# ASSESSING RISK FACTORS OF SPOILAGE AND PATULIN CONTAMINATION IN FRUIT PRODUCTS BY PAECILOMYCES NIVEUS

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# ASSESSING RISK FACTORS OF SPOILAGE AND PATULIN CONTAMINATION IN FRUIT PRODUCTS BY PAECILOMYCES NIVEUS

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In 1909, the first specimen of *Paecilomyces niveus* Stolk & Samson (*Byssochlamys nivea* Westling) was isolated from a specimen of *Geaster* that was stored in alcohol, a prelude to the remarkably persistent properties of this fungus food makers would later come to grips with. In the century since, this heat resistant mold *P. niveus* has continued to spoil normally shelf-stable fruit products. This is particularly alarming contamination because the fungus produces patulin, a known and regulated mycotoxin. It has been long assumed that *P. niveus* spoilage inoculum originates from environmental sources like air, soil, and infested equipment, but recently, the fungus was found to cause the postharvest apple disease Paecilomyces rot, capable of infecting and growing in live apple fruits. Processing infected apples can result in highly infested and mycotoxin-contaminated cider, a finding that fundamentally changes how we view sources of *P. niveus* spoilage inoculum.

In the first chapter of this dissertation, I review previous work and new developments regarding research on *P. niveus*, focusing on 1). The economic impact and extremotolerant properties of this fungus and 2). How the new understanding that *P. niveus* causes plant disease can drive future research in this field. In chapters two and three, I investigate the host range of Paecilomyces rot and the infection biology of *P. niveus*. I determined that various rosaceous and citrus fruits are susceptible to infection by *P. niveus,* and established cultivar-based susceptibility in apple fruits. In

chapter 4, I developed species-specific primers based on the *patK* gene, encoding a 6-methylsalicylic acid synthase, and validated a new qPCR system designed to detect *P. niveus* in food, fruit, and soil. The qPCR system was used to investigate questions regarding *P. niveus* disease biology using apple blossom and strawberry bioassays. Lastly, in chapter 5 I assess risk of spoilage and contamination in hard apple cider by four patulin-producing apple pathogens. Our results support my hypothesis that bench-top fermentation does not sufficiently preclude *P. niveus* spoilage and patulin contamination in finished product. Results from these studies will aid food producers in better understanding and assessing risk factors of *P. niveus* spoilage and patulin contamination in fruit products.

# BIOGRAPHICAL SKETCH

Tristan Wei Wang was born and raised in Granada Hills, California. He attended Granada Hills Charter High School and graduated from Harvard University in 2016 with a bachelor's degree in Organismic and Evolutionary Biology. His undergraduate thesis project was advised by Dr. Donald Pfister and focused on the ectoparasitic fungus *Herpomyces*. After spending a year at Alabama A&M University working as a laboratory assistant, he committed to pursuing his doctoral research under the advisement of Dr. Kathie T. Hodge on the postharvest pathogen and food spoiling agent, *Paecilomyces niveus*.

For my family and friends and everyone who’s never stop rooting for me

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# CHAPTER 1

## PAECILOMYCES NIVEUS: RECENT ADVANCEMENTS ON A CENTURY-OLD FOOD SPOILAGE PROBLEM

### Introduction

*Paecilomyces niveus* is a notorious food spoiling agent that is among the most commonly found heat-resistant molds (HRM) in fruit products and their derivatives (Tournas, 1994). Spoilage by *P. niveus* is a human health concern because the fungus produces patulin, a mycotoxin that is regulated by several countries and commissions (Affairs, 2020; Commission, 2003; Commission Regulation, 2006).

It has been long assumed that entry of *P. niveus* spoilage inoculum is entirely environmental: that the mold enters foods through air, soils, and infested equipment (Tournas, 1994). However, it has been recently shown that *P. niveus* can infect and grow in live apple fruits, causing the postharvest disease Paecilomyces rot, and that processing infected fruits can lead to contaminated product (Biango-Daniels and Hodge, 2018; Biango-Daniels et al., 2019b). This finding fundamentally changes how we view *P. niveus* spoilage because infected fruits may constitute a previously overlooked route of *P. niveus* entry into foods and reveals questions about the role the disease may have in food spoilage (Biango-Daniels et al., 2019b).

This review synthesizes recent developments and research directions in the field of spoilage and patulin contamination by *P. niveus*. It includes: 1. The economic impact of *P. niveus* in foods and the fungus' extraordinary ability to tolerate various stresses, and 2. The disease biology of *P. niveus* and principles and methods in Paecilomyces rot disease management.

## Food spoilage

Postharvest operations, including losses due to pathogens and food spoilage, account for a loss of almost a third of available US food (133 billion pounds) with this proportion significantly higher in less developed countries (Alkan and Fortes, 2015; Kumar and Kalita, 2017). Mycotoxigenic pathogens are particularly problematic as they can contaminate food and render downstream products unusable. For example, *Aspergillus carbonarius* and *Penicillium expansum* are two important fruit pathogens that can infect grapes and apples and contaminate them with ochratoxin and patulin respectively (Baert et al., 2007; Battilani et al., 2004; Bellí et al., 2006). Mycotoxigenic pathogens that infect fruits threaten two central tenets of food security: availability and utilization. *P. niveus* is both a patulin-producer and postharvest pathogen of apples (Biango-Daniels and Hodge, 2018). However, this fungus is uniquely problematic, especially for food producers, as it produces thermotolerant ascospores that can survive some temperatures of pasteurization allowing it to spoil finished product (Biango-Daniels et al., 2019b).

Presence of HRM ascospores is widespread in food processing environments and spoilage by *Paecilomyces* is traditionally considered a persistent problem of heat-treated fruit products (Rico-Munoz, 2017). Because of the sporadic nature of food spoilage, surveys of HRMs are limited to just a handful of studies. One survey by Santos et al. (2018) found that *P. niveus* is relatively common, present in 27.6% batches of processed strawberries, 13.3% batches of orange juice, and 42.3% batches of apple puree in tested facilities across three countries. In addition, anecdotal evidence of *P. niveus* spoilage in a variety of fruit products suggests that spoilage may be even more widespread than assumed. There have been reports of *P. niveus* in tomato paste, rhubarb juice, cucumber brine, and passion fruit nectar,

among other products (Ferreira et al., 2009; Ismail, 2006; Kotzekidou, 1997; Tournas, 1994; Yates and Ferguson, 1963). Not limited to just fruit products and their derivatives, the fungus has also been found in spoiled milk, soft drinks, and maize silages (Engel and Teuber, 1991; Penagos-Tabares et al., 2022; Silva et al., 2022). While patulin contamination has been established as a recurring problem in fruit juices and products, few studies have established direct links between spoilage agents and patulin contamination (Harris et al., 2009; Spadaro et al., 2007). It is traditionally assumed that the majority of patulin contamination cases are caused by *Penicillium expansum*, causal agent of Blue mold, as it causes severe infections of apple fruits and produces significant amounts of patulin (Baert et al., 2012; Morales et al., 2008). However, *P. niveus* may be overlooked as a major causal agent of patulin contamination and it is important to determine the spoiling agent responsible for patulin contamination in individual cases because different spoilage agents require different methods of control.

## Taxonomy

*P. niveus* Stolk & Samson (*Byssochlamys nivea* Westling) (Ascomycota, Eurotiales, Thermoascaceae) is able to persist in various food and agricultural environments due to its extremotolerant properties, a quality shared by several relatives. The filamentous fungus was described by Westling in 1909, originally isolated from a specimen of *Geaster* stored in spirit (J. O. Roland and Beuchat, 1984). The fungus belongs to the large and diverse order of Eurotiales, which includes other impactful postharvest pathogens like *Penicillium digitatum* and *Penicillium italicum* on citrus fruits, *Penicillium expansum* on various fruits, and *Aspergillus carbonarius* on grapes (Battilani et al., 2004; Bhatta, 2022). Members of its family, Thermoascaceae, are often noted for their thermotolerant properties and as

food spoiling molds (*Thermoascus spp.* and *Paecilomyces fulvus*) and some are occasional opportunistic pathogens of plants and people (Heidarian et al., 2018; Hosoya et al., 2014; Houbraken et al., 2008, 2006; Marques et al., 2019; Scaramuzza and Berni, 2014). It is generally assumed that *Paecilomyces spp.* and most HRMs are saprobic by nature (Tournas, 1994). *P. niveus* forms abundant naked, globose asci with ellipsoidal ascospores and conidia measuring 3.1-4.3 x 2.6-3.4 µm (Samson et al. 2009). On potato dextrose agar, *P. niveus* forms white colonies that yellow with age (Fig. 1.1). A key to *Byssochlamys* and corresponding *Paecilomyces* anamorphs was made available by Samson et al. (2009).

