Paecilomyces Rot: A New Apple Disease

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Abstract

Paecilomyces niveus is an important food spoilage fungus that survives thermal processing in fruit products, where it produces the mycotoxin patulin. Spoilage of products has been attributed to soil contamination; however, little is known about the ecology of this organism. In this study, orchard soils and culled apple fruit were surveyed and the ability of *P. niveus* to infect apple was tested on two popular apple varieties. *P. niveus* was found in 34% of sampled orchard soils from across New York. Completing Koch's postulates, *P. niveus* was demonstrated to cause postharvest disease in Gala and Golden Delicious apple. Symptoms of this disease, named Paecilomyces rot, resemble several other apple diseases, including

black rot, bitter rot, and bull's-eye rot. External symptoms of Paecilomyces rot include brown, circular, concentrically ringed lesions, with an internal rot that is firm and cone-shaped. Both Gala and Golden Delicious apple fruit inoculated with *P. niveus* developed lesions ≥ 43 mm in size at 22 days after inoculation. There is some evidence that the size of lesions and rate of infection differ between Gala and Golden Delicious, which may indicate differing resistance to *P. niveus*. This work shows that *P. niveus* is common in New York orchard soil and can cause a novel postharvest fruit disease. Whether infected fruit can serve as an overlooked source of inoculum in heat-processed apple products requires further study.

Paecilomyces niveus Stolk & Samson (*Byssosclamyces nivea* Westling) (Ascomycota, Eurotiomycetes, Eurotiales, Thermoascaeaceae) is an important food spoilage fungus (Houbraken and Samson 2017). It causes disintegration of thermally processed foods while on the shelf, and can produce the federally regulated mycotoxin patulin (Houbraken et al. 2006; Puel et al. 2010; Sant'Ana et al. 2008). Not only does it produce conidia and chlamydospores but, being homothallic, it also readily produces abundant, durable ascospores that can survive high heat (Samson et al. 2009). Ascospores of *P. niveus* can survive temperatures of 90°C, leading to spoilage of thermally processed syrups and canned fruit (Beuchat and Toledo 1977; Tournas 1994). This hard-to-kill species and its relatives in the genus *Paecilomyces* grow in harsh conditions, including on leather, in alcohol, and under low-oxygen conditions (Beuchat and Rice 1979; Beuchat and Toledo 1977; Brown and Smith 1957; Taniwaki et al. 2009, 2010).

P. niveus can grow in apple products containing up to 40 g of added sucrose per 100 ml (40% weight by volume) and at a water activity of 0.92 (Beuchat and Toledo 1977). A water activity this low inhibits the growth of most other spoilage organisms by limiting the water availability and creating an osmotic gradient that dehydrates growing fungi (Beuchat and Toledo 1977; Grant 2004). Spoilage of thermally processed foods by *P. niveus* has been speculated to result from soil contamination (Brown and Smith 1957; King et al. 1969). The fungus has been found in orchard soils and on decaying fruit and vegetable matter but its ecology is not well understood (Beuchat and Rice 1979; Splittstoesser et al. 1971).

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One clue to the ecology of *P. niveus* may be its production of patulin, which is not only a mycotoxin but also a well-established virulence factor. It enhances the ability of the distantly related fungus *Penicillium expansum* to invade apple fruit (Sanzani et al. 2012; Snini et al. 2014), leading to Penicillium blue mold rot, an important postharvest disease of apple. Some studies have raised questions about the potential of *Paecilomyces niveus* to act as an apple pathogen, which could give it a direct route into apple-based foods (Dombrink-Kurtzman and Engberg 2006; Salomão et al. 2014; Splittstoesser et al. 1971).

This study aimed to elucidate the ecology of *P. niveus*. A selective isolation protocol was used to survey for it in orchard soils and culled apple fruit, and the ability of *P. niveus* to act as a postharvest pathogen of apple was tested.

