pathogens will be used in inoculations and natural incidence of decay will be evaluated.
    i. Evaluation of application technologies for postharvest fungicides (e.g., dips, drenches, and sprays in the packinghouse).
    ii. Evaluation of treatment additives such as Tween 80 when lower fungicide rates are used.
    iii. Treatment of fruit at different temperatures with fungicide solutions at selected temperatures to find out if fungicide uptake into fruit is temperature-dependent.
2) Evaluation of the fitness of naturally occurring isolates of *P. expansum* that are resistant to fludioxonil or pyrimethanil.
    i. Co-inoculation of pear fruit with pyrimethanil-sensitive and -resistant isolates and determination of the proportion of sensitive and resistant spore progeny produced on the decaying fruit.
3) Evaluation of captan, sodium hypochlorite, potassium hypochlorite, and acidified hydrogen peroxide (Perasan) as sanitizers of fungicide drench solutions, other water tank systems (e.g., float tanks), or of fruit surfaces.
    i. Experimental packingline treatments with sanitizers used alone or in mixtures with fungicides.
    ii. Evaluation of application technologies for sanitizers without using pear float (e.g., elevators in aqueous dump tanks, dry dumps on impact-absorbing foam rollers, etc.).
    iii. Evaluation of sanitizers as fruit surface disinfectants.

MATERIALS AND METHODS

Efficacy of postharvest treatments and application methods using single fungicides and mixtures. The efficacy of difenoconazole (formulation A8574D), Scholar 230SC, as well as a mixtures of these two fungicides were evaluated using different rates and were compared to treatments with Penbotec, Alumni (TBZ), or Scholar+Alumni. Bartlett or Bosc pears were wound-inoculated with TBZ-resistant isolates of *B. cinerea* or *P. expansum* (low or high inoculum concentrations - 10^5 or 10^6 conidia/ml - were used for *P. expansum*), incubated

for 16-17 h at 13C or 20C, and then treated. For studies on bull's eye rot, Granny Smith apples were used (pears were not available for this study) and were inoculated with *N. perennans* and *N.* sp. *nova* (10^6 conidia/ml), 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.

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 all species-specific primers are used in a single amplification reaction. Amplification products were then 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 the 50% inhibitory concentrations for mycelial growth were determined as described previously.

RESULTS AND DISCUSSION OF 2011 RESEARCH

Efficacy of postharvest treatments and application methods using single-fungicides, mixtures, and pre-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-TBZ mixtures to control decay caused by TBZ-resistant isolates, and the effect of temperature between inoculation and treatment on treatment efficacy.

Rates of difenoconazole used by itself or in mixtures with fludioxonil. Difenoconazole was not effective against gray mold at any of the rates tested (Fig. 1), but was highly effective against blue mold of Bartlett and Bosc pear, similar to Scholar or Penbotec. Rates of 200 ppm used in in-line drench applications reduced the incidence of blue mold to very low levels as compared to the untreated control (Figs. 1-3). Scholar-difenoconazole mixtures were evaluated. Blue mold and gray mold were reduced to very low levels using 180 ppm Scholar and 180-270 ppm difenoconazole (Figs. 1,4). 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 for the manufacturer and user.

Treatment efficacy was very high using these low fungicide rates even when treatments were applied 16-17 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.

Comparison of fruit incubation temperatures after inoculation. The fungicides tested were similarly highly effective against blue mold when fruit were incubated between inoculation and treatment (16 to 17 h) at 13C (55F) or at 20C (68F) (Figs. 2-4). In 2010, however, we noticed that low-volume spray applications were sometimes less effective when the incubation temperature was at 20C. This indicates that there is some flexibility in the timing of postharvest fungicide treatments. In locations with low temperatures during harvest and packing, gray mold and blue mold can be effectively controlled in a delayed postharvest treatment. Under California conditions where temperatures can still be high in late summer and fall, a timely application is more important, and optimum application methods should be used.