## Reproduction

Both sexual and asexual reproductive modes have been observed in *P. niveus,* and individual colonies arising from a single spore readily produce ascospores, indicative of its homothallic lifestyle (Houbraken et al., 2008). It is unclear how often *P. niveus* outcrosses, if at all. Recombination, whether by selfing or outcrossing, has been observed to help lower rates of deleterious mutations in fungi, but the consequences of selfing for evolutionary fitness are assumed to be limited as the nuclei that fuse to form meiotic products are identical (Bruggeman et al., 2003). Most importantly in the context of food safety, a single spore of *P. niveus* can give rise to ascospores, the resistant spore structure that leads to *P. niveus* spoilage.

Homothallism is relatively common within the Eurotiales as several relatives are able to self-fertilize including *Aspergillus nidulans*, *Neosartorya fischeri*, *Thermoascus aurantiacus*, *Monascus* spp., and Eupenicillia among others (Babitha et al., 2007; Bruggeman et al., 2003; Gabriel et al., 2020; Pöggeler et al., 2011). The term homothallism in fungi encompasses any reproductive mode that can result in a spore giving rise to a sexually reproducing colony (Wilson et al., 2015). Homothallic

reproductive modes include having: both mating types in one genome (primary homothallism), opposite mating types occupying two different nuclei in the same cell (pseudohomothallism), and the ability to switch mating types (bidirectional and unidirectional mating type switching) (Bennett and Turgeon, 2016; Wilson et al., 2015). In each case, complementary *MAT* idiomorphs are needed for sexual reproduction. The genome of *P. niveus* CO7, a strain isolated from culled apples, has been published and is publicly available (Biango-Daniels et al., 2018), but it has been noted that the genome does not contain the *MAT1-1* (*mat*$\alpha$) idiomorph, only the *MAT1-2* (*mat**a***) idiomorph (Wilson et al., 2021). As a result, the fungus has been proposed to be unisexual: able to undergo sexual reproduction regardless of absence of a mating type, a rare form of homothallism (Wilson et al., 2021).

## Stress tolerance

*P. niveus* demonstrates extremotolerant properties, in its ability to withstand temperatures well above 85˚C, high levels of alcohol, and cold stress (Beuchat and Toledo, 1977; Brown and Smith, 1957; Houbraken et al., 2006). The thermotolerance of *P. niveus* ascospores has been of considerable scientific interest and has been investigated in various substrates including apple juice, grape juice, strawberry puree, tomato paste, and fruit syrups (Beuchat and Toledo, 1977; Evelyn and Silva, 2015; King et al., 1969; Kotzekidou, 1997; Menezes et al., 2020). *P. niveus* thermotolerance has also been shown to increase with ascospore age and to vary by strain (Evelyn and Silva, 2017; Santos et al., 2018). Similar to other HRMs, *P. niveus* ascospore germination has been shown to be heat-activated, with heat shocks of 75˚C stimulating germination (Tournas, 1994; Yates et al., 1968). This means that light heat treatments may inadvertently spur *P. niveus* germination and spoilage of fruit products. In one case study, Wareing (2016) remarked that a mycelial mat of *P.*

*niveus* formed in finished rhubarb-mixed fruit juice even after three instances of pasteurization including a flash pasteurization at 95˚C for 30 sec and a tunnel pasteurization at 75˚C for 20 minutes. It was suggested that vegetative cells of *P. niveus* were killed during pasteurization but thermotolerant ascospores survived, were heat-activated, and grew to produce enough spores to survive the successive heat treatments (Wareing, 2016). This raises the question of whether processing environments and food treatment are selecting for strains with higher thermotolerance. Several studies have taken advantage of these thermotolerant properties as a way to selectively isolate the fungus from soils (Biango-Daniels and Hodge, 2018; Put, 1964). Because of its significant tolerance to heat and other food treatment processes, Silva and Evelyn (2020) proposed *P. niveus* be incorporated into methods of testing food processing, as a standard for spoiling agents. For a comprehensive review of previously established D-values (time in minutes required for a log-reduction at a specific temperature) of *P. niveus*, see Dijksterhuis (2019).

The genus *Paecilomyces* includes facultative anaerobes, which are highly tolerant of both low oxygen conditions and high $CO_2$ levels (Pitt and Hocking, 2022; Taniwaki et al., 2009). Several studies have looked into the ability of *P. niveus* to tolerate and grow under modified or controlled atmospheric conditions, practices used to maintain food quality, often by reducing changing $O_2$ and $CO_2$ levels (Dumont et al., 2016; Nguyen Van Long et al., 2017). In one study that evaluated growth of eight fungi in varying $O_2$ and $CO_2$ levels, researchers found that only *P. niveus* was able to grow on cheeses under conditions of 40% $CO_2$ with less than 0.5% $O_2$ (Taniwaki et al., 2001). This is concerning for fruits and foods stored under limited oxygen conditions as *P. niveus* growth may not be completely inhibited (Drusch et al., 2007; Dumont et al., 2016).

In food microbiology, “hurdle technology” combines factors like pH, water activity, and preservatives to create a hostile environment for food-borne microbes (Putnik et al., 2020). *P. niveus,* however, exhibits remarkable cross-stress resistance to various abiotic and chemical treatments. Table 1.1 provides a comprehensive list of literature resources detailing *P. niveus* tolerance to various stresses.

**Table 1.1. Review of various treatments and stress tolerance assays on ascospore germination and growth of *Paecilomyces niveus***

| Treatment/stress | Reached conclusion | Reference |
|---|---|---|
| Chemical | Resistance to postharvest fungicides difenoconazole, fludioxonil, and pyrimethanil | Biango-Daniels et al. 2019a |
| | Growth inhibition in SO2 (300 ppm), potassium sorbate (400 ppm), and diethylpyrocarbonate (600 ppm) in grape juice | Beuchat, 1976 |
| | Capable of tolerating and degrading pentachlorophenol | Bosso et al., 2015 |
| | Ascospore survival in chlorine dioxide (1000 ppm) | Dijksterhuis et al., 2018 |
| | Minimum inhibitory concentration established for sorbic acid and sorbic acid treated with ethylenediamine tetraacetic, no effect of propionic acid on growth inhibition | Razavi-Rohani and Griffiths, 2007 |
| | Various log reductions determined using biguanide, iodine, ortho-phenylphenol, sodium hypochloite, peracetic acid, and iodine | Stefanello et al., 2020 |
| Thermosonication | High pressure thermal processing with thermosonication (600MPa, 24 kHz, 0.33 W/mL) for 30 minutes at 75˚C resulted in 2.7 and 2 log reduction for 4- and 12-week-old spores | Evelyn and Silva, 2017 |
| | Spore inactivation under power ultrasound (24 kHz, 0.33 W/mL) and thermal processing (75˚C) can in strawberry puree | Evelyn and Silva, 2015 |
| Cold and heat-stress | Ascospore survival in fruit products stored at 7˚C and -30˚C and in grape juice heated to 75˚C | Beuchat and Toledo, 1977 |
| | Survival at 90˚C for 20 minutes in tomato juice | Kotzekidou, 1997 |
| | Survival at 90˚C for 2 minutes in canned fruits | Luthi and Hochstrasser, 1952 |
| | Growth from 10.58˚C to 43.21˚C | Panagou et al., 2010 |
| | Survival at 87.5˚C for 10 minutes, but not at 90 or 92.5˚C in strawberry juice for 10 minutes | Put and Kruiswijk, 1964 |
| Reduced water activity | Growth and ascospore production in juice with $a_w$ at .90 | Beuchat and Toledo, 1977 |
| | Growth at aw at .892 to .992 | Panagou et al., 2010 |
| | Minimum $a_w$ for growth at 21˚C and 30˚C is .914 and .886 respectively | Roland and Beuchat, 1983 |
| | Minimum $a_w$ for growth established above .9 | Valle et al., 2022 |
| | Growth in pineapple juice with $a_w$ ranging from .99 to .9 | Zimmerman et al., 2011 |
| High acid levels | 70% $CO_2$ and 5% $O_2$ conditions were insufficient at preventing spore germination | Nguyen Van Long et al., 2017 |
| Low $O_2$, high $CO_2$ levels | Resistance to high acid levels | Silva and Evelyn, 2020 |
| | Growth observed at 20 and 40% $CO_2$ with < 0.5% $O_2$ on cheese | Taniwaki et al., 2001 |
| | Growth observedat 20%-60% $CO_2$ and < 0.5% $O_2$ on CYA and PDA | Taniwaki et al., 2009 |
| | Detected growth in pure $CO_2$ | Yates et al., 1967 |
| High pressure processing-thermal treatments | 3.2 log reductions under 700MPa, 70˚C for 15 minutes in grape juice | Butz et al., 1996 |
| | 1.7 and 2.0 log reductions under 600MPa, 75˚C for 15 and 20 minutes in strawberry puree | Evelyn and Silva, 2015 |
| | 1.5 log reductions under 600MPa, 70˚C for 15 minutes in pineapple nectar | Ferreira et al., 2009 |
| | Ascospore inactivation achieved after 3 or 5 cycles of oscillatory pressurization (689 Mpa) at 60˚C in apple and cranberry juice concentrates | Palou et al., 2003 |
| Hyperbaric | Ascospore development in apple juice was inhibited after storage at 25-150 Mpa for 30 days at room temperature | Pinto et al., 2023 |
| UV-C treatment | 4 log reductions at 36W/m2 for 8 minutes in apple juice | Menezes et al., 2020 |