Materials and Methods

Identification methods. Putative isolates of *P. niveus* were morphologically identified using the Ascomycete key developed by Samson et al. (2000). To distinguish *P. niveus* from closely related species, genomic DNA was extracted and the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA), or the BenA region of the β -tubulin gene was amplified and sequenced. Mycelium was harvested from *P. niveus* cultures grown on potato dextrose agar (PDA; Criterion). Genomic DNA was extracted using the PowerSoil DNA Isolation Kit (MoBio), following the recommended protocol, and stored in elution buffer at -20°C. Genomic DNA was the target for polymerase chain reaction (PCR) amplification of the ITS region of the rDNA repeat, a widely accepted barcode for fungi, using forward ITS5 (5'-GGAGTAAAAGTCGTAACAAGG-3') and reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (Schoch et al. 2012; White et al. 1990). The BenA region of the β -tubulin gene was amplified using forward Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and reverse Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') primers (Visagie et al. 2014).

Sigma-Aldrich REDTaq genomic DNA polymerase with MgCl₂ was used in a DNA Engine PTC-200 thermal cycler (MJ Research). The ITS amplification was programmed as follows: initial denaturation at 95°C for 2 min; then, 26 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min; and final extension of 72°C for 15 min. The BenA region was amplified using a program of initial denaturation at 95°C for 2 min; then, 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 3 min; and final extension of 72°C for 15 min. PCR amplicons were cleaned with the E.N.Z.A. Cycle-Pure kit (Omega Bio-tek), following the manufacturer's instructions. Amplicons were sequenced with the forward PCR primers, using an Applied Biosystems Automated 3730xl DNA analyzer, at the Cornell University Biotechnology

Resource Center. The sequences were compared with reference sequences at the National Center for Biotechnology Information (NCBI) using BLASTn (Agostino 2012; Altschul et al. 1990), and they were subject to phylogenetic analyses.

Phylogenetic analyses. To confirm morphological identification of isolates and place them phylogenetically, two phylogenetic analyses were conducted, one using the partial β -tubulin alone and the other using a concatenation of the partial β -tubulin and ITS regions for isolate CO7 (this study). The identities of isolates used in this study were inferred by comparing their phylogenetic relationship with those of Samson et al. (2009). Accession numbers provided by Houbraken et al. (2008) and Samson et al. (2009) were used to acquire sequences for the partial β -tubulin and ITS regions from NCBI GenBank (Table 1). Only isolate sequences that were used in partial β -tubulin and ITS region tree construction of Samson et al. (2009) were used in the phylogenetic analysis (Table 1), apart from *P. fulva*

(*B. fulva*) CBS 113954, for which only the partial β -tubulin sequence was included.

For the partial β -tubulin analysis, sequences from putative *P. niveus* isolates generated in this study were aligned to those used by 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. The total numbers of taxa and characters in the partial β -tubulin analysis were 47 and 437, respectively. For the concatenated partial β -tubulin and ITS region analysis, the partial β -tubulin and ITS regions of isolate CO7 (this study) were aligned to those isolates used by Samson et al. (2009), for which both gene sequences were available. Each alignment was subsequently trimmed, as previously described. The partial β -tubulin and ITS region alignments were concatenated using SequenceMatrix, v1.8 (Vaidya et al. 2011). The total numbers of taxa and characters in