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 highly effective against gray mold sensitive to TBZ (Fig. 1), but not against blue mold resistant to TBZ (Figs. 1,2). When fruit, however, were inoculated with lower concentrations of *P. expansum* resistant to TBZ, decay was significantly reduced from the control (Fig. 2A), indicating that TBZ can still have partial efficacy when disease pressure is low.

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 (Fig. 6). In additional trials, Penbotec at 500 ppm was similarly effective 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 to the lack of efficacy of Penbotec treatments.

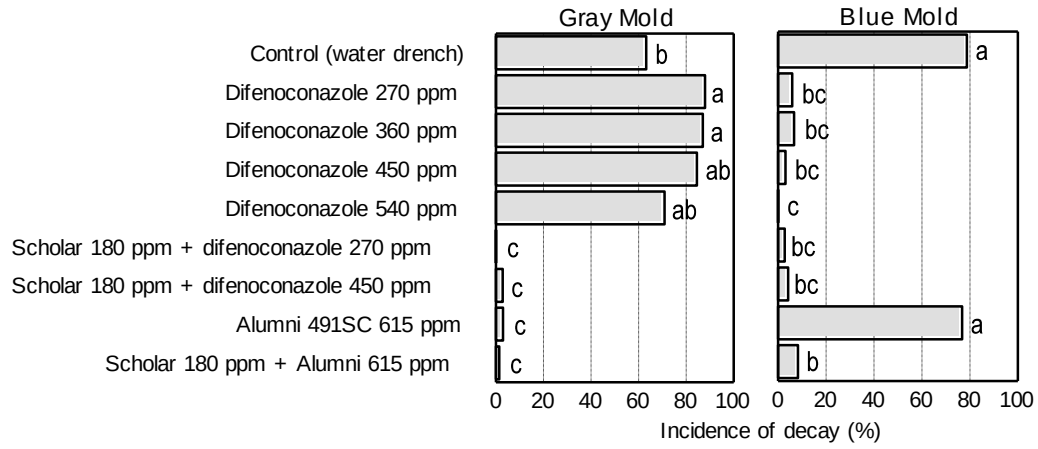
Summary of new postharvest fungicide treatments. In our postharvest studies we found that mixtures of Scholar with difenoconazole were highly effective in managing gray and blue molds of pear. Although difenoconazole is not effective against gray mold, and generally did not provide an additive effect in blue mold control when they were 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. Additionally, because difenoconazole is also 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 pears through the IR-4 program.

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 *Cryptosporiopsis kienholzii*. Banding patterns of PCR products from reference and newly collected isolates after agarose gel separation are shown in Fig. 5.

Baseline studies for sensitivity of *N. perennans* to difenoconazole. The in vitro sensitivity of mycelial growth for 74 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. 7A). Sensitivity of isolates was normally distributed and skewed with most isolates sensitive between 0.009 and 0.02 ppm in a frequency histogram (Fig. 7B). Thus, difenoconazole has a high in vitro activity against this pathogen and the sensitivity range can be used in the monitoring of resistance development.

