# Annual Report - 2012

*Prepared for the California Pear Board*

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| Project Title: | Evaluation of Postharvest Treatments for Management of Gray Mold, Blue Mold, and other Decays of Stored Pears in California |
| Project Leader: | Dr. James E. Adaskaveg, Department of Plant Pathology, University of California, Riverside, CA 92521 (951) 827-7577 |
| Cooperators: | Dr. H. Förster, D. Felts and D. Cary (UC Riverside) |
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## MAIN ACHIEVEMENTS IN 2012 RESEARCH

- 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. This was done to ultimately provide a pre-mixture that is both highly efficacious and cost-effective.
- Difenoconazole was not effective against gray mold, but was highly effective against blue mold of Bartlett and Bosc pear, 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.
- 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 Bosc pear, as well as two other pome fruit crops.
- 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.
- 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.
- 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

Gray mold, caused by *Botrytis cinerea*, and blue mold, caused by *Penicillium expansum* and 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 caused by *Neofabraea* spp. 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 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, P. expansum*, as well as *Mucor, Alternaria*, 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. These treatments, however, may increase the likely-hood of selecting for resistance of postharvest pathogens over-usage (number of applications per season) and incomplete coverage. Furthermore, they are not as effective as when used as postharvest treatments (i.e., Elevate vs. Judge). 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 treatments are just recently being utilized in packinghouses because many countries had to establish maximum residue limits (MRLs) that allow import of California grown fruit that were postharvest treated with these fungicides.

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, repeated selection 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 (Scholar, Penbotec) are highly effective against TBZ-resistant blue mold. Our laboratory selection studies indicated that the latter two fungicides have a similar high risk to develop resistance. For difenoconazole, the resistance potential has not been determined. 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 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*. We are also currently developing resistance potential estimates for difenconazole in *P. expansum*.

None of the currently registered postharvest treatments with high efficacy is approved for organic production. Recently, however, the bio-fungicide polyoxin-D they 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 evaluations with polyoxin-D. Because pear fruit were not available, the study was done using apples.

**Objectives**

1) Comparative evaluation of postharvest fungicides (fludioxonil - Scholar, pyrimethanil – Penbotec, difenoconazole, polyoxin-D, as well as difenoconazole-fludioxonil and TBZ-fludioxonil pre-mixtures) for postharvest management of gray mold, blue mold, Alternaria decay, and bull's eye rot. 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).
    ii. Evaluation of treatment additives such as fermentation products for decay management.
    iii. Treatment of fruit of different temperatures with fungicide solutions at selected temperatures to find out if fungicide uptake into fruit is temperature-dependent.
    iv. Evaluate treatment effects on fungicide residues on pear fruit
2) Evaluation of the resistance potential to difenoconazole in populations of *P. expansum*
    i. Exposure of large populations of conidia to a gradient of fungicide concentrations in the laboratory and potentially in the packinghouse using the SGD method.
3) Determination of baseline sensitivities of fludioxonil and difenoconazole against *Alternaria* spp. from decaying pear fruit.

## 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 mixtures and pre-mixtures (i.e., A20171A) of these two fungicides were evaluated using different rates (in mixtures rates are based on the total active ingredient or for both materials) and were compared to treatments with Penbotec, or Scholar. Bartlett or Bosc pears were wound-inoculated with TBZ-resistant isolates of *B. cinerea* ($10^5$ conidia/ml) or *P. expansum* ($10^6$ conidia/ml), incubated for 16-17 h at 20C, and then treated. For studies on bull's eye rot, Bosc pears were used and were inoculated with *N. perennans* ($10^6$ conidia/ml). For the evaluation of Ph-D, Granny Smith apples were used (pears were not available for this study) and fruit were inoculated with *B. cinerea* and *P. expansum* as indicated above. 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, 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. 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. 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 OF 2012 RESEARCH

**Evaluation of postharvest treatments using single-fungicides, mixtures, and pre-mixtures.** Experimental packingline studies were conduced to evaluate single-fungicide, mixture, and pre-mixture treatments. Scholar at 180 ppm in in-line drench applications effectively reduced blue mold and gray mold (Figs. 1,2). Although this rate of Scholar is very low, it was highly effective in these studies using recently harvested fruit. We found that with more senescent fruit that is more susceptible to pathogen infection and development, higher rates of Scholar are required to obtain adequate decay control. The high efficacy of all treatments in these studies can also 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. As previously established, difenoconazole was not effective against gray mold but highly effective against blue mold and also bull's eye rot. Scholar was previously shown to be not very efficacious against bull's eye rot because of its contact and non-systemic characteristics on fruit.

