Paecilomyces niveus: Pathogenicity in the Orchard and Sensitivity to Three Fungicides

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Abstract

Paecilomyces rot of apples is a postharvest disease caused by the thermotolerant fungus *Paecilomyces niveus* (*Byssoschlamys nivea*). The etiology of disease and the activity of fungicides against *P. niveus* are not yet well understood. This study evaluated the ability of *P. niveus* to infect ‘Gala’ apples growing in a conventionally managed orchard. In addition, the sensitivity of *P. niveus* isolates to postharvest fungicides difenoconazole, fludioxonil, and pyrimethanil was characterized for isolates from both agricultural and non-agricultural environments. Apples were wounded and mock-inoculated or inoculated with *P. niveus* in early July. At the time of harvest, 8 weeks after wounding, the inoculated apples had significantly larger lesions than mock-inoculated apples ($P < 0.005$). The average diameter of lesions on wound-inoculated apples was $11.17 \text{ mm} \pm 6.82 \text{ SD}$, while the average diameter of mock-inoculated lesions was $3.34 \text{ mm} \pm 1.85 \text{ SD}$. Disease symptoms in the orchard were similar to postharvest symptoms of *Paecilomyces* rot. Symptoms included a brown, flattened, circular lesion with faint concentric rings.

The necrosis of mesocarp was firm and roughly U-shaped. Baseline isolates of *P. niveus*, from nonagricultural environments, were used to determine the effective fungicide dose at which growth was inhibited by 50% (EC_{50}). Furthermore, fungicide sensitivity of *P. niveus* isolates was examined using relative growth assays at the mean baseline EC_{50} values to compare baseline isolates with isolates obtained from commercial apple orchards where they were likely exposed to fungicides. Among the exposed isolates, reduced sensitivity to all fungicides was observed, but significant differences between baseline and exposed isolates were only observed with fludioxonil ($P < 0.0001$). This is the first report demonstrating that *P. niveus* can infect apples that are wound-inoculated in the orchard and that isolates from agricultural environments are less sensitive to common fungicides, especially fludioxonil. This finding may have implications for the control of this postharvest diseases. Whether natural infections of apples by *P. niveus* is initiated in the orchard or during postharvest has yet to be determined.

The United States is the world’s second-largest apple producer. This economically important crop has an estimated annual wholesale value of \$4 billion (US Apple Association 2018). Commercial apple producers face numerous fungal diseases, especially during postharvest, when apples are stored for periods exceeding 6 months (Robinson et al. 2014; Rosenberger 2014). While average annual losses of apples during postharvest storage due to factors including disease in the United States are on the order of 10 to 15%, losses are typically greater in markets in developing countries (Jurick and Cox 2016).

Paecilomyces niveus Stolk & Samson (*Byssoschlamys nivea* Westling) (Ascomycota, Eurotiomycetes, Eurotiales, Thermoascaceae) has long been recognized as an important heat-resistant mold implicated in spoilage (Houbraken and Samson 2017; Houbraken et al. 2006; Tournas 1994). Along with its relatives, *P. variotti* (*B. spectabilis*) and *P. fulvus* (*B. fulva*) (Samson et al. 2009), *P. niveus* is one of the most difficult heat-resistant molds to inactivate during thermal processes such as pasteurization (Bayne and Michener 1979; Tournas 1994). *Paecilomyces* species are the most abundant heat-resistant molds in apple cider production (Swanson et al. 1985).

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Ascospores of *P. niveus* survive temperatures of $\geq 85^{\circ}\text{C}$, enabling it to spoil processed foods that range from fruit concentrate to syrup (Brown and Smith 1957; Tournas 1994). Recently, this species has been described as a postharvest pathogen of apples, causing *Paecilomyces* rot (Biango-Daniels and Hodge 2018).

Paecilomyces rot resembles other common apple diseases, which may make it difficult to detect. External symptoms include brown circular flattened lesions, with faint concentric rings that resemble black rot or bull’s-eye rot (Biango-Daniels and Hodge 2018; Spotts 2014; Sutton 2014a). The internal necrosis caused by *Paecilomyces* rot is bruise-like. It most closely resembles bitter rot disease, but the tissue is considerably firmer (Biango-Daniels and Hodge 2018; Phillips et al. 2007; Sutton 2014b). Although little is known about the ecology of *P. niveus*, it has been isolated from both orchard and vineyard soils (Biango-Daniels and Hodge 2018; King et al. 1969).

P. niveus is of additional concern because it can produce patulin, the most hazardous mycotoxin associated with apples (Houbraken et al. 2006). Patulin, which is also produced by the postharvest apple pathogen, *Penicillium expansum*, is one of only a few internationally regulated mycotoxins (Houbraken and Samson 2006; Sanzani et al. 2012). Patulin concentrations in processed apple products must not exceed 50 ppb according to the U.S. Food and Drug Administration and Health Canada (Health Canada 2016; US FDA 2001), and 10 ppb by the EU (Puel et al. 2010). *P. niveus* differs from other patulin-producing species in its ability to produce patulin in packaged apple juice under shelf-storage conditions (Rice et al. 1977; Roland and Beuchat 1984; Sant’Ana et al. 2010).

Fungicides are one of the few tools available to prevent or mitigate these losses caused by postharvest fungal pathogens. Three common fungicides used to control postharvest diseases are difenoconazole (a.i. 20.9%), fludioxonil (a.i. 12.5%), and pyrimethanil, which represent FRAC groups 3, 9, and 12 respectively, as defined by the Fungicide Resistance Action Committee (FRAC) (FRAC 2018). Difenoconazole and fludioxonil are found in the commercial products Academy (Syngenta, Basel, Switzerland) and Scholar & Academy (Syngenta), respectively. Pyrimethanil can be found in Scala (Bayer CropScience; Monheim am Rhein, Germany) and PenBoTec

(Janssen Pharmaceutica NV). Some fungicides in these FRAC groups are also used early in the apple growing season to manage diseases like apple scab and powdery mildew, in the summer to manage diseases like sooty blotch and flyspeck, and as postharvest treatments against rot diseases (Cox 2017). While fungicides can be highly effective in controlling fungal diseases of apple, reductions in sensitivity or complete resistance has limited their overall efficacy, creating problems for management (Karaoglaniadis et al. 2011; Villani et al. 2015).

The published description of Paecilomyces rot on apples has led some to question the traditional wisdom that *P. niveus* is introduced solely through soil contamination or unsanitary conditions during food production (Brown and Smith 1957; Biango-Daniels and Hodge 2018); could infected apples also be a source of contamination? Critical research is needed to understand the etiology of Paecilomyces rot in the orchard and the activity of fungicides against *P. niveus*, related to the risk of resistance development. This study aimed to evaluate the ability of *P. niveus* to infect apples in orchards, and to characterize the sensitivity of *P. niveus* isolates to three different fungicide active ingredients, pyrimethanil, difenoconazole, and fludioxonil. Specific objectives were to: (1) determine pathogenicity of *P. niveus* in an apple orchard, (2) compare any symptoms resulting from inoculation in an orchard to symptoms of postharvest Paecilomyces rot, (3) determine the in vitro efficacy of postharvest fungicides against *P. niveus*, and (4) compare fungicide sensitivity of baseline and exposed *P. niveus* isolates.

Materials and Methods

Identification of *P. niveus* isolates. To identify all *P. niveus* isolates used in the fungicide study (Table 1), as well as the fungi reisolated from infected apples in the orchard disease trial, pure cultures were first morphologically identified using the Ascomycete key by Samson et al. (2000). To confirm morphological identities, and distinguish *P. niveus* from close relatives, the genomic DNA of each

isolate was extracted with the PowerSoil DNA Isolation Kit (MoBio) following the recommended protocol, and the β -tubulin region (*benA* gene) was subject to PCR amplification as previously described (Biango-Daniels and Hodge 2018; Visagie et al. 2014). Resulting amplicons were sequenced with the forward PCR primer, using an Applied Biosystems Automated 3730xl DNA analyzer at the Cornell University Biotechnology Resource Center, and accession numbers were deposited in NCBI (Table 1).

Morphological identities of isolates confirmed using *benA* sequences of isolates in this study were compared using phylogenetic analysis to well-studied isolates in the same genus (Samson et al. 2009). Accession numbers for reference sequences, provided in Samson et al. (2009), were used to acquire sequences for *benA* from NCBI GenBank. Next, the sequences from putative *P. niveus* isolates generated in this study were aligned to those used in Samson et al. (2009) using MUSCLE (Edgar 2004) via the EMBL-EBI online bioinformatics resource (Li et al. 2015), with poorly aligned, divergent regions trimmed from the alignment using GBlocks version 0.91b (Castresana 2000), specifying the less stringent criteria, which allows for smaller blocks, gap positions within the final blocks, and less strict flanking positions. The total number of taxa and characters in the partial β -tubulin analysis were 67 and 429, respectively.

A tree was constructed by Bayesian inference using MrBayes v3.2.6 (Ronquist et al. 2012). The partial β -tubulin analysis was conducted using a mixed model. Posterior probabilities at branch nodes were inferred using two runs of 1,000,000 generations, with 25% burn-in. Phylogenetic trees were visualized in R v3.2.3, using the Ape package (Paradis et al. 2004), in the RStudio v1.1.383 environment, rooted with *Thermoascus crustaceus* as the outgroup.

Preparation of *P. niveus* inoculum. Inoculum on colonized toothpick pieces was prepared as previously described for characterization of *P. niveus* postharvest infection and for *Penicillium* species that cause wet core rot in apples (Biango-Daniels and Hodge 2018; van der Walt et al. 2010). *P. niveus* CO7 (NRRL 66824), an isolate previously used to study postharvest Paecilomyces rot that was originally isolated from a decaying apple at Cornell Orchards, Ithaca, NY, was used to generate all inoculum (Biango-Daniels and Hodge 2018).

In short, inoculated toothpick pieces (16 mm) were first sterilized and incubated on PDA Petri dishes (100 mm), with an agar plug (5 mm) of a 1-week old *P. niveus* CO7 culture (Biango-Daniels and Hodge 2018). After 2 weeks of incubation (30°C, dark), toothpicks were completely colonized by *P. niveus*. Toothpick pieces used for the mock-inoculation were treated the same, but they were placed on PDA Petri dishes without the fungus.

Inoculation of apples in the orchard. Apples were wound-inoculated on trees in a 17-year-old orchard planting of 'Gala' trees on B.9 rootstocks at the New York State Agricultural Experiment Station in Geneva, NY. The orchards were planted as a randomized block design with four replicate blocks for each plot designated for treatment. On 13–15 July, apples were wound-inoculated with *P. niveus* ($n = 55$) or mock-inoculated with an uncolonized toothpick piece ($n = 42$). The bloom was from 27 April to 9 May and at the time of inoculation, fruit were green pre-color and 25 to 40 mm. One toothpick piece was inserted approximately halfway between the stem and calyx of immature apples. Inoculated and mock-inoculated apples were marked with different colored flags, tied near stems. At the time of inoculation, apples weighed an average of $44.7 \text{ g} \pm 11.85 \text{ SD (sd)}$ ($n = 34$) and had no visible defects or diseases.

Measurement of *P. niveus* symptoms in the orchard. After 1 month, a subsample of apples (treatment $n = 5$, control $n = 4$) were harvested from across the four replicate plots to assess the efficacy of the inoculation method and progression of infection. Symptoms (i.e., lesion diameter) were evaluated, apple weight and roundness were recorded, and fungi were isolated from several inoculated apples for identification. The remaining apples were harvested 8 weeks later, on 9 September, giving lesions time to fully develop. At harvest, the apples were fully mature for the fresh market.

Immediately following harvest, apples were refrigerated for 48 h, and subsequently evaluated for symptoms and apple development in

Table 1. Origins of exposed and baseline *P. niveus* isolates used to characterize fungicide sensitivity and NCBI accession numbers for *benA* region DNA sequence.

Group	Isolate	Origin	NCBI accession
Exposed	100-10	Orchard soil, Ithaca, NY	MG008853
Exposed	101-3	Orchard soil, Geneva, NY	MG008854
Exposed	102-1	Orchard soil, Geneva, NY	MG008855
Exposed	104-22	Orchard soil, Peru, NY	MG008856
Exposed	106-3	Orchard soil, Peru, NY	MG008857
Exposed	108-11	Orchard soil, Peru, NY	MG008859
Exposed	109-21	Orchard soil, Peru, NY	MG008860
Exposed	110-3	Orchard soil, Peru, NY	MG008861
Exposed	111-1	Orchard soil, Peru, NY	MG008862
Exposed	112-22	Orchard soil, Chazy, NY	MG008863
Exposed	125-31	Orchard soil, Sodus, NY	MG008864
Exposed	140-11	Orchard soil, Shenandoah, NY	MG008865
Exposed	141-11	Orchard soil, New Paltz, NY	MG008866
Exposed	146-311	Orchard soil, Newark, NY	MG008867
Exposed	AF01	Alfalfa field soil, Owego NY	MH046877
Exposed	CF4	Corn field soil, Berkshire, NY	MH046873
Exposed	CFO3	Corn field soil, Owego, NY	MH046874
Exposed	CO7	Cornell Orchards apple, Ithaca NY	MG008852
Exposed	KRA4	Processed food, Geneva, NY	MH046875
Exposed	STE	Decaying 'Gala' Apple, Ithaca, NY	MH046876
Baseline	CC8,	Cornell compost soil, Ithaca, NY	MH046878
	CC12		MH046882
Baseline	MC8, MC5,	Garden soil, Ithaca, NY	MH046879
	MC4		MH046880
			MH046881
Baseline	EP1, EP3	Wetland soil, Seneca Falls, NY	MH046883
			MH046884
Baseline	BY8, BY3,	Barnyard soil, Speedsville, NY	MH046885
	BY11		MH046886
			MH046887

terms of lesion diameter, apple weight, and roundness. Lesion diameter was measured at two perpendicular angles, using digital calipers (VWR Carbon Fiber Composite), and averaged. Roundness of apples was measured as the difference between the diameter of the uninoculated axis and inoculated axis (Fig. 1). To confirm that *P. niveus* was present in infected apples, fungal isolations were made from lesions on inoculated apples. The fungi isolated were grown in pure culture and identified (See section: Identification of *P. niveus* Isolates).

To test the effect of *P. niveus* inoculation on three apple properties (lesion diameter, weight, and roundness), ANOVA models were used that included fixed effects of orchard block, treatment, and their interaction. Lesion diameter data were log-transformed to meet the assumptions of normality. The models were evaluated in the R statistical software with posthoc analyses using the lsmeans package. The data were visualized using ggplot2 package.

Characterization of fungicide sensitivity in baseline isolates of *P. niveus*. The baseline sensitivity of *P. niveus* isolates to postharvest fungicides containing difenoconazole, fludioxonil, or pyrimethanil was determined for 10 *P. niveus* isolates that originated from non-agricultural environments (Table 1). Being collected from non-agricultural settings, these isolates were unlikely to have been exposed to agricultural fungicides. Difenoconazole, fludioxonil, and pyrimethanil were chosen because they represent different FRAC groups and are frequently used to manage postharvest diseases on apple.

The effective concentration needed to inhibit mycelial growth by 50% (EC_{50}) was determined for baseline *P. niveus* isolates using PDA amended with commercial formulations of difenoconazole (Inspire; Syngenta Agrochemical Company), fludioxonil (Scholar; Syngenta Agrochemical Company), and pyrimethanil (Scala; Bayer Crop Science) dissolved in distilled water. Plugs (3 mm) of *P. niveus* isolates were transferred from the edge of actively growing 3-week-old cultures (dark, $25 \pm 2^\circ\text{C}$) to PDA plates that had been amended with each fungicide at the following doses: 0 (control), 0.001, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and $10 \mu\text{g ml}^{-1}$. Plates were incubated at room temperature ($24 \pm 2^\circ\text{C}$). The mycelial growth on each fungicide-amended plate was measured after 1 week with two replicate colonies per isolate.

The percent relative growth was calculated for each fungicide concentration as previously described (Frederick et al. 2014; Villani et al. 2015). A probit analysis was performed using the Statistical Analysis System (SAS) Version 9.4 (Cary, NC) to calculate the effective

concentration needed for 50% inhibition based on the PDA control as previously described (Kreis et al. 2016).

Comparisons of fungicide sensitivity between baseline and exposed *P. niveus* isolates. Twenty *P. niveus* isolates that originated from commercial orchards where fungicides were likely used were designated as exposed isolates (Table 1); 10 others from non-agricultural sources were designated as baseline isolates. Some exposed *P. niveus* isolates originated from production soils other than apple as previously described (Biango-Daniels and Hodge 2018).

To allow for direct comparisons of fungicide sensitivity between baseline and exposed isolates, isolates were subjected to a relative growth assay using discriminatory doses based on the mean EC_{50} value for baseline isolates. Plugs (3 mm) of exposed *P. niveus* isolates were transferred from the edge of actively growing 3-week-old cultures (dark, $25 \pm 2^\circ\text{C}$) to PDA plates that had been amended with the approximate EC_{50} concentrations of each fungicide (difenoconazole $0.1 \mu\text{g ml}^{-1}$, fludioxonil $0.01 \mu\text{g ml}^{-1}$, and pyrimethanil $0.5 \mu\text{g ml}^{-1}$), as well as a control PDA plate, which had no added fungicide. Plates were incubated, diameter of growth was measured, and percent relative growth was calculated for two replicate colonies for each isolate as described above.

To visualize the relationship among exposed and baseline isolates of *P. niveus*, the percent relative growth data at the EC_{50} concentrations of fungicides were used to construct a heat map. The R-package heatmap.2 was used with the package defaults, including hierarchical clustering algorithm and Euclidean distances. A linear mixed effects model was used to compare percent relative growth of baseline and exposed *P. niveus* isolates on difenoconazole, fludioxonil, and pyrimethanil. This model included fixed effects of fungicide active ingredient and group (exposed and baseline), and a random effect of *P. niveus* isolate. The response was percent relative growth after 1 week. Posthoc analyses were done with Tukey's honest significance test to determine pairwise differences among treatment combinations. This model was evaluated in R v3.2.3 using the lme4 and lmerTest packages. The posthoc analyses were done using the lsmeans package and data were visualized using ggplot2 package.

Results

Identification of *P. niveus* isolates. *P. niveus* that were isolated from inoculated apples at the mid- and endpoint of the orchard experiment were grown in pure cultures, all of which were morphologically identified as *P. niveus*. DNA sequences of the β -tubulin (*benA*) gene were deposited in NCBI (MH04688–MH046893). The morphological identities of these isolates (isolates A–F in Supplementary Fig. S1), as well as all isolates used in the fungicide sensitivity assays (Table 1), were supported by phylogenetic analysis, in which all isolates clustered with well-studied *P. niveus* (*B. nivea*) isolates (Samson et al. 2009).

Fig. 1. A representative image of an inoculated 'Gala' apple, illustrating how roundness was measured; as the difference between the diameter of the uninoculated axis (a) and inoculated axis (b).

Fig. 2. The diameter of inoculated and mock-inoculated lesions on 'Gala' apples at harvest (8 weeks post-inoculation). In each orchard block, inoculated lesions were significantly larger than mock-inoculated lesions ($P < 0.005$).

Characteristics of symptoms caused by *P. niveus* in the orchard. After 8 weeks, the average lesion diameters of mock-inoculated ($n = 35$) and inoculated ($n = 42$) apples were $3.34 \text{ mm} \pm 1.85 \text{ SD}$, and $11.17 \text{ mm} \pm 6.82 \text{ SD}$, respectively. The inoculated apples in all four experimental blocks had lesions that were significantly larger than mock-inoculated lesions ($P < 0.005$) (Fig. 2). Lesions on wound-inoculated apples were brown and circular with faint concentric rings of alternating browns (Fig. 3A). The center of the lesion was a lighter brown, which progressively darkened to the margins, which were dark-brown and diffuse (Fig. 3A). No external sporulation was observed. The internal necrotic symptoms included a medium-brown, firm, and U-shaped rot (Fig. 3B). In the mesocarp of inoculated apples, darker tissue, which may be a callus, was observed at the interface of necrotic and healthy tissue (Fig. 4A). When the rot extended to the core, white mycelia and asci were produced in the air space around the seeds (Fig. 4B).

At harvest, there was no significant difference in weight between inoculated and mock-inoculated apples ($P = 0.229$). The average weight of mock-inoculated and inoculated apples was $134 \text{ g} \pm 55.6 \text{ SD}$ and $133.6 \text{ g} \pm 47.1 \text{ SD}$, respectively. There was a significant difference in roundness between mock-inoculated and inoculated apples ($P = 0.009$). Wound-inoculated apples were visibly flattened on the face of the apple that had been wounded, giving them an asymmetrical, lopsided shape (Fig. 1, Fig. 3).

Sensitivity of *P. niveus* isolates to fungicides. To assess current or future shifts toward insensitivity in exposed populations, baseline sensitivity of *P. niveus* was determined for difenoconazole, fludioxonil, and pyrimethanil. The EC_{50} values for baseline *P. niveus* isolates to difenoconazole, fludioxonil, and pyrimethanil were variable. They ranged from 0.0137 to $0.593 \mu\text{g/ml}^{-1}$, 0.000587 to $0.02789 \mu\text{g/ml}^{-1}$, and 0.261 to $0.868 \mu\text{g/ml}^{-1}$ for difenoconazole, fludioxonil, and pyrimethanil, respectively. Fludioxonil had the highest level of activity

Fig. 3. Representative image of symptoms of *Paecilomyces* rot on 'Gala' apples 8 weeks postinoculation. In cross section, a firm brown U-shaped necrotic area developed beneath a flattened surface where the inoculated toothpicks were inserted, leading to notable growth asymmetry (A). A brown lesion forming around the point of inoculation, with faint concentric rings and diffuse margins (B).

Fig. 4. Representative images of the mesocarp of inoculated apples with darkened tissue at the edge of the lesion, scale bar = 1 mm (A). Asci and ascospores of *Paecilomyces niveus* (*Byssoschlamys nivea*) produced inside the core of an inoculated apple, scale bar = $10 \mu\text{m}$ (B).

against baseline isolates of *P. niveus*. The mean EC₅₀ for fludioxonil was determined to be 0.015 µg/ml⁻¹. Pyrimethanil had the lowest activity against baseline isolates, with a mean EC₅₀ of 0.464 µg/ml⁻¹. Difenoconazole had intermediate activity against baseline isolates and the mean EC₅₀ was 0.194 µg/ml⁻¹.

When comparing fungicide sensitivity between baseline and exposed isolates, exposed isolates had decreased sensitivity to all three fungicides (Fig. 5). The median percent relative growth for exposed isolates were 68.2%, 106.7%, and 67.5% for difenoconazole, fludioxonil, and pyrimethanil, respectively (Fig. 5). Exposed isolates

Fig. 5. The percent relative growth of baseline and exposed *P. niveus* isolates after one week at adjusted mean EC₅₀ concentrations of fungicides. Groups with a different letter are significantly different ($P < 0.05$).

had greater percent relative growth than baseline isolates. The difference in percent relative growth between baseline and exposed isolates was statistically significant only for fludioxonil ($P < 0.0001$) (difenoconazole $P = 0.57$, pyrimethanil $P = 0.053$) (Fig. 5). For exposed isolates, the percent relative growth ranged from 58.8 to 124.4%, 41.1 to 166.7%, and 40.5 to 108.5% for difenoconazole, fludioxonil, and pyrimethanil, respectively. The heatmap visualizing comparisons among exposed and baseline *P. niveus* isolates indicated that baseline isolates grouped together and that they were more sensitive to the three fungicides as a group (Fig. 6).

Discussion

This is the first report demonstrating that *P. niveus* can infect wounded apples in the field in midsummer. Symptoms of *Paecilomyces* rot on these wound-inoculated apples included lesions and rot like those described under postharvest conditions (Biango-Daniels and Hodge 2018). This is also the first study to assess the sensitivity of isolates of *P. niveus* to three fungicides with uses in pre- and post-harvest apple production: difenoconazole, fludioxonil, and pyrimethanil. Baseline isolates were more sensitive to these fungicides than isolates from production environments where they may have been exposed to fungicides ($P < 0.0001$). Sensitivity was highest to fludioxonil, which is exclusively used postharvest in apple production.

Paecilomyces (*Byssoschlamys*) species are the most common heat-resistant molds in cider production (Swanson et al. 1985). *P. niveus* is common in orchard soils, and the only member of its genus known to be a plant pathogen (Biango-Daniels and Hodge 2018; Splittstoesser et al. 1971). Given that heat-resistant asci have been observed in infected apples, spoilage associated with this fungus could originate on infected fruit used as ingredients. Whether apples are infected post-harvest or preharvest when *P. niveus* encounters wounds requires further investigation, as both could be possible routes to infection.

Fig. 6. Heatmap of relationships among exposed and baseline *P. niveus* isolates based on percent relative growth at EC₅₀ concentrations of difenoconazole, fludioxonil, and pyrimethanil.

Future research should also investigate *P. niveus* as an opportunist wound pathogen. In this study, it was observed that the difference in lesion size between mock-inoculated and inoculated apples was most marked on trees with fire blight (data not shown). There is currently no explanation for why the apple trees used from experimental block three, which had severe fire blight symptoms, appeared to have more severe *P. niveus* infections.

The external disease symptoms on orchard apples from *P. niveus* inoculation were similar to postharvest symptoms described for this organism (Biango-Daniels and Hodge 2018). The lesions around the wounds were circular, with faint concentric rings of medium-brown color. The wounded faces of the inoculated apples were flattened, giving them a lopsided appearance when compared with mock-inoculated apples. This was not observed in postharvest infections and the malformation of apples is likely the effect of infection on growth. Internal symptoms of *P. niveus* infection were like postharvest symptoms, with the noteworthy difference that the necrosis, which was firm and medium brown in color, was a distorted U-shape due to the asymmetrical growth of inoculated apples. The necrotic mesocarp tissue did not easily separate from healthy tissue. Unlike postharvest symptoms of *Paecilomyces* rot, there was a dark layer of tissue at the interface of the healthy and diseased tissue, which was a solid medium-brown color (Biango-Daniels and Hodge 2018).

As *P. niveus* has been shown to successfully infect wound-inoculated apples in the orchard and under postharvest conditions, it is important to consider how to best manage this disease. Three different classes of fungicides, represented by difenoconazole, fludioxonil, and pyrimethanil, were found to have good efficacy against baseline populations of *P. niveus* during the mycelial growth stage, suggesting potential use in disease management. However, isolates from environments likely to have been exposed to fungicides showed reduced sensitivity to fludioxonil and pyrimethanil ($P < 0.0001$ and $P = 0.053$, respectively). The significant reduction in sensitivity to fludioxonil among exposed isolates was surprising, as this chemical has only postharvest uses and a low to medium fungicide resistance risk (FRAC 2018). Resistance to fludioxonil in *Botrytis cinerea* has been documented in berries, but is considered sporadic (Li et al. 2014). Pyrimethanil carries a medium risk of fungicide resistance and remains effective against *Venturia inaequalis* in both New York and Chile (FRAC 2018; Henríquez et al. 2011; Köller et al. 2005).

Compared with baseline isolates, exposed isolates exhibited a wider range of relative growth values in fungicide trials. What appear to be outliers may instead reflect differences in quantitative resistance among isolates resulting from different levels of selective pressures in the environments of origin. The baseline isolates group together, with lower percent relative growth than most of the exposed isolates. Among exposed *P. niveus* isolates, 108-11, 146-311, and 141-11 grouped with baseline isolates according to their growth on fungicides despite their origins from orchard soils. This may indicate that reduced sensitivity, especially among exposed isolates, is not yet widespread.

If use of fungicides becomes warranted to manage *P. niveus*, products with difenoconazole may provide better control than the other fungicides analyzed in this study. Currently, difenoconazole is registered in New York for use in apple to manage summer diseases such as flyspeck and sooty blotch (Cox 2017). Therefore, summer cover applications of difenoconazole could also serve to manage *P. niveus* along with other diseases. Careful fungicide resistance management practices should be followed if application is required to avoid any decrease in fungicide sensitivity, as difenoconazole insensitivity has been noted in other systems (Villani et al. 2015).

In summary, *P. niveus* can infect wound-inoculated developing apples in the orchard. If the population of *P. niveus* from agricultural environments has already been exposed to these chemicals and responded to the selection pressure, it may be difficult for growers to manage this pathogen with fungicides containing pyrimethanil, difenoconazole, or fludioxonil. Background levels of selected quantitative resistance in *P. niveus* may be present and mandate higher rates of fungicides for optimal control. The development of *Paecilomyces*

rot may be difficult to predict and will remain so until more research elucidates the distribution and prevalence of *P. niveus* in commercial orchards.

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