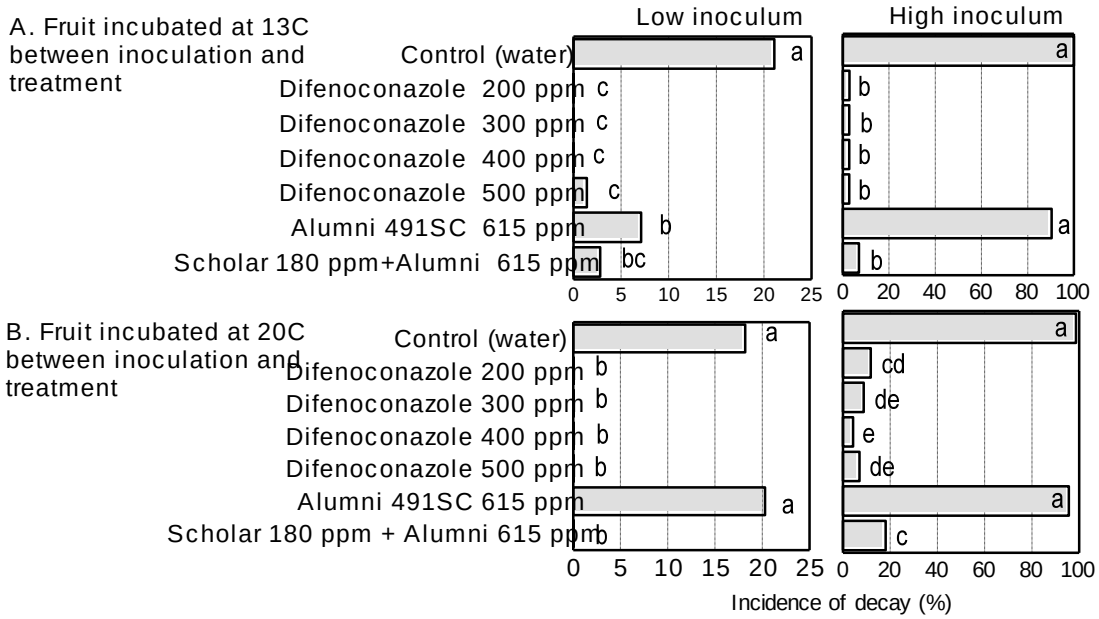
Evaluation of captan, sodium hypochlorite, potassium hypochlorite, and acidified hydrogen peroxide (Perasan) as sanitizers of fungicide drench solutions, other water tank systems (e.g., float tanks), or of fruit surfaces. Potassium hypochlorite was not supplied by the registrant due to regulatory issues and thus, was not evaluated. Acidified hydrogen peroxide (Perasan) as a sanitizer of fungicide drench or dip solutions was tested in previous studies and shown to successfully sanitize fungicide solutions while not affecting the fungicide active ingredient in solution. Sanitizers evaluated, however, were ineffective in sanitizing lignosulfate-based pear float material. Because of this, captan at 2 lb/100 gal was evaluated as an additive to lignosulfate and was shown to be effective against gray mold but not Penicillium decays. Other registered postharvest fungicides (e.g., Penbotec, Scholar) could be tested but may not be economical depending on the gallonage and tank size used. Still, captan was used because of its multiple-site mode of action. Using single-site mode of actions fungicides like Penbotec and Scholar would therefore be a high-risk for selecting populations resistant to these fungicides. Thus, the lignosulfate pear float remains one of the critical steps that results in the inoculation of fruit and increase of fruit decay in storage. The high-cost pear-float alternatives, sodium silicate and sodium carbonate, have been reported elsewhere to be compatible with chlorine sanitizers but these compounds have not been evaluated by us.

Fig. 1. Evaluation of new postharvest in-line drench applications with fungicides for management of blue and gray mold decay of Bosc pears in experimental packingline studies - Effect of difenoconazole rate, and mixtures -



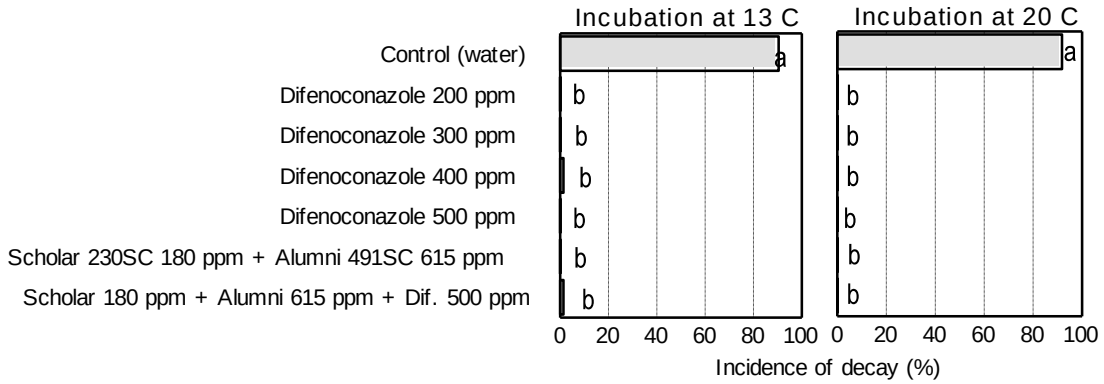
Fruit were inoculated with conidia of a TBZ-resistant isolate of *Penicillium expansum* (10^6 conidia/ml) or a TBZ-sensitive isolate of *B. cinerea* (10^5 conidia/ml) and were incubated for 16-17 h at 20C. Treatments with aqueous fungicide solutions were done by in-line re-circulating drench applications that were followed by a CDA application with carnauba fruit coating (Decco 231). Fruit were then incubated at 20 C for 6 days. Difenoconazole = A8574D, Alumni = A10466G.