## *Paecilomyces niveus* and patulin

Patulin is a highly regulated mycotoxin, noted for its carcinogenic, cytotoxic, and neurotoxic properties, among other acute and chronic effects (Ramalingam et al.,

2019; Ülger et al., 2020). Moake et al. (2005) provides a comprehensive list of acute, cellular, and chronic effects of patulin. The mycotoxin was originally designated as an antibiotic because of its observed inhibitory effects on various bacterial species (Chain et al., 1942; Korzybski et al. 1976). However, further research into its toxic effects led to the mycotoxin's regulation by multiple countries, and it is now considered the most important mycotoxin in apple juice production (Affairs, 2020; Commission, 2003; Commission Regulation, 2006). Patulin is one of the only two mycotoxins to have an official action level by the United States Food and Drug Administration, limiting the mycotoxin to 50 µg/kg in apple juices (Affairs, 2020; Nutrition, 2022). The European Commission also limits patulin to 50 µg/kg in fruit juices and concentrates and adds further limitations of 25 µg/kg in solid apple products and 10 µg/kg for apple products intended for infant consumption (Commission Regulation, 2006).

While over a hundred species of filamentous fungi have been purported as capable of producing patulin, about 29 have been confirmed to be able to do so (Frisvad, 2018). Of the confirmed patulin producers, almost all are from the Eurotiales including *Penicillium spp.* and a handful of *Aspergillus* species (Frisvad, 2018). Interestingly, an unrelated fungus, *Xylaria longiana,* has also been demonstrated to produce patulin (Frisvad, 2018; Lykakis et al., 2009). Within *Paecilomyces*, *P. niveus*, *P. fulvus, P. laguncullariae*, and *P. saturatus* have been reported to be patulin producers, the former two of which are considered important food spoiling molds in heat-treated products; however, Puel et al. (2007) demonstrated that some isolates of *P. fulvus* lack key genes in the patulin biosynthetic gene cluster, including missing polyketide synthase and isoepoxydon dehydrogenase genes and it is unclear if all strains of the species can produce patulin (Frisvad, 2018; Rico-Munoz, 2017).

Patulin is a polyketide-derived secondary metabolite that is synthesized via about 10 enzymatic reactions (Fig. 1.2.) (Moake et al., 2005; Puel et al, 2010; Tannous et al., 2014). Some level of sequence conservation within the gene cluster exists among patulin-producing species and synteny between the putative patulin gene clusters of *Aspergillus clavatus* NRRL1 and *P. niveus* CO7 is conserved (Tannuous et al., 2014). The isoepoxydon dehydrogenase gene (*patN*) in *P. niveus* has been observed to have high sequence homology to those belonging to *Penicillium expansum* and *Penicillium griseofulvum*, two other patulin producers (Dombrink-Kurtzman and Engberg, 2006).

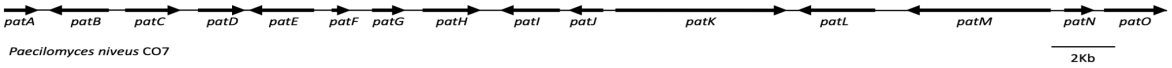


**Figure 1.1. Putative patulin gene cluster in *P. niveus* CO7.** Representation of the patulin biosynthetic gene cluster. Orientation of arrowheads indicates direction of transcription. Genes are drawn to scale.

Previous research has investigated the effects of preservative treatment, water activity, temperature, and packaging on *P. niveus* patulin production (J. O. Roland and Beuchat, 1984a; J. O. Roland and Beuchat, 1984b). In addition, Sant'Ana et al. (2010) determined that lower storage temperatures resulted in lower mycelium dry weight and patulin levels in apple juices. Roland and Beuchat (1984a) established a positive relationship between water activity and patulin production in *P. niveus*. In addition to patulin, other mycotoxins are produced by *P. niveus,* including byssochlamic acid, byssochlamysol, and mycophenolic acid (Mori et al., 2003; Puel et al., 2005).

## Paecilomyces rot and infection biology

*P. niveus* spoilage inoculum has traditionally been assumed to enter food through environmental sources like air, water, and equipment (Tournas, 1994). Recently, Biango-Daniels and Hodge (2018) demonstrated that the fungus is able to infect and grow in apple fruits, causing the postharvest disease Paecilomyces rot. Processing apple fruits infected with Paecilomyces rot leads to apple juice infested with *P. niveus* spoilage inoculum and contaminated with patulin (Biango-Daniels et al., 2019b).

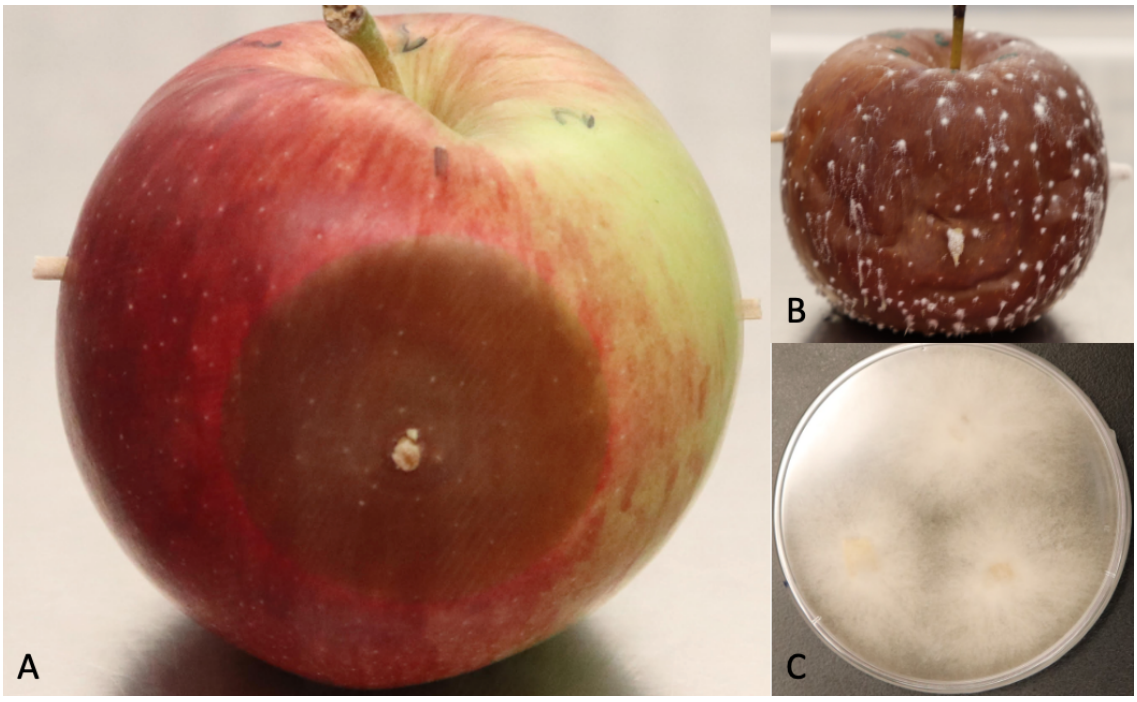


**Figure 1.2. Symptomatic apple fruits infected with *P. niveus*.** A) Infected Fuji apple 2 weeks post inoculation. B) Infected Fuji apple 7 weeks post inoculation. C) Culture of *Paecilomyces niveus* on PDA grown from three initial inocula.

Development of Paecilomyces rot in lab-inoculated apples follows similar development to other wound-infecting postharvest pathogens. Symptoms initially appear as brown to dark brown lesions that radiate from the point of inoculation (Fig.

1.2A) (Biango-Daniels and Hodge, 2018). Concentric circles of alternating light brown and brown may also appear (Biango-Daniels and Hodge, 2018). Internal rot is dark brown and at advanced stages of the disease (>3 weeks), *P. niveus* hyphae can be seen on the surface of apple fruits and in the core of the apple (Biango-Daniels and Hodge, 2018). However, unlike Blue mold infection, lesion development associated with Paecilomyces rot is slower and infection by *P. niveus* results in a hard rot in apple fruits, not a soft rot – lesions are firm and spongy (Biango-Daniels and Hodge, 2018). It has been hypothesized that rot infections caused by *P. niveus* may be more common than we assume but are often masked by other postharvest rot (Biango-Daniels and Hodge, 2018). The host range of *P. niveus* is potentially even broader, as the fungus has been reported to occur on peas and aphids (Dragić et al., 2016; Zawadneak et al., 2015).

Several studies have investigated the role of patulin in pathogenicity and have hypothesized that the mycotoxin is an important virulence agent (Chen et al., 2021; Sanzani et al., 2012). Chen et al. (2021) observed that *Pe. expansum* mutants with a knocked out PeMetR gene, encoding a bZIP transcription factor, showed a large reduction in patulin production and complete loss of virulence. Similarly, when both *patL* and *patK* genes were deleted or disrupted in *Pe. expansum* the result was a significant decrease in patulin production and loss of virulence, unless additional patulin was added (Snini et al., 2016; Sanzani et al. 2012). Likely the most compelling argument for patulin as a virulence agent comes from Bartholomew et al. (2022), who reproduced Blue mold symptoms by treating fruits with patulin alone, and observed the mycotoxin's effect to be both cultivar-independent and dose-dependent. They suggested that the mycotoxin aided the breakdown of fruit tissue and caused tissue damage in the surrounding areas of patulin application (Bartholomew et al., 2022). While the role of patulin is unexplored in the *P. niveus* system, previous studies have

demonstrated that the fungus produces large quantities of patulin during fruit infection (Biango-Daniels et al., 2019)

It is generally assumed that pathogens of postharvest rots are necrotrophic, killing and feeding off dead host cells (Alkan and Fortes, 2015). Many postharvest pathogens occur as latent infections during fruit growth and remain quiescent until environmental cues like pH and total soluble sugars activate their transition into a necrotrophic lifestyle (Alkan and Fortes, 2015; Prusky et al., 2013). Like *Penicillium expansum*, *P. niveus* seems to act as an opportunistic wound-infecting pathogen of fruits although it is interesting to note that the ability to act as a postharvest pathogen is unusual for species of *Paecilomyces*. However, apples naturally infected with Paecilomyces rot were observed to display diffuse symptoms suggesting internal rot may have resulted from a latent infection (Khokhar et al., 2019). Because soils can be rich reservoirs of *P. niveus* disease inoculum, care has to be taken to ensure that harvested fruits do not come in contact with the ground (Biango-Daniels and Hodge, 2018; Put, 1964). This can be a challenge for fruits that are commonly grown close to the ground like strawberries, and for fruit producers that still use drop-apples for cider production (Miles et al., 2020). Little is known about the disease mechanisms of *P. niveus* outside of wound-infection bioassays and how often the disease occurs naturally in orchards is unclear.

## Conclusions

Prevention of spoilage and patulin contamination by *P. niveus* remains a priority from both a food safety and brand reputation standpoint. Spoilage by this fungus is a long-standing problem in food processing environments and *P. niveus* appears to have a global distribution, being commonly found in soil surveys and various food processing plants (Biango-Daniels and Hodge, 2018; Put, 1964; Santos

et al., 2018). The description of Paecilomyces rot reveals a potentially overlooked source of *P. niveus* spoilage inoculum in the form of infected fruits and food and juice producers lack effective tools for detecting and controlling the spoilage fungus (Biango-Daniels et al., 2019b).

Physical and chemical treatments such as bioactive plant compounds, hydrostatic pressure, ultrasounds, irradiation, hypobaric pressure, and various combinations may be promising alternatives to pasteurization (Aneja et al., 2014; Chen et al., 2023; Usall et al., 2016). However, food makers need to be wary to not reduce food quality while controlling postharvest diseases and spoilage. To avoid severe food treatments, food processing systems can invest in robust detection systems and strategies for preventing spoilage. PCR-based detection systems including quantitative PCR, loop-mediated isothermal amplification, and droplet digital PCR remain reliable tools for sensitive detection (Martínez et al., 2011; Niessen et al., 2013). Other novel methods including metabolomic profiling and nanosensors are among technologies currently explored for use in monitoring food spoilage and adulteration (Mohammadi and Jafari, 2020; Pinu, 2016). Further research is needed to develop and determine effective methods for *P. niveus* detection.

There is an advantage in approaching *P. niveus* food spoilage as a plant pathogen problem because processing apples infected with Paecilomyces rot leads to infested product (Biango-Daniels et al., 2019b). In addition to standard sanitation measures, spoilage prevention of *P. niveus* also requires either preventing Paecilomyces rot development in apples or effective screening for diseased fruits. However, very little is known about this newly described disease and no detection system has been developed for this fungal spoiler. Outstanding questions regarding Paecilomyces rot include its potential host range, whether cultivar-based resistance exists, and whether the fungus can only enter the host via wounds. Understanding

the risk factors that allow *P. niveus* to infect fruits may lead to the development of tools or strategies for combating the disease and as a result, aiding the control of food spoilage by *P. niveus*.

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# CHAPTER 2

## SUSCEPTIBILITY OF ROSACEOUS POME AND STONE FRUITS TO POSTHARVEST ROT BY PAECILOMYCES NIVEUS[1]


### Abstract

Paecilomyces rot of apples is a postharvest disease caused by *Paecilomyces niveus*, a problematic spoiling agent of fruit juices and derivatives. Processing fruits infected with Paecilomyces rot can lead to juices contaminated with *P. niveus* ascospores. These ascospores are heat resistant and may survive food processing and germinate in finished products. Because the fungus produces the mycotoxin patulin, juice spoilage by *P. niveus* is a health hazard. Little is known about the disease biology and control mechanisms of this recently described postharvest disease. The range of fruit products contaminated by *P. niveus* and patulin led us to hypothesize that the host range of Paecilomyces rot is broader than previously thought. Following Koch's postulates, we confirmed that multiple previously untested rosaceous fruits and popular apple cultivars are susceptible to Paecilomyces rot infection and that these infected fruits contain significant levels of patulin. We also observed that two closely related food spoiling fungi, *Paecilomyces fulvus* and *Paecilomyces variotii*, were unable to infect, cause symptoms in, or grow in wounded fruits. Therefore, we challenge the assumption that *P. niveus* spoilage inoculum is introduced to foods solely through environmental sources, and we show that other economically important rosaceous fruits, peaches, pears, sweet cherries, and sour cherries, are susceptible to infection and can also be sources of spoilage inoculum.


[1] Wang, T.W., Hodge, K.T., 2022. Susceptibility of Rosaceous Pome and Stone Fruits to Postharvest Rot by Paecilomyces niveus. Plant Dis. 106, 121–126. https://doi.org/10.1094/PDIS-04-21-0855-RE

Our results highlight the unique abilities of *P. niveus* to infect a variety of fruits, produce patulin, and form resistant spores capable of spoiling normally shelf-stable products.

## Introduction

Patulin is a serious mycotoxin that can cause gastrointestinal, immunological, and neurological damage and is an important contaminant of apple products and their derivatives (Puel et al. 2010). One 2009 survey detected patulin in 23% of apple juice and cider samples from Michigan retail grocery stores, with 11.3% of these samples containing patulin over the U.S. Food and Drug Administration (FDA) limit of 50 ppb (Harris et al. 2009). Contamination by this mycotoxin is thought to occur through postharvest fruit infection and food spoilage by patulin-producing microbes. At least 18 *Aspergillus*, *Paecilomyces*, and *Penicillium* species are known to produce patulin (Puel et al. 2010). Patulin contamination has been detected in a wide variety of fruit products including pear, peach, cherry, apricot, orange, and mango juices and jams (Erdoğan et al. 2018; Hussain et al. 2020; Moake et al. 2005; Spadaro et al. 2008).

One important patulin producer, *Paecilomyces niveus* Stolk & Samson (*Byssochlamys nivea* Westling), is uniquely equipped to threaten juice production because it is also a thermotolerant mold that creates durable ascospores that can lead to food contamination (Biango-Daniels et al. 2019). Spoilage by *P. niveus* is a long-standing problem, and the fungus has been found in a wide variety of fruit products including apple-based products, concentrated orange juice, strawberry puree, and tomato paste (Kotzekidou 1997; Santos et al. 2018). *P. niveus* is a common soil fungus, found in a third of New York orchard soil samples by Biango-Daniels and Hodge (2018). It is thought that *P. niveus* spoilage of food products is environmental in origin, originating from soil, air, and equipment. Even small

quantities of this homothallic fungus pose a hazard for juice production because a single *P. niveus* spore can give rise to heat-resistant ascospores.

Recently, *P. niveus* was identified as the causal agent of Paecilomyces rot, a postharvest disease of apples (Biango-Daniels and Hodge 2018) and citrus fruits (Wang and Hodge 2020). Biango-Daniels et al. (2019) showed that processing apple fruits infected with Paecilomyces rot can result in apple juice significantly contaminated with patulin and viable *P. niveus* ascospores. Since the description of Paecilomyces rot (Biango-Daniels and Hodge 2018), *P. niveus* has been found infecting apples in a fruit market in China (Khokhar et al. 2019). In addition, the fungus was observed to be acting as a seed borne pathogen on peas (*Pisum sativum*) in Serbia (Dragić et al. 2016).

The broad range of fruit products in which *P. niveus* and patulin have been found led us to hypothesize that the fungus may be able to cause disease in other rosaceous fruits, as well as a range of apple cultivars (‘Empire’, ‘Fuji’, ‘Granny Smith’, and ‘Golden Delicious’). We also asked whether pathogenesis in our wound inoculations was unique to *P. niveus* CO7, testing if other strains and close relatives of *P. niveus* share its wound-infecting abilities. Like *P. niveus*, two closely related heat-resistant molds known to contaminate fruit products, *P. fulvus* and *P. variotii*, produce heat-resistant ascospores capable of surviving temperatures >85°C (Houbraken et al. 2006; Samson et al. 2009). We hypothesized that both species can also cause symptoms, reproduce in apple fruits, and spoil apple products via infected apple fruits.

To test the preceding hypotheses, we tested the wound-infecting ability of *P. niveus* in fruits of a variety of apple relatives: peaches, pears, sweet cherries, and sour cherries. In addition, we inoculated and compared lesion development in four popular apple cultivars. Lastly, we inoculated apple fruits with *Paecilomyces variotii*

and *Paecilomyces fulvus* and observed them for symptom development.

## Materials and Methods

### Fruit

Detached peach fruits (*Prunus persica* 'Lori Anne') (*n* = 26) and pear fruits (*Pyrus communis* 'Green D'Anjou') (*n* = 31) were selected at a local supermarket based on their uniformity in size and absence of both wounds and disease symptoms. Sour cherries (*Prunus cerasus* 'Montmorency') (*n* = 40) and sweet cherries (*Prunus avium* 'Kristin') (*n* = 31) were freshly picked from a local New York orchard. For apple cultivar susceptibility testing, Empire (*n* = 31), Fuji (*n* = 28), Golden Delicious (*n* = 31), and Granny Smith (*n* = 31) apples were purchased from a local supermarket. These four cultivars were chosen based on their popularity in U.S. markets. Three of these cultivars (Empire, Fuji, and Granny Smith) were not previously tested for susceptibility to Paecilomyces rot. Fruits and cultivars used for patulin analysis were sourced similarly to the fruits used in infection assays with the exception of the sweet cherries, for which the cultivar is unknown. For susceptibility tests involving *P. fulvus* and *P. variotii*, 30 Empire apples from a local supermarket were inoculated with each fungus. To test fruit susceptibility to other *P. niveus* strains, *P. niveus* MC4 and 106-3 were each used to inoculate two Empire, Fuji, and Granny Smith apples in addition to three D'Anjou pears for each strain.

### Fungal pathogens

*P. niveus* strain CO7, a patulin-producing strain isolated from culled apple fruits in New York, was used to inoculate fruits according to the method described by Biango-Daniels and Hodge (2018). We sequenced the full genome of this strain

(Biango-Daniels et al. 2018) and found that sequences from internal transcribed spacer (*ITS*) and *β-tubulin* regions can reliably be used to confirm identity as *P. niveus*. *P. niveus* strain MC4, isolated from New York residential garden soil and *P. niveus* strain 106-3, isolated from New York orchard soil, were also used in apple and pear inoculation and identified via *ITS* and *β-tubulin* regions (Biango-Daniels and Hodge 2018). *P. fulvus* strain 7, obtained from the Worobo lab collection (Cornell University) and originally isolated from spoiled food, and *P. variotii* strain 103-2, isolated from New York soil, were identified morphologically and by sequencing of the *ITS* region.

To produce inoculum, fungi were grown from 5mm plugs taken from the edge of 2-week-old colonies. Five plugs of *P. niveus* were cultured on potato dextrose agar. The fungus was allowed to grow in the dark for 2 weeks at 25°C, covering toothpicks that had been tyndallized: autoclaved once in water and again in potato dextrose broth the next day. Control toothpicks were similarly treated but in the absence of the fungus. Both *P. fulvus* and *P. variotii* were grown as described previously to produce toothpick inoculum.

**Fruit handling, inoculation, and measurement**

Fruits were sanitized before fungal inoculation with *P. niveus* CO7: Peach fruits were sanitized for 30 s in 70% ethanol, 2 min in 1% sodium hypochlorite, and 15 s in 70% ethanol and then left to dry in a class IIB biological safety cabinet. Two treatment toothpicks and two control toothpicks were inserted on opposite sides of the fruit's equator, as described by Biango-Daniels and Hodge (2018) and van der Walt et al. (2010). The toothpicks served to both wound (1 cm deep) and inoculate the fruits. Individual fruits and wounds were numbered. The peaches were placed in moist chambers (≥95% humidity) in a dark incubator at 25°C. Over the course of 2 to

3 weeks, fruits and disease symptoms were observed. Horizontal and vertical diameters of lesions were quantified with a digital caliper (VWR Carbon Fiber Composite) every other day. Fruits displaying colonization or symptoms such as blue-green or brown sporulation, indicative of other common postharvest diseases, were removed from the study and further analysis.

Pear and apple fruits were similarly sanitized and inoculated with *P. niveus* CO7. Individual fruits of the four apple cultivars were randomized in moist chambers. Both sour and sweet cherry fruits were wounded only two times on opposite sides (5 mm deep), once with a *P. niveus* CO7 treatment toothpick and once with a control toothpick before being placed in moist chambers (≥95% humidity). The sanitation and inoculation process was repeated for apple and pear fruits inoculated with *P. niveus* strains MC4 and 106-3.

**Patulin quantification**

To quantify patulin in diseased fruits, 12 peaches, 12 pears, 60 sour cherries, and 60 sweet cherries were either inoculated with *P. niveus* CO7–infested toothpicks (treatment) or mock inoculated with control toothpicks according to the methods described previously (control). After 2 weeks of incubation (1 week for sweet and sour cherries), whole fruits were blended, and puree from both treatment and control fruits was submitted to Trilogy Analytical Laboratory (Washington, MO) for high-performance liquid chromatography patulin quantification via official AOAC International methods for apple juice (method 995.10 in Brause et al. 1996).

**Statistical analysis**

To test for significance of lesion diameters compared with the controls, we performed statistical analysis via two separate linear mixed-effects models at day 6 and day 20 for apple fruits, at day 2 and day 12 for pear fruits, and at day 2 and day 8

for peach and cherry fruits infected with *P. niveus* CO7. Bioassays of apple infection by *P. niveus* strains MC4 and 106-3 yielded similar results to infection by CO7. As a result, statistical analysis was done on the representative CO7 strain infection. Mixed-effects models were constructed in R statistical programming software with the lmerTest and emmeans packages. Ggpubr and ggplot2 packages were used to construct models and visualize results. The models took into consideration the fixed effects of the moist chamber and the random fruit identification number. To test for differences in cultivar resistance, we evaluated differences in lesion growth via a one-way analysis of variance, followed by a post hoc Tukey honestly significant difference test.

## Results

### Lesion growth in peaches, pears, and cherries

In peaches, pears, and cherries tested for wound infection with *P. niveus* CO7, spreading lesions were observed and lesion diameters (± standard error) were measured every other day (Fig. 2.1). In peaches, pears, and cherries, lesions developed at every inoculation point and grew rapidly over the course of 2 weeks. The average lesion diameters in peaches, sweet cherries, and sour cherries at day 2 were compared with average lesion diameters at day 8 to measure lesion development ($P < 2e\text{-}16$). Pear lesions at day 2 and 12 were similarly compared ($P < 2e\text{-}16$). At 2 days, average treatment lesion diameters were 3.76 ± 0.54 mm ($n = 26$) in Lori Anne peaches, 2.93 ± 0.25 mm ($n = 31$) in D'Anjou pears, and 13.32 ± 0.86 mm ($n = 40$) in Montmorency cherries. No lesions were detected in Kristin cherries at 2 days. At 8 days post-inoculation, average treatment lesion diameters were 30.01 ± 1.98 mm ($n = 14$) in Lori Anne peaches, 16.51 ± 0.45 mm ($n = 29$) in Montmorency

cherries, and 13.32 ± 0.86 (*n* = 18) in Kristin cherries. At 12 days, average treatment lesion diameter was 49.15 ± 0.41 mm (*n* = 30) in D'Anjou pears.

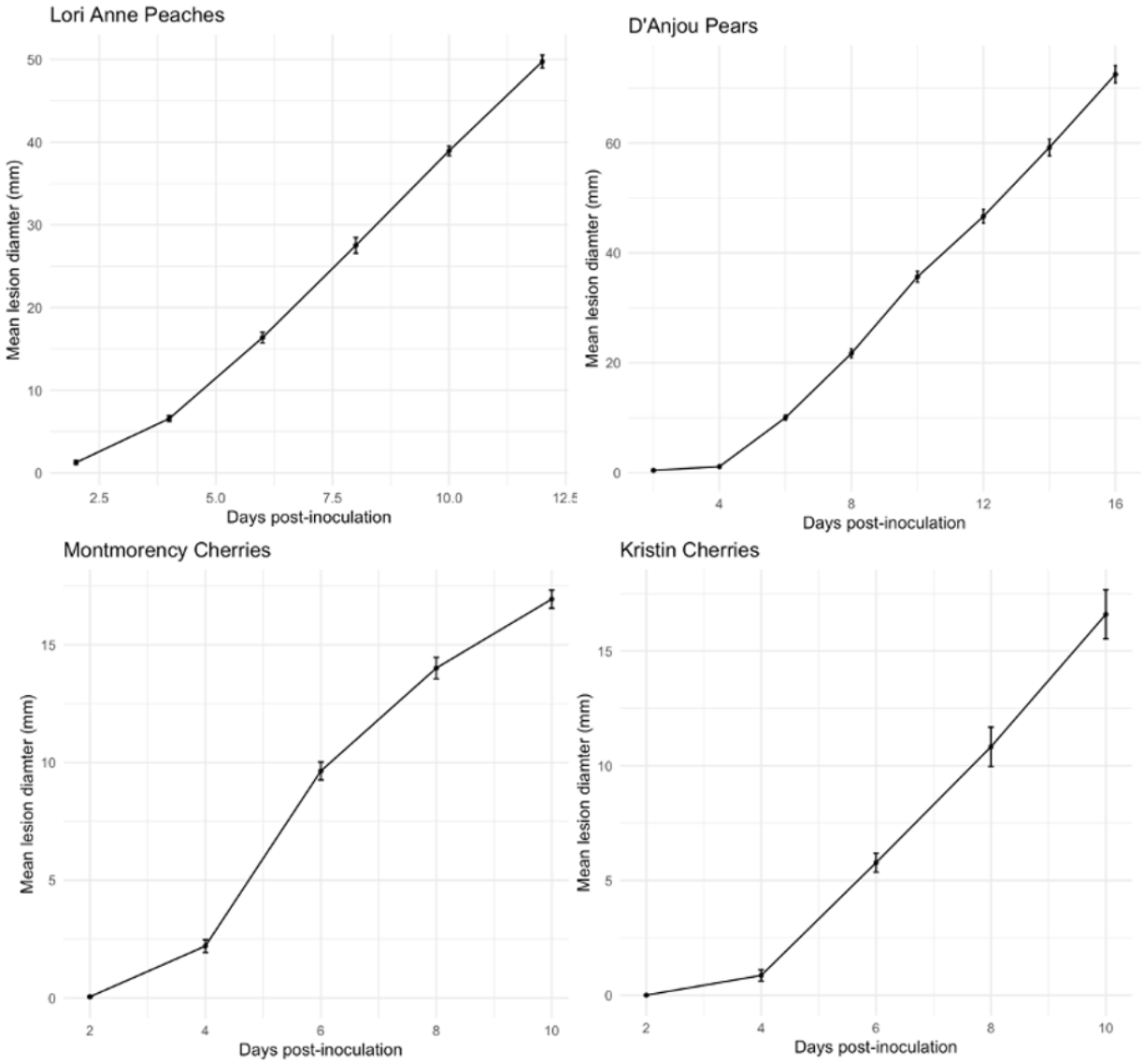


**Fig. 2.1. Lesion development on infected fruits.** Measurements of mean lesion diameter (± 1 SE) over the course of 2 to 3 weeks incubation (25˚C, ≥95% humidity) on Lori Anne peaches, D'Anjou pears, Montmorency sour cherries, and Kristin sweet cherries inoculated with *Paecilomyces niveus CO7.*

Any fruits that displayed symptoms of other postharvest infections during the

study, particularly Lori Anne peaches and Kristin sweet cherries, were removed from the experiment and further statistical analysis. This included three control peach fruits and one control pear fruit on days 12 and 14, respectively. Control peach and pear fruits showed little or no detectable lesion development, and no lesions developed on control sweet or sour cherries throughout the experiment.

**Characterization of Paecilomyces rot in rosaceous fruits**

In Lori Anne peaches, circular lesions developed at the site of inoculation (Fig. 2.2A). At 7 days post-inoculation, discoloration of the epidermis was light brown and the lesion was semifirm to the touch. Unlike the defined lesion borders found in Paecilomyces rot in apples, lesion borders in peaches were less sharp. White, branching surface hyphae could be seen in early stages of the disease (1-week postinoculation) and extended deeply into the fruit. Fruit flesh discoloration ranged from yellow brown to brown. Necrotic tissue was not easily separable from healthy tissue.

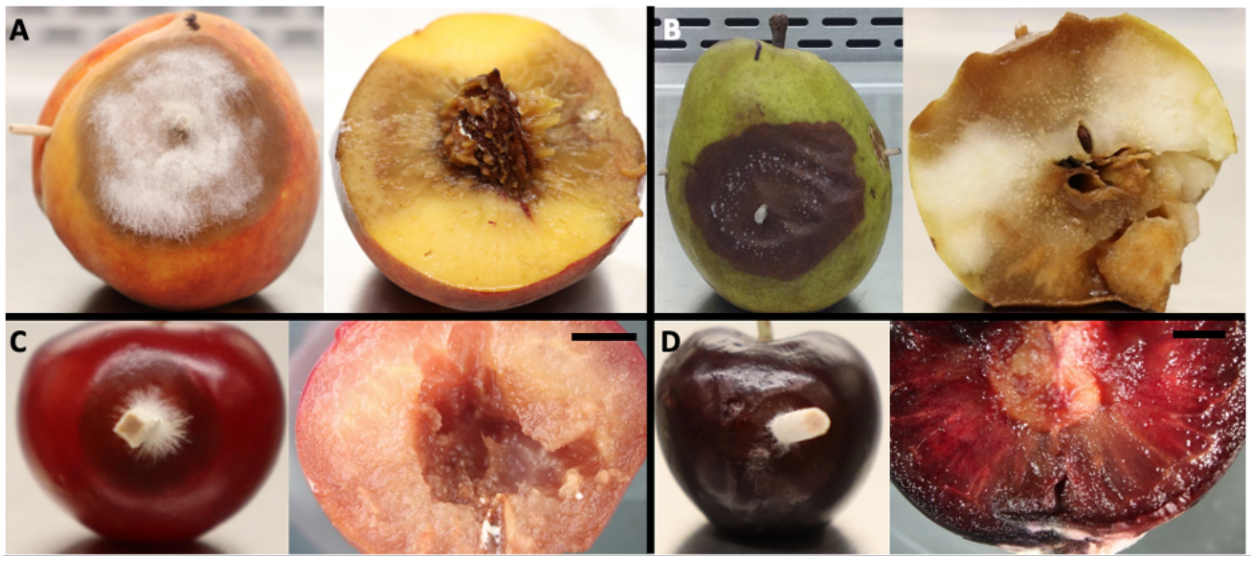


**Fig. 2.2. External and internal symptoms of infected rosaceous fruits.** Infected fruits, 2 weeks after inoculation with *Paecilomyces niveus* CO7 after incubation in dark, moist chambers (25˚C, ≥95% humidity) of A) Lori Anne peaches and B) D'Anjou pear, with cross-sections of each. Close-up view of external developing lesion and cross-section showing internal rot on C) Montmorency cherry and D) Kristin cherry. Scale bars are 3 mm.

D'Anjou pear infections resulted in dark brown lesions that were generally circular and even in discoloration (Fig. 2.2B). Lesions with sharp, distinct edges expanded radially from the point of inoculation. Unlike Paecilomyces rot in apples, which manifests as a hard rot that is spongy to the touch, necrotic tissue in pear fruits was soft, wet, and fragile. At 7 days postinoculation, fruit epidermis on the lesion surface became papery and brittle. At 14 days postinoculation, faint concentric rings of varying brown shades were first seen and tufts of white hyphae were observed at lenticels. Yellow-brown internal rot spread deeply into the fruit. Diseased tissue was easily separable from healthy tissue. Unique to pears, diseased flesh became translucent, which allowed clear visibility of pear sclereids.

In both Montmorency sour and Kristin sweet cherries (Fig. 2.2C and 2.2D), circular lesions developed at the site of inoculation. Epidermal discoloration was brown, sometimes light-brown close to the center of the lesion in sour cherry lesions

and yellow brown in sweet cherry lesions. Whereas lesion edges were distinct in sour cherries, lesion edges were less pronounced in sweet cherries. At 14 days postinoculation, dense tufts of mycelia appeared on the surface of sweet cherries. Rot extended deeply into the fruit in both sweet and sour cherries, resulting in an orange brown to brown flesh discoloration. Necrotic tissue was soft and watery and not easily separated from healthy tissue.

**Completing Koch's postulates**

For peaches, pears, and cherries newly tested for Paecilomyces rot, lesion surfaces of two individual symptomatic fruits were sanitized with 70% ethanol. Infected interior flesh was cultured for fungus, and the isolated culture was identified morphologically as *P. niveus* through its naked asci and white colonies that yellow with age when grown on potato dextrose agar (Samson et al. 2009). Reisolation and identification of *P. niveus* were also performed on apples and pears infected with *P. niveus* MC4 and 106-3.

**Apple cultivar-based susceptibility and *P. niveus* lesion size**

All four apple cultivars inoculated with *P. niveus*, Golden Delicious, Empire, Granny Smith, and Fuji (the latter three had not previously been tested for susceptibility to Paecilomyces rot), showed clear signs of lesion development 4 to 6 days postinoculation. Over the next 2 to 3 weeks, lesions continued to grow rapidly, and average diameters of treatment lesions were measured every other day. At 6 days postinoculation, lesion diameters (± standard error) were 6.44 ± 0.68 mm ($n$ = 31) on Empire, 9.30 ± 0.61 mm ($n$ = 28) on Fuji, 6.86 ± 0.47 mm ($n$ = 31) on Golden Delicious, and 10 ± 0.73 mm ($n$ = 29) on Granny Smith apples (Fig. 2.3A). At 20 days postinoculation, lesion diameters were 51.98 ± 4.28 mm ($n$ = 31) on Empire, 33.52 ±

1.62 mm (*n* = 26) on Fuji, 14.49 ± 1.36 mm (*n* = 31) on Golden Delicious, and 33.38 ± 2.57 mm (*n* = 29) on Granny Smith apples (Fig. 2.3A and 2.3B).

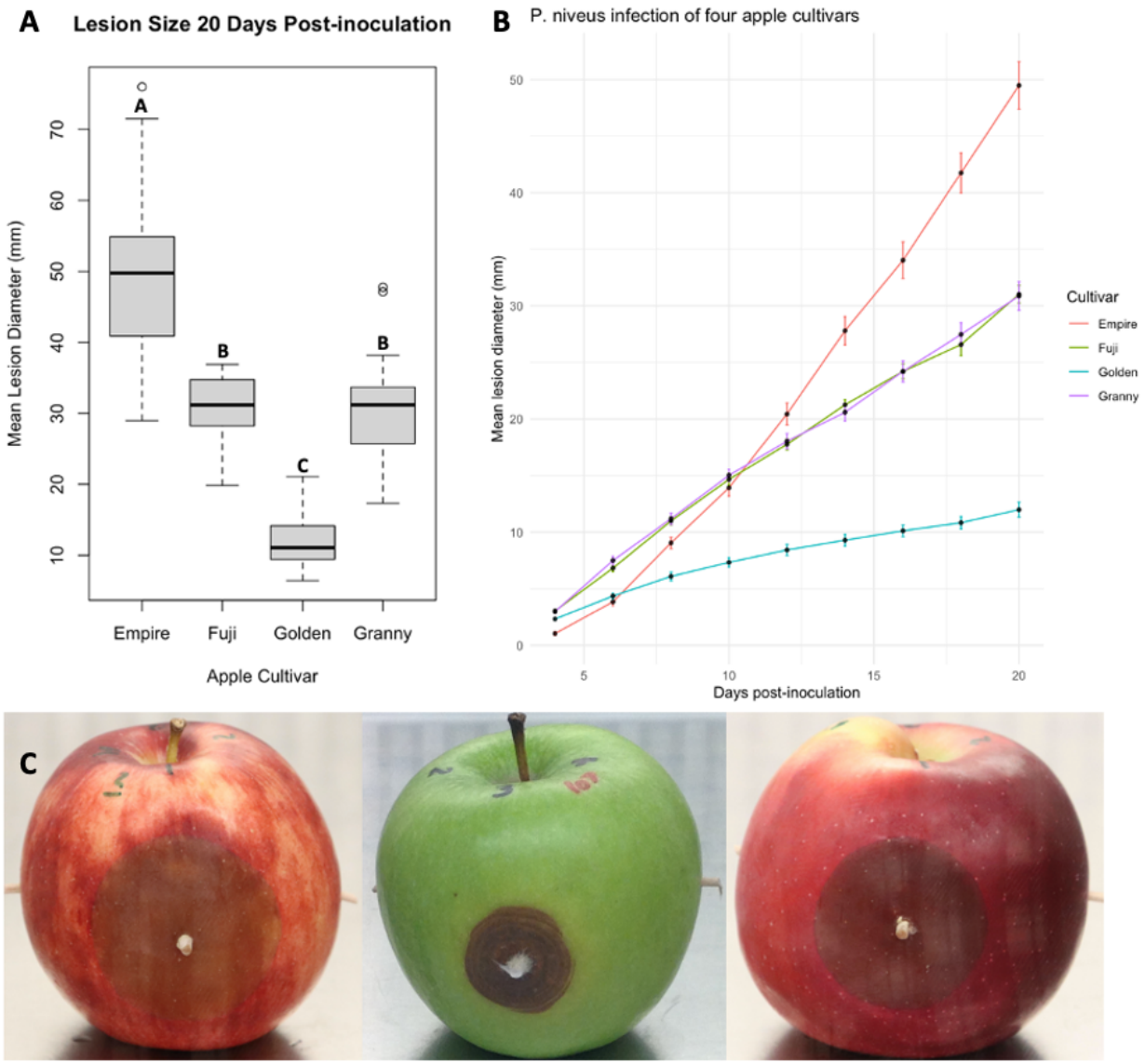


**Fig. 2.3. Comparison of infected apple cultivars.** A) Boxplot of mean lesion diameters on infected apple cultivars at 20 days postinoculation. Lettering represents statistical groups. B) 16-day progression of mean lesion diameter of four apple cultivars stored in the dark (25˚C, ≥95% humidity) infected with *Paecilomyces niveus* CO7. C) From left to right: infected Fuji, Granny Smith, and Empire apples after two weeks postinoculation.

### Pathogenicity of *P. fulvus* and *P. variotii* in apple fruits

For two closely related *Paecilomyces* species, *P. fulvus* and *P. variotii*, no noticeable symptoms developed in inoculated Empire apples after 3 weeks. Apple fruits remained intact, and at the end of the 3-week period they were cut in half for further observation. The wound created by fungus-infested toothpick insertion remained identical to the control toothpick wound, and no rot or lesions developed. There was no evidence these fungi were able to infect apples.

### Patulin production by *P. niveus* in wounded fruits

No patulin was detected in pureed, mock-inoculated peaches, pears, or sweet cherries (detection limit >10 ppb); pureed mock-inoculated sour cherries did contain patulin, at 250 ppb. Puree from inoculated fruits contained significantly higher concentrations of patulin: 43,945 ppb for sour cherries and 5,817 ppb for sweet cherries. Patulin levels in pureed peaches and pears were determined to be >50,000 ppb.

## Discussion

Rosaceae is a large plant family that includes economically important fruits such as peaches, pears, cherries, strawberries, and apples, the latter being the most consumed fruit in the United States (U.S. Apple Association 2021). Each year the United States produces 240 million bushels of apples, a third of which are processed into juices and other apple products (U.S. Apple Association 2021). In 2020 and 2021, the United States also produced 658,000 tons of pears, 720,000 tons of fresh peaches and nectarines, and 383,000 tons of cherries (USDA-FAS 2020a, b). Postharvest diseases are responsible for a 20 to 25% loss of harvested fruits and

vegetables in developed countries, resulting in 10 to 15% losses in apples during storage, and even more in developing countries (Jurick and Cox 2016; Nunes 2012). A 2018 study reported that 92% of juice manufacturers surveyed have experienced fungal spoilage in finished products (Snyder and Worobo 2018).

*Paecilomyces* is a notorious fungal genus that includes both important food spoiling fungi (*P. niveus*, *P. fulvus*, *P. variotii*) and emerging opportunistic pathogens of humans (*P. variotii* and *P. formosus*) (Heshmatnia et al. 2017). Unlike common postharvest pathogens of apples, *P. niveus* uniquely threatens juice production because it not only produces the FDA-regulated mycotoxin patulin but also infects apples as a postharvest disease and persists through pasteurization and fruit processing. Spoilage by *P. niveus* directly leads to contamination of processed fruit products with patulin and potentially other mycotoxins, including byssochlamic acid, byssochlamysol, and mycophenolic acid (Houbraken et al. 2006).

Data presented in this study suggest that *P. niveus*, a patulin-producing (Biango-Daniels et al. 2019; Frisvad 2018), heat-resistant mold, can also be a wound-infecting pathogen of rosaceous fruits other than apples. *P. niveus* strain CO7, isolated from decaying apples, was able to grow, reproduce, and cause symptoms in other fruits including pears, peaches, and both sweet and sour cherries. Two additional *P. niveus* strains, MC4 and 106-3, were also confirmed through Koch's postulates to be able to infect and cause symptoms in Empire, Fuji, and Granny Smith apples and D'Anjou pears. Preliminary data suggest that *P. niveus* can also rapidly infect strawberries and raspberries, but these infections often co-occurred with other postharvest diseases, complicating analysis. Paecilomyces rot manifested similarly across all these fruits, causing lesions and internal rot at wound sites, mostly consistent with the original description of the disease (Biango-Daniels and Hodge 2018). However, key differences in symptom development included profuse mycelial

growth on the surface of peaches, dense tufts of mycelia on the surface of cherries, and notable disintegration of fruit flesh in pears. Disintegration from *P. niveus* infection occurred at a faster rate in peach and pear fruits than it did in apple fruits. In addition, all apple cultivars inoculated with *P. niveus* were susceptible to Paecilomyces rot. Interestingly, the rate of lesion growth in inoculated Golden Delicious apples was slower than previously observed (Biango-Daniels and Hodge 2018), suggesting that external factors like sugar levels, acidity, phenolic compounds, or water content may influence lesion development. Patulin quantification of fruits inoculated with *P. niveus* CO7 showed that the fungus is fully capable of producing large amounts of patulin in infected fruits. Notably, patulin content in infected peaches and pears was ≥1,000 times higher than the FDA limit of 50 ppb. We also observed that sour cherry puree from uninoculated fruits contained patulin at 250 ppb. This finding may reflect infection by an undetected patulin-producing fungus but is still significantly lower than that of pureed infected sour cherries: 43,945 ppb. We also tested the infection potential of two close relatives of *P. niveus: P. fulvus* and *P. variotii*. Neither was observed to cause disease, despite their close phylogenetic relationship with *P. niveus*, and their status as common heat-resistant spoilage fungi of processed fruit products. These data provide a contrast to the infectious abilities of *P. niveus*, a pathogen that can grow and reproduce in living fruits.

Our findings have implications for fruit growers and juice producers as they expand the known host range of Paecilomyces rot, revealing that diseased peaches, pears, and cherries may harbor a significant amount of *P. niveus* spoilage inoculum. This finding is problematic because *P. niveus* ascospores tolerate high heat and may survive certain thermal processes, especially when suspended in fruit products such as strawberry puree (Evelyn and Silva 2015), pineapple juice (da Rocha Ferreira et al. 2009), canned tomato paste (Kotzekidou 1997), apple, and cranberry juice (Palou

et al. 1998). This study disproves the prevailing idea that *P. niveus* contamination results solely from environmental sources and suggests that diseased fruits can be a source of spoilage inoculum. Although the sporadic nature of food spoilage makes it difficult to demonstrate this route of contamination in real-world processes, our results highlight the risks of patulin contamination from even small quantities of *P. niveus*-infected fruits in food processing. Although *P. niveus* spoilage and patulin contamination are commonly associated with apples and apple products, our study suggests a need for vigilance about contamination in other rosaceous fruit products. Future work should assess and quantify the incidence of natural infection of Paecilomyces rot in fruits and examine agricultural practices and risk factors that may contribute to *P. niveus* spoilage of foods. To assess risks presented by *P. niveus* to the food industry, next steps should include assessing the prevalence of the fungus on farms and in food processing plants, understanding interactions of *P. niveus* with other postharvest pathogens that we speculate may mask infection, and adjusting food processing protocols to reliably kill heat-resistant spores that can lead to spoilage and patulin production in processed fruit products.

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# CHAPTER 3

## PAECILOMYCES NIVEUS AS A WOUND-INFECTING PATHOGEN OF CITRUS: ORANGES AND CLEMENTINES[2]

### Introduction

The United States is the fourth-largest producer of oranges and produced over 330,000 tons of orange juice in 2019 (USDA-FAS 2020). Heat-resistant molds are an important source of losses in juice production. Santos et al. (2018) found that 46.7% of juice samples from one processing line were contaminated with living heat-resistant molds, including ascospores of *Paecilomyces niveus*, which contaminated 13.3% of juice batches. These heat-resistant molds were often assumed to be soil-borne contaminants. However, we recently demonstrated that *P. niveus* is a postharvest pathogen of apples that causes Paecilomyces rot (Biango-Daniels and Hodge, 2018a; Khokhar et al., 2019) and can be introduced to processed fruit products via infected fruits (Biango-Daniels et al., 2019).

*Paecilomyces niveus* is a white mold most often found in either soils or fruit products. As a wound-infecting pathogen, it spreads within the flesh of the fruit, producing great numbers of heat resistant ascospores that can germinate and grow in low pH environments (Biango-Daniels and Hodge, 2018a; Butz et al., 1995). It poses a further threat to juice production because it produces patulin, a federally regulated mycotoxin with neurotoxic and carcinogenic properties, and ascospores resistant to typical heat-processing approaches (Biango-Daniels et al., 2019; Puel et al. 2010). Its high incidence in orange juice led us to hypothesize that oranges, too, may be

[2] Wang, T.W., Hodge, K.T., 2020. Paecilomyces niveus as a Wound-Infecting Pathogen of Citrus: Oranges and Clementines. Plant Health Prog. 21, 333–334. https://doi.org/10.1094/PHP-05-20-0044-BR

susceptible to Paecilomyces rot, and that fruit infection may be an overlooked source of orange juice contamination and spoilage.

## Methods

To determine the ability of *P. niveus* to infect citrus fruits, we wounded and inoculated ripe, detached orange (*Citrus* x *sinensis*, Navel cultivar) and clementine fruits (*Citrus* x *clementina*) and watched for developing signs and symptoms. *Paecilomyces niveus* isolate CO7 (Biango-Daniels et al., 2018b), a patulin-producing strain isolated from a decaying apple from Ithaca NY, was grown on potato dextrose agar from hyphal tips for 14 days (Biango-Daniels et al., 2018a). The identity of isolate CO7 was first determined based on the ITS and beta-tubulin regions, and the fungus was later fully characterized by full genome sequencing (Biango-Daniels et al., 2018b; genome available at NCBI). Toothpick halves used to wound and inoculate the fruits were tyndallized via two autoclave cycles, the first in water and the second in potato dextrose broth 24 h later to kill germinating heat-resistant spores, following the protocol of Biango-Daniels and Hodge (2018a) and van der Walt et al. (2010). Sterile toothpick halves were placed on potato dextrose agar inoculated with *P. niveus* until completely overgrown. Twenty orange fruits and 36 clementine fruits were purchased from a local supermarket and washed in 70% ethanol for 30 s, 1% NaClO for 2 min, 70% ethanol for 15 s, then left to dry in a biological safety cabinet. Each fruit was wounded four times (1 cm deep), twice by inserting two infested toothpick halves and twice by inserting two control toothpick halves, which were treated similarly but not infested (Biango-Daniels and Hodge, 2018). Each fruit received both treatment and control toothpicks, on opposite ends