Table 1. Table of isolates and accession numbers used in phylogenetic analyses^a

Species identification	Isolate	Source	ITS ^b	β -tubulin
<i>Byssoschlamys fulva</i>	CBS 113954	Samson et al. 2009	...	FJ389985
<i>B. fulva</i>	CBS 132.33	Samson et al. 2009	FJ389939	FJ389988
<i>B. fulva</i>	CBS 135.62	Samson et al. 2009	FJ389943	FJ389989
<i>B. fulva</i>	CBS 146.48	Samson et al. 2009	FJ389940	FJ389986
<i>B. fulva</i>	CBS 604.71	Samson et al. 2009	FJ389941	FJ389997
<i>B. lagunculariae</i>	CBS 110378	Samson et al. 2009	FJ389946	FJ390006
<i>B. lagunculariae</i>	CBS 373.70	Samson et al. 2009	FJ389944	FJ389995
<i>B. lagunculariae</i>	CBS 696.95	Samson et al. 2009	FJ389945	FJ389996
<i>B. nivea</i>	CBS 100.11	Samson et al. 2009	FJ389934	FJ389999
<i>B. nivea</i>	CBS 113245	Samson et al. 2009	FJ389936	FJ389998
<i>B. nivea</i>	CBS 133.37	Samson et al. 2009	FJ389935	FJ390000
<i>Paecilomyces niveus</i>	CO7	This study	MG008851	MG008852
<i>P. niveus</i>	100-10	This study	...	MG008853
<i>P. niveus</i>	101-3	This study	...	MG008854
<i>P. niveus</i>	102-1	This study	...	MG008855
<i>P. niveus</i>	104-22	This study	...	MG008856
<i>P. niveus</i>	106-3	This study	...	MG008857
<i>P. niveus</i>	107-1	This study	...	MG008858
<i>P. niveus</i>	108-11	This study	...	MG008859
<i>P. niveus</i>	109-21	This study	...	MG008860
<i>P. niveus</i>	110-3	This study	...	MG008861
<i>P. niveus</i>	111-1	This study	...	MG008862
<i>P. niveus</i>	112-22	This study	...	MG008863
<i>P. niveus</i>	125-31	This study	...	MG008864
<i>P. niveus</i>	140-11	This study	...	MG008865
<i>P. niveus</i>	141-11	This study	...	MG008866
<i>P. niveus</i>	146-311	This study	...	MG008867
<i>B. spectabilis</i>	CBS 101075	Houbraken et al. 2008	EU037051	EU037069
<i>B. spectabilis</i>	CBS 102.74	Houbraken et al. 2008	EU037055	EU037073
<i>B. spectabilis</i>	CBS 121581	Houbraken et al. 2008	EU037062	EU037080
<i>B. spectabilis</i>	CBS 338.51	Samson et al. 2009	FJ389930	FJ390007
<i>B. zollerniae</i>	CBS 374.70	Samson et al. 2009	FJ389933	FJ390008
<i>P. brunneolus</i>	CBS 370.70	Houbraken et al. 2008	EU037050	EU037068
<i>P. divaricatus</i>	CBS 110429	Samson et al. 2009	FJ389932	FJ389991
<i>P. divaricatus</i>	CBS 284.48	Samson et al. 2009	FJ389931	FJ389992
<i>P. formosus</i>	CBS 113247	Samson et al. 2009	FJ389921	FJ390009
<i>P. formosus</i>	CBS 296.93	Samson et al. 2009	FJ389928	FJ389994
<i>P. formosus</i>	CBS 371.70	Samson et al. 2009	FJ389920	FJ389982
<i>P. formosus</i>	CBS 372.70	Samson et al. 2009	FJ389926	FJ389990
<i>P. formosus</i>	CBS 628.66	Samson et al. 2009	FJ389927	FJ389983
<i>P. formosus</i>	CBS 990.73B	Samson et al. 2009	FJ389929	FJ389993
<i>P. saturatus</i>	CBS 251.55	Samson et al. 2009	FJ389951	FJ390002
<i>P. saturatus</i>	CBS 323.34	Samson et al. 2009	FJ389947	FJ390005
<i>P. saturatus</i>	CBS 368.70	Samson et al. 2009	FJ389948	FJ390001
<i>P. saturatus</i>	CBS 492.84	Samson et al. 2009	FJ389949	FJ390003
<i>P. saturatus</i>	CBS 990.73A	Samson et al. 2009	FJ389950	FJ390004
<i>Thermoascus crustaceus</i>	CBS 181.67	Samson et al. 2009	FJ389925	FJ389981

^a All CBS isolate identifications are per table 1 in Samson et al. (2009). Species identifications for isolates derived in the present study are based on phylogenetic affiliation with those of *P. niveus* (*B. nivea*) sensu Samson et al. (2009).

^b Internal transcribed spacer.

the concatenated partial β -tubulin and ITS region analysis were 31 and 984, respectively.

For both the partial β -tubulin and the concatenated partial β -tubulin and ITS region analyses, trees were constructed by Bayesian inference using MrBayes, v3.2.6 (Ronquist et al. 2012). The partial β -tubulin analysis was conducted using a K80+G model, selected by jModelTest 2.1 (Darriba et al. 2012) with the Bayesian information criterion. The Bayesian model averaging approach of rjMCMC was applied to the concatenated partial β -tubulin and ITS region analyses (Huelsenbeck et al. 2004). In the concatenated analysis, each gene was considered a separate partition, allowing for independent estimation of substitution rates, character state frequencies, gamma shape distributions, and proportions of invariable sites per partition. 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, v3.4.2, using the Ape package (Paradis et al. 2004) in the RStudio v1.1.383 environment, rooted with *Thermoplasma crustaceus* as the outgroup.

Detection of *P. niveus* in New York orchard soil. To determine whether *P. niveus* was present in New York state orchard soils, in summer 2016, soil samples were taken from the top 6 in. (15.24 cm) of soil in orchards across the state by Cornell Cooperative Extension agents and independent consultants. This depth was chosen because it represents the soil that may contact apple fruit during harvest or if they are on the ground. Soils were sampled and mailed to Cornell University, refrigerated (4°C) upon receipt, and processed within 2 weeks. In total, 32 different orchards were sampled across New York state. Due to miscommunication, four of the orchards were sampled in multiple places, which led to 48 soil samples being received by the Hodge Lab.

Soils were thermally processed by mixing them with sterile deionized (DI) water (1:2, wt/vol). The resulting dilute soil samples were placed in 2-ml microcentrifuge tubes and heated in a water bath at 70°C for 30 min. This process reduces the number of microorganisms present and has been shown to increase germination of *P. niveus* ascospores (Splittstoesser et al. 1970). For each cooled sample, 200 μ l was spread with a sterile L-shaped rod on five PDA Petri plates (90 mm) supplemented with streptomycin sulfate at 30 μ g ml⁻¹. After a 3-week incubation at 25°C, all emerging colonies with a morphology consistent with *P. niveus* were subcultured on PDA at 25°C (Beuchat and Pitt 2001). As described above, isolates were morphologically identified and the identity was confirmed by phylogenetic analysis of sequences of the BenA region of the partial β -tubulin gene, a recognized fungal barcode that differentiates closely related species of *Paeclomyces* (Schoch et al. 2012).

Isolation of *P. niveus* from culled apple fruit. In addition to surveying for *P. niveus* in orchard soils, stored apple fruit with signs of postharvest diseases were sampled to detect any *P. niveus* present. In spring 2017, Cornell Orchards staff selected apple fruit displaying symptoms of postharvest diseases as bins were pulled from cold storage. Cornell Orchards, located in Ithaca, NY, is a teaching and retail facility that grows apple for fresh-market and cider production. The apple fruit, which were cultivars Mutsu, Goldrush, Empire, and Shizuka, had been stored at 1°C since harvest the previous autumn.

Each week for 5 weeks, a 5-gal. (18.9 liter) bucket of culled apple fruit was received and apple lesions were dissected to harvest infected tissue. To reduce the possibility of surface soil contaminants, apple fruit were first surface sanitized with 70% ethanol. The pooled weekly sample, weighing between 40 and 51 g, was frozen and stored at -20°C for 2 to 3 months. Additionally, a separate sample from an apple, inoculated with *P. niveus* in the lab as described below, was aseptically dissected and its infected tissue was included as a positive control. Its tissue was subjected to approximately the same freezing time.

To isolate any *P. niveus* present, tissue samples taken from culled apple fruit were combined with sterile DI H₂O (1:1, wt/vol). The suspensions were homogenized in sterile stomacher bags (Seward Stomacher 400 circulator) for 2 min at 230 rpm (Beuchat and Pitt 2001). Next, samples were divided among five 10-ml glass test tubes; four were incubated at 75°C for 30 min in a closed water bath, while the fifth was not heated. The unheated sample was used to estimate the background number of nonheat-resistant fungi present. Duplicate

aliquots (100 μ l) of apple homogenate from each of the four heated test tubes per sample were spread on Petri dishes (90 mm) of Rose Bengal agar (HIMEDIA). Dilutions from 10⁻¹ to 10⁻⁴ were made of the unheated samples, and aliquots (100 μ l) of each dilution were spread on Rose Bengal agar. All Petri plates were incubated at 30°C for 1 month (in the dark), and emerging colonies were counted.

Koch's postulates. To test whether *P. niveus* can cause disease in apple, the four steps of Koch's postulates were completed: (i) *P. niveus* isolate CO7 was isolated from a rotting apple discovered at Cornell Orchards in fall 2014, (ii) *P. niveus* was grown in pure culture and morphologically and genetically identified (Samson et al. 2000), (iii) the culture of CO7 was used to inoculate healthy apple fruit through a wound and disease symptoms were observed on the inoculated apple fruit using a dissecting microscope (Olympus SZX12), and (iv) the fungus was reisolated from within the lesions of infected apple fruit and confirmed to be *P. niveus* using morphological and genetic approaches.

Inoculation method. In all, 15 Gala and 15 Golden Delicious apple fruit were sanitized (30 s in 70% ethanol, 2 min in 1% sodium hypochlorite, and 15 s in 70% ethanol) and dried in a biological safety cabinet (Geisler 2013; van der Walt et al. 2010). Following sanitization, apple fruit were numbered and inoculated with *P. niveus* CO7, which was identified morphologically (Samson et al. 2000), and confirmed through phylogenetic analysis of sequences of the ITS and BenA regions (MG008851 and MG008852).

Colonized toothpicks were used to wound and inoculate mature Gala and Golden Delicious apple fruit. These apple varieties were selected because they are among the top 10 commercial cultivars (US Apple Association 2018). Toothpicks were inoculated with *P. niveus* CO7 as described for *Penicillium* spp. on apple (van der Walt et al. 2010). Briefly, toothpick pieces (16 mm) were sterilized by autoclaving them five times in DI water, which was replaced between cycles. They were next transferred to potato dextrose broth (Teknova) and autoclaved a final time. Toothpick pieces were aseptically transferred to PDA Petri dishes (90 mm) containing agar plugs (5 mm) of a 1-week-old *P. niveus* CO7 culture. After 2 weeks of incubation (30°C, in darkness), toothpick pieces were well colonized by *P. niveus*. Control toothpicks were similarly treated, except they were placed on uncolonized PDA Petri dishes (90 mm). Toothpick pieces were inserted at four points, each halfway between the stem and calyx, to wound the sanitized apple fruit. Two treatments were applied to each apple: (i) two opposite wounds inoculated with *P. niveus* CO7 and (ii) two opposite wounds that received control toothpicks. Inoculated Gala and Golden Delicious apple fruit were incubated in separate dark, moist chambers (25°C, $\geq$ 95% humidity) for 3 weeks.

Disease characterization. To describe the disease symptoms that developed following inoculation with *P. niveus*, Gala and Golden Delicious apple fruit in moist chambers were monitored. Lesions were measured three times a week during the 3-week incubation period. Using digital calipers (VWR Carbon Fiber Composite), lesion diameters were measured at two perpendicular angles and averaged. To allow for comparisons of each wound over time, individual wounds and apple fruit were tracked with an identification number. To eliminate the possibility of lesion size being influenced by contaminating fungi, any apple fruit that showed signs of contamination during incubation, specifically by *Penicillium* spp., were removed from the experiment. Several apple fruit of each variety were maintained in moist chambers for an extended period to observe disease symptoms at an advanced stage.

Statistical analysis. Two separate linear mixed-effects models, one at day 7 and one at day 22, were used to test for significant differences between lesion diameters for both treatments (control or inoculated) on both varieties of apple fruit. Fixed effects of apple variety (which was confounded with chamber), treatment, and their interaction were included and a random effect of apple identification number was included, which accounted for the multiple wounds on each apple. These models were executed using the lme4 and lmerTest packages in R statistical programming software. Posthoc comparisons were carried out using the lsmeans package. The data were visualized using the ggplot2 package.

Identification and phylogeny. BLASTing (Blastn) of the sequenced regions of CO7 (MG008851 and MG008852) showed that CO7 closely matched *P. niveus* isolates in the NCBI GenBank. The ITS and BenA regions of CO7 were concatenated and the placement of the isolate within the *P. niveus* clade further confirmed its identity (Supplementary Fig. S1). The *P. niveus* isolates from New York state orchard soils were morphologically identified, and their identities were supported by phylogenetic analysis (Fig. 1). All isolates from soil clustered with *P. niveus* (*B. nivea*) that were predominately isolated from spoiled foods (Samson et al. 2009) (Fig. 1).