Fig. 2. Evaluation of new postharvest in-line drench applications with fungicides for management of blue mold decay of Bartlett pears in experimental packingline studies - Effect of inoculum concentration, incubation temperature, and difenoconazole rate -



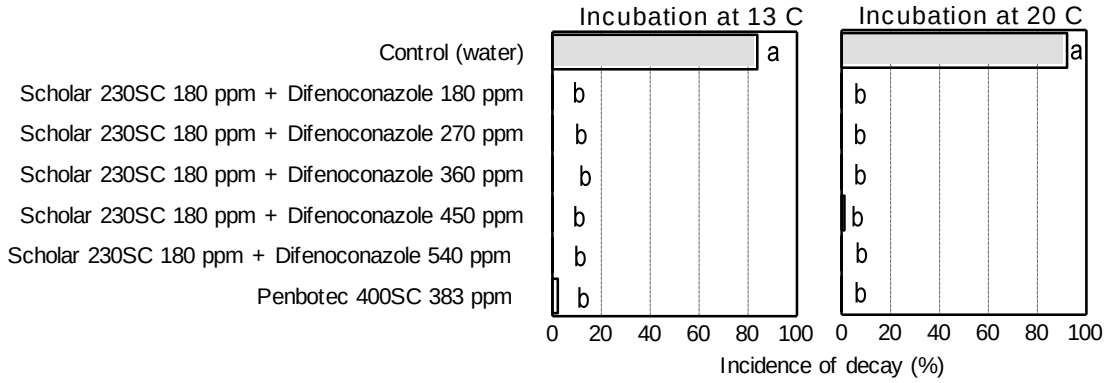
Fruit were inoculated with conidia of a TBZ-resistant isolate of *Penicillium expansum* (10^5 conidia/ml - low inoculum, 10^6 conidia/ml - high inoculum) and were incubated for 16-17 h at 13 or 20C. Treatments with aqueous fungicide solutions were done by in-line re-circulating drench applications that were followed by a CDA application with carnauba fruit coating (Decco 231). Fruit were then incubated at 20 C for 6 days. Difenoconazole = A8574D, Alumni = A10466G.

Fig. 3. Evaluation of new postharvest in-line drench applications with fungicides for management of blue mold decay of Bartlett pears in experimental packingline studies - Effect of incubation temperature, difenoconazole rate, and mixtures -



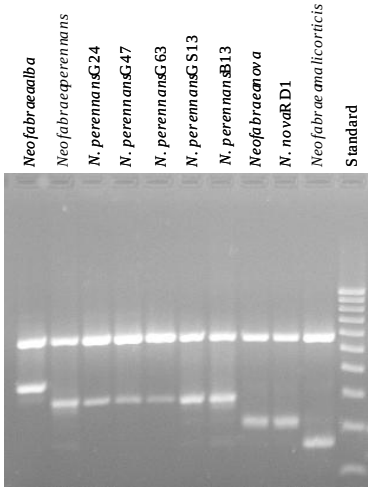
Fruit were inoculated with conidia of a TBZ-resistant isolate of *Penicillium expansum* (10^6 conidia/ml - high inoculum) and were incubated for 16-17 h at 13 or 20C. Treatments with aqueous fungicide solutions were done by in-line re-circulating drench applications that were followed by a CDA application with carnauba fruit coating (Decco 231). Fruit were then incubated at 20 C for 6 days. Difenoconazole = A8574D, Alumni = A10466G.