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 and on both Bartlett and Bosc pear, the pre-mixture treatments reduced the incidence of the three decays to very low levels (Figs. 1,2). 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 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. 3). 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. 4). 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 $3.6 \times 10^{-5}$ for fludioxonil and from $1.2x10^{-8}$ to $1.8 \times 10^{-6}$ 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. 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. 4A,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. 5). 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 1 shows maximum residue limits (MRLs) for several fungicides in selected countries. Scholar has received MRLs and Codex tolerances in most countries of the world and 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. 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.

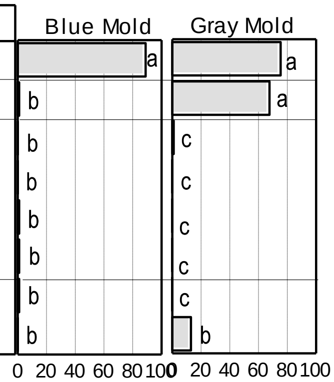
Table 1. Maximum residue limits (MRLs) for four fungicides in the United States (US), Codex (Cod), European Union (EU), Hong Kong (HG), Japan (Jpn), Korea (Kor), and Taiwan (Tai).

| Crop | Fungicide | US *1* | Cod *2* | EU *3* | HK *4* | Jpn *5* | Kor *6* | Tai *7* |
|---|---|---|---|---|---|---|---|---|
| Pear | Difenoconazole | 1 | {0.5} | {0.5} | {0.5} Cod | 1 | 1 | {0.5} |
| | Fenhexamid | 10 | --- | {0.05} | --- | --- | {1} | --- |
| | Fludioxonil | 5 | 5 | 5 | 5 Cod | 5 | {1} | 5 |
| | Pyrimethanil | 14 | {7} | {5} | {7} Cod | {1} | {3} | {2} |

*-MRL values in brackets are more restrictive than in the United States.
Note: Hong Kong deafaults to Codex.

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

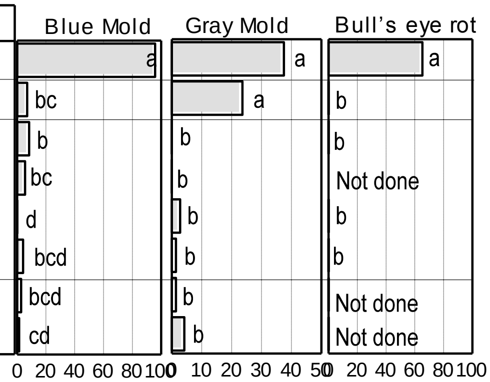
| Fungicide | Active ingredients | Rate |
|---|---|---|
| Control (water) | --- | --- |
| A8574D 360SC | difenoconazole | 300 PPM (10.7 fl oz / 100 gal/) |
| Scholar 230SC + A8574D | fludiox. + difenoc. | 180 PPM (10 fl oz / 100 gal.) + (10.7 oz / 100 gal/) |
| A20171A 384SC | fludiox. + difenoc. | 360 PPM (12 fl oz / 100 gal.) |
| A20171A 384SC | fludiox. + difenoc. | 420 PPM (14 fl oz / 100 gal.) |
| A20171A 384SC | fludiox. + difenoc. | 480 PPM (16 fl oz / 100 gal.) |
| Scholar 230SC | fludioxonil | 180 PPM (10 fl oz / 100 gal.) |
| Penbotec | pyrimethanil | 383 PPM (12.8 fl oz / 100 gal.) |




Fruit were inoculated with conidia of TBZ-resistant isolates 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.

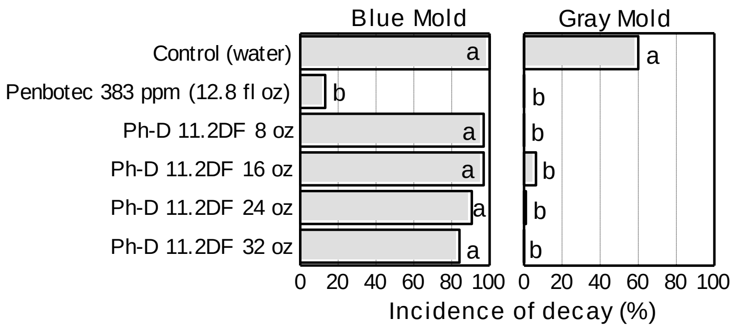
Fig. 2. Evaluation of postharvest in-line drench applications with new fungicides for management of blue mold, gray mold, and bull's eye rot decay of Bosc pears in experimental packingline studies
- Effect of difenoconazole in mixtures with fludioxonil -