After 1 month of incubation at 30°C, three samples from the not-heat-treated culled apple fruit had fungal colonies that were counted. Fungal counts for these samples ranged from 1,000 to 22,000 CFU ml⁻¹. *Penicillium* spp. were most common on these plates. The other two not-heat-treated culled apple samples showed no fungal growth. Among the five heat-treated samples, only one isolate of a heat-resistant mold was found. It was determined not to be a *Paecilomyces*

***P. niveus* causes disease in apple.** Isolate CO7, taken from rotting apple fruit at Cornell Orchards in 2014, was morphologically identified as *P. niveus* and its identity was supported by phylogenetic analysis. It was grown in pure culture and then used to inoculate healthy apple fruit. After the infected apple fruit began to show symptoms of disease, a fungus was reisolated, grown in pure culture, and identified as *P. niveus*. Koch's postulates were completed by using the fungus to infect healthy apple fruit.

After an extended incubation period (44 days), advanced disease symptoms included the complete discoloration of the apple epidermis to light tan-brown (Fig. 2E and F). The surface of these apple fruit was observed to be sparsely covered by branching white hyphae, concentrated around lenticels in floccose tufts (Fig. 2F). After 44 days, the internal necrotic tissue extended to the cores of both apple varieties (Fig. 2D), which were filled with white mycelium and asci containing ascospores (Fig. 3). At this late stage of necrosis, tissue was marbled in shades of tan and dark brown (Fig. 2D).

Size of *P. niveus* lesions. Wounded apple fruit that were inoculated had lesions that grew rapidly. The average diameters of treatment lesions ($\pm$ standard error), measured 7 days after inoculation were 13.3 ± 0.4 mm ($n = 30$) and 17.1 ± 0.3 mm ($n = 30$) on Gala and Golden Delicious, respectively (Fig. 4). The average diameters of treatment lesions ($\pm$ standard error), measured 22 days after inoculation, were 47.6 ± 0.7 mm ($n = 24$) and 43.2 ± 0.9 mm ($n = 26$) on Gala and Golden Delicious, respectively (Fig. 4). The diameter of control lesions remained near the size of the original inoculation wound (1.8 mm) throughout the study. The average diameters of control lesions ($\pm$ standard error), measured 7 days after inoculated, were 2.0 ± 0.3 mm ($n = 30$) and 2.4 ± 0.4 mm ($n = 30$) on Gala and Golden Delicious, respectively. After the 22-day incubation, control lesions ($\pm$ standard error) increased from the initial 1.8-mm diameter of the toothpick to an average of 3.4 ± 0.9 mm ($n = 24$) and 3.2 ± 0.9 mm ($n = 26$) on Gala and Golden Delicious, respectively (Fig. 4).

Isolate	Orchard	Location	
		County	Town
100-10	1	Tompkins	Ithaca
101-3	2	Clinton	Geneva
102-1	3	Clinton	Geneva
104-22	4	Clinton	Peru
106-3	5	Clinton	Peru
107-1	5	Clinton	Peru
108-11	6	Clinton	Peru
109-21	6	Clinton	Peru
110-3	6	Clinton	Peru
111-1	6	Clinton	Peru
112-22	7	Clinton	Chazy
125-31	8	Wayne	Sodus
140-11	9	Dutchess	Shenandoah
141-11	10	Ulster	New Paltz
146-311	11	Wayne	Newark

The diameters of *Paecilomyces* rot treatment lesions on both Gala and Golden Delicious were significantly larger than control lesions on days 7 and 22 ($P < 0.0001$). Although confounded by chamber, treatment lesions on the Gala were smaller than those on Golden Delicious early in the disease ($P < 0.0001$, day 7), however, this trend reversed by day 22, when Gala treatment lesions were larger than those on the Golden Delicious ($P = 0.005$) (Fig. 4). There were no significant differences in control lesion diameters on days 7 or 22 on Gala or Golden Delicious. Over the course of the experiment, six Gala and four Golden Delicious apple fruit were removed from their respective incubation chambers because they showed signs of being colonized by other fungi. Once removed, these apple fruit were excluded from further measurements and statistical analysis.

Discussion

This is the first report that *P. niveus* can infect apple fruit, which are affected by many other postharvest fungal diseases (Houbraken et al. 2006; Jurick and Cox 2016; Sutton et al. 2014). At least 10 to 15% of apple fruit are lost during storage because of disease (more in developing markets) (Jurick and Cox 2016). In the United States, postharvest losses cost an estimated \$6.5 million annually (Jurick and Cox 2016). This work demonstrates that *P. niveus* is commonly found in orchard soils in New York, and can cause a novel postharvest

fruit disease, *Paecilomyces* rot, on two popular varieties of apple (Piecková et al. 1994; Splittstoesser et al. 1971).

Prior research suggested that soil was the source of food contamination and, indeed, *P. niveus* was present in many New York orchard soils (Table 2). Not only was *P. niveus* in more than a third of the orchards sampled but also isolates were found at multiple points in two of those orchards (Table 2). This finding demonstrates that *P. niveus* is widespread across New York and that it may be common throughout orchards. All *P. niveus* isolates that were found in orchard soils shared a clade with other isolates of this species in the phylogenetic analysis (Fig. 1). Very little is known about the ecology of *P. niveus* and its relatives; however, none of these food spoilage species have been previously described as plant pathogens. *Paecilomyces* spp. are known for the food spoilage problems they cause in a variety of thermally processed foods but their biology and niches in nature are virtually unstudied. *P. niveus* and some *P. saturatus* are the only species in the genus known to produce patulin (Samson et al. 2009). Perhaps this toxin is responsible for the ability of *P. niveus* to infect apple, because patulin is a virulence factor in apple in the distantly related blue rot pathogen, *Penicillium expansum* (Ascomycota, Eurotiomycetes, Eurotiales,

Fig. 2. Disease symptoms caused by *Paecilomyces niveus* (*Byssoschlamys nivea*) after incubation in dark, moist chambers (25°C, ≥95% humidity). External symptoms 12 days postinoculation (dpi) on **A**, Golden Delicious and **B**, Gala fruit. Insets show a control apple of each variety. Scale bars are 1.8 mm. **C**, Cross-section of Gala apple at 12 dpi, showing representative cone-shaped internal rot. **D**, Longitudinal section of Gala apple, showing internal marbling at 44 dpi. **E**, Gala apple with advanced *Paecilomyces* rot at 44 dpi. **F**, Close-up view of tufts of white mycelia produced on the surface of an infected Gala apple.

Fig. 3. Representative image of *Paecilomyces niveus* (*Byssoschlamys nivea*) asci containing ascospores, observed in the cores of infected apple fruit. Scale bar = 10 μm.

Fig. 4. Increase, over a 22-day incubation (25°C, ≥95% humidity), in the mean diameter of lesions (± 1 standard error) on Gala and Golden Delicious apple fruit inoculated with *Paecilomyces niveus* (*Byssoschlamys nivea*). Treatment lesions were significantly larger than control lesions on Gala and Golden Delicious at days 7 and 22 ($P < 0.0001$)

Trichocomaceae) (i.e., they are in different families) (Baert et al. 2007; Sanzani et al. 2012).

Examining the *Paecilomyces niveus* isolates found in this study in New York orchard soil and on a decaying apple (CO7) (Table 1) within a phylogenetic context, the *P. niveus* that were isolated were in the same clade as *P. niveus* cultured from food environments (Fig. 1), including pasteurized fruit juice in Europe (CBS 113245) and milk in North America (CBS 133.37) (Samson et al. 2009). *P. fulvus* spoilage isolates, cultured from bottled fruit and fruit juice (CBS 132.33 and CBS 135.62), were sister to *P. niveus* from orchard soil in our analysis, in agreement with previous work (Fig. 1) (Samson et al. 2009). Because *P. niveus* and *P. fulvus* are close relatives that similarly spoil thermally processed fruit, future studies should evaluate whether *P. fulvus* can infect the fruit that it often spoils.

We hypothesize that infected fruit may be an overlooked source of spoilage of heat-treated apple products, when *P. niveus* survives thermal processing. The harmful practice of using “drops” (fallen apple fruit) must be discouraged. Not only could “drops” passively transport spores in adhering soil particles but also apple fruit may also be wounded and infected through soil contact. The ascospores that develop in infected fruit (Fig. 3) may be able to survive thermal processing. Future work should ask whether fruit with *Paecilomyces* rot introduce this spoilage organism to thermally processed apple products.

In addition to spoiling food, *P. niveus* produces the mycotoxin patulin in food (Moake et al. 2005; Sant’Ana et al. 2008). Patulin is not eliminated by thermal processing and is a major concern for the apple industry. Patulin acute toxicity includes convulsions, subacute toxicity can include gastrointestinal disorders, and there are concerns that it is carcinogenic (Puel et al. 2010). For these reasons, patulin is limited to 50 µg liter⁻¹ by the U.S. Food and Drug Administration, 50 ppb by Health Canada, and 10 to 25 µg liter⁻¹ by the European Union in apple juices and other products, of which children are the biggest consumers and most at risk from exposure (Bennett and Klich 2003; Health Canada 2018; Moake et al. 2005; US FDA 2001). Although variety differences between Gala and Golden Delicious were confounded by chamber effects in our study, hints of differences in lesion size and symptoms between the varieties could have implications for contamination. Blue rot lesion diameter positively correlates with patulin accumulation in apple fruit (Sant’Ana et al. 2008). Future research should investigate the accumulation of patulin in apple fruit during the development of *Paecilomyces* rot and further evaluate disease progression of different apple varieties.

Paecilomyces rot has characteristics that make it difficult to distinguish from other apple diseases: bull’s-eye rot, apple ring rot, black rot, and bitter rot. It may have been overlooked because it is masquerading as these other diseases. It causes lesions like those seen in early stages of black rot, caused by *Diplodia seriata*, and bull’s-eye rot or apple ring rot, caused by *Neofabraea* spp. and *Botryosphaeria dothidea*, respectively (Sutton et al. 2014). The internal symptoms of *Paecilomyces* rot resemble bitter rot disease, caused by *Colletotrichum gloeosporioides*; however, in cross-section, the infected cone-shaped tissue has edges that are more outwardly bowed (U-shaped) (Phillips et al. 2007; Sutton et al. 2014) (Fig. 2C). *Paecilomyces* rot symptoms, especially on Golden Delicious apple, were reminiscent of bull’s-eye rot or black rot symptoms on apple fruit (Sutton et al. 2014). Like both rots, the lesions caused by *P. niveus* were slightly flattened and firm (Fig. 2A and B). Unlike bull’s-eye and black rots, *P. niveus* produces no external fruiting bodies such as pycnidia, pseudothecia, or acervuli (Fig. 2F). Although possibly confounded by a chamber effect in our experiment, surface hyphae were more often observed on Gala than Golden Delicious at the site of inoculation; the lenticels on Gala were often colonized with floccose, white mycelia with asci (Fig. 2B). The sparse white surface mycelium noted within *Paecilomyces* rot lesions (Fig. 2B), distinguished it from bitter rot, which is known for copious quantities of conidia produced on the surface of lesions (Sutton et al. 2014). Compared with postharvest diseases such as blue mold, which has a characteristic musty odor (Sutton et al. 2014), apple fruit infected with *P. niveus* smelled sweet.

Another reason that *P. niveus* has not been previously described as an apple pathogen or isolated from apple storage environments could

be due to sampling methods that are biased for fast-growing *Penicillium* spp. (Amiri and Bompeix 2005). To test this idea, we attempted to isolate *P. niveus* from culled apple fruit. After a recommended heat treatment to reduce other species and activate *P. niveus* ascospores (Houbraken and Samson 2006), no *P. niveus* was recovered, even from apple tissue known to be infected. This finding may indicate that the temperature used was too high or that combined freezing and heating damaged *P. niveus*. Younger ascospores are known to be less resilient (Bayne and Michener 1979; Dijksterhuis 2007). If *P. niveus* was present in the culled apple fruit at low concentrations, we may have failed to isolate it because of low sample weights. New postharvest sampling approaches, involving the addition of a mild heat treatment to increase germination, might be more successful (Yates et al. 1968).

P. niveus is both important and unusual, because it infects apple fruit, produces patulin, causes spoilage, and resists high temperatures, creating multiple risks for processed apple products. To avert food safety problems caused by *P. niveus*, apple products may benefit from higher processing temperatures. More research is needed on the incidence of *Paecilomyces* rot, to trace a pathway from infected fruit to spoiled processed foods, and to investigate patulin production in infected apple fruit.

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