Fig. 4. Evaluation of new postharvest in-line drench applications with fungicides for management of gray and blue mold decay of Bartlett pears in experimental packingline studies - Effect of mixture rates -



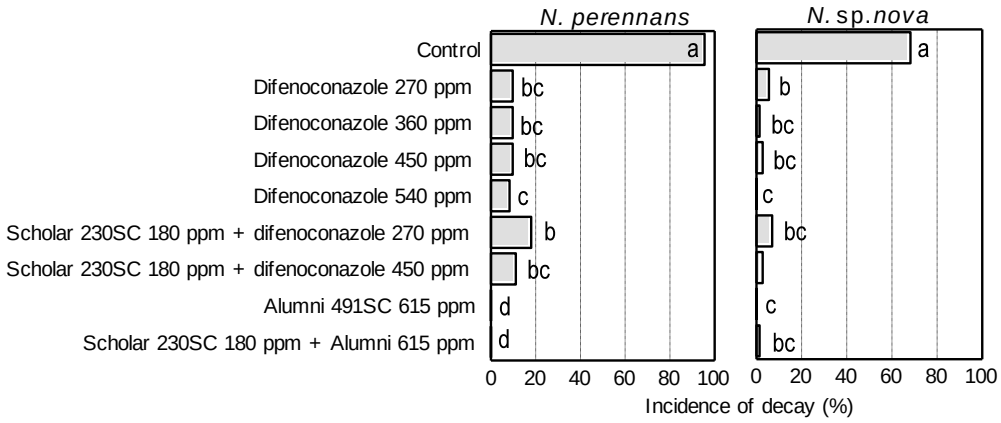
Fruit were inoculated with conidia of TBZ-resistant isolates of *Penicillium expansum* (10^6 conidia/ml - high inoculum) or *B. cinerea* (10^5 conidia/ml) and were incubated for 16-17 h at 20C. Treatments with aqueous fungicide solutions were done by in-line re-circulating drench applications that were followed by a CDA application with carnauba fruit coating (Decco 231). Fruit were then incubated at 20 C for 6 days. Difenoconazole = A8574D.

Fig. 5. Molecular identification of *Neofabraea* species using species-specific primers



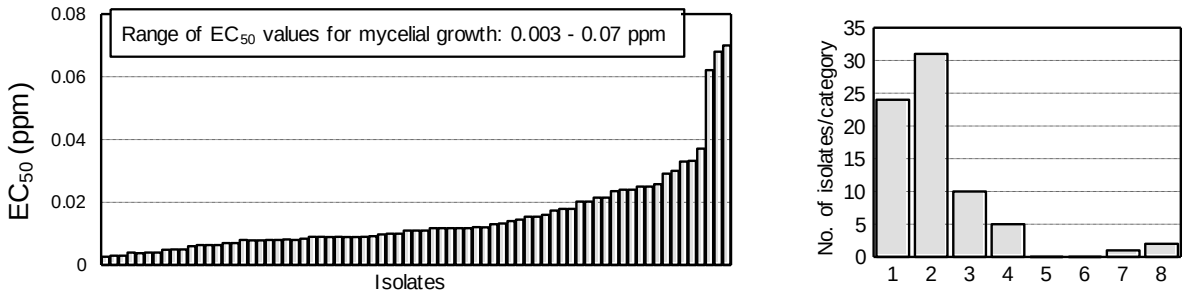
Reference isolates are the specific taxonomic names; whereas isolates from our collections are followed by accession numbers.

Fig. 6. Evaluation of new postharvest in-line drench applications with fungicides for management of bull's eye rot of Granny Smith apples in experimental packingline studies - Effect of difenoconazole rate, and mixtures -



Fruit were inoculated with conidia of *N. perennans* and *N.* sp. *nova* (10^6 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. Difenoconazole = A8574D, Alumni = A10466G.

Fig. 7. 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.