| Fungicide | Active ingredients | Rate |
|---|---|---|
| Control (water) | --- | --- |
| A8574D 360SC | difenoconazole | 300 PPM (10.7 fl oz / 100 gal/) |
| Scholar 230SC + A8574D | fludiox. + difenoc. | 180 PPM (10 fl oz / 100 gal.) + (10.7 oz / 100 gal/) |
| A20171A 384SC | fludiox. + difenoc. | 360 PPM (12 fl oz / 100 gal.) |
| A20171A 384SC | fludiox. + difenoc. | 420 PPM (14 fl oz / 100 gal.) |
| A20171A 384SC | fludiox. + difenoc. | 480 PPM (16 fl oz / 100 gal.) |
| Scholar 230SC | fludioxonil | 180 PPM (10 fl oz / 100 gal.) |
| Penbotec | pyrimethanil | 383 PPM (12.8 fl oz / 100 gal.) |



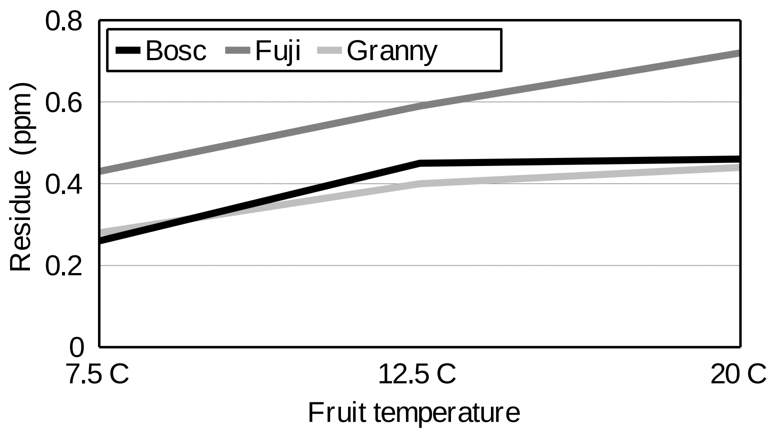

Fruit were inoculated with conidia of TBZ-resistant isolates of *Penicillium expansum* ($10^6$ conidia/ml), *B. cinerea* ($10^5$ conidia/ml), or *Neofabraea perennans* ($10^6$ conidia/ml) and were incubated for 16-17 h at 20C. Treatments with aqueous fungicide solutions were done by in-line re-circulating 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.

Fig. 3. Evaluation of postharvest in-line drench applications with Ph-D (polyoxin-D) for management of blue mold and gray mold decay of Granny Smith in experimental packingline studies



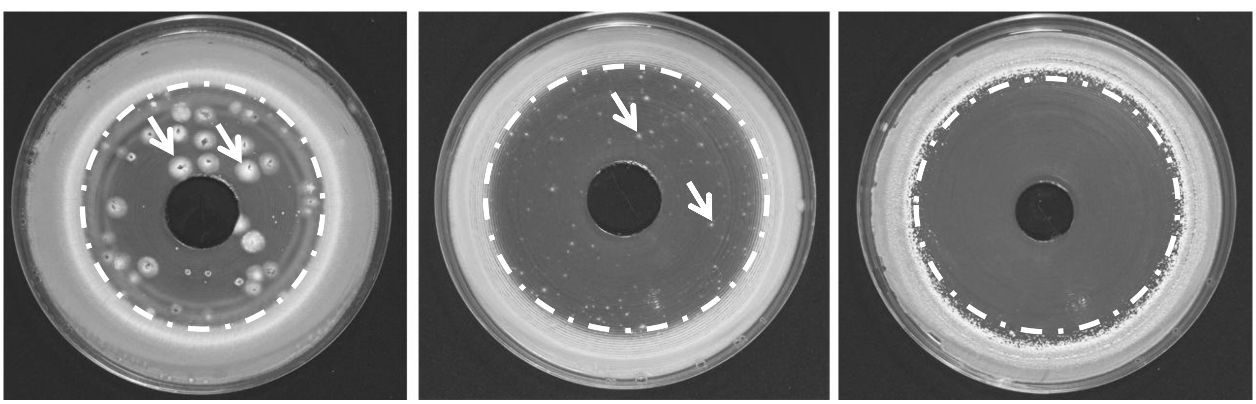

Fruit were inoculated with conidia of *Penicillium expansum* ($10^6$ conidia/ml) or *B. cinerea* ($10^5$ conidia/ml) and were incubated for 16-17 h at 20C. Treatments with aqueous fungicide solutions were done by in-line re-circulating drench applications that were followed by a CDA application with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

Fig. 4. Effect of fruit temperature on fludioxonil residue after dip treatments



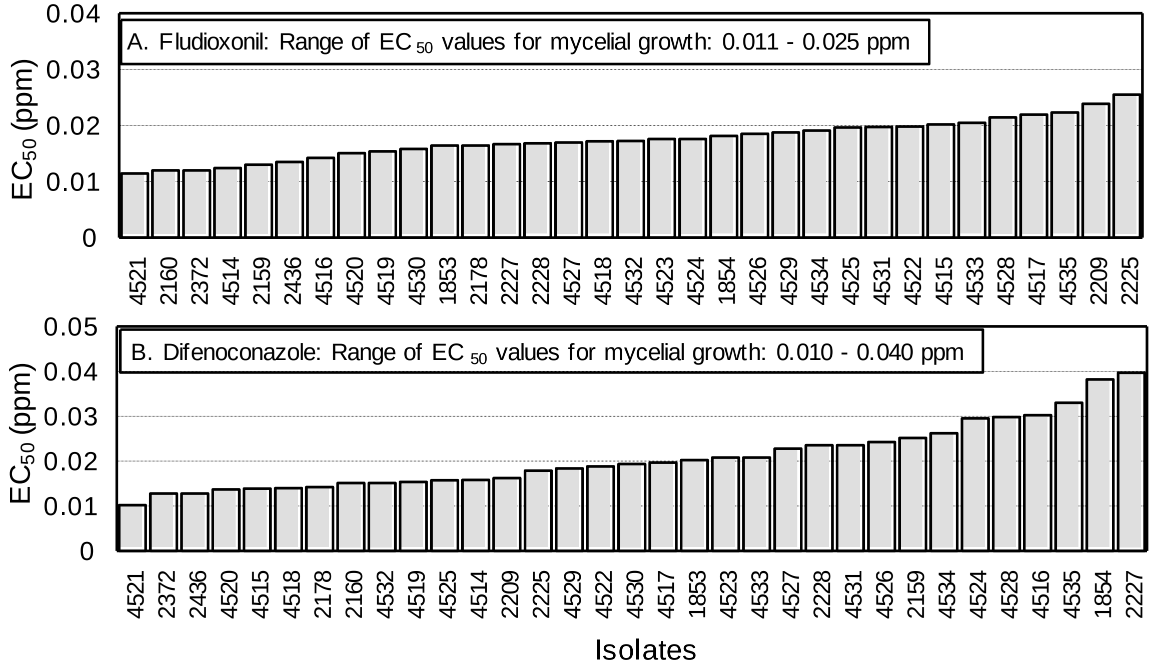

Bosc pear fruit or Fuji and Granny Smith apple fruit were equilibrated to temperatures of 1.5, 12.5, or 20 C and then dipped for 30 sec in an aqueous solution of 180 ppm fludioxonil at 10C. Fruit were then air dried and processed for residue analysis.

Fig. 5. Fungicide resistance potential assay for *Penicillium expansum* using the SGD method



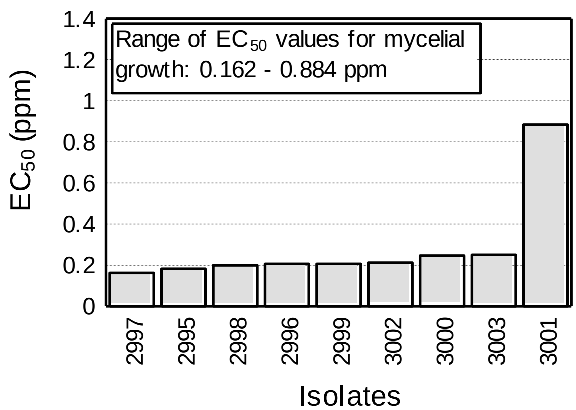
The assay was done for A. Fludioxonil on PDA; B. Pyrimethanil on AP agar; and C. Difenoconazole on PDA. High concentrations of the fungicides are near the center; whereas lower concentrations are near the perimeter of each plate. $EC_{95}$ concentrations are shown as a dotted circle. Arrows indicate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

Fig. 6. 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. 7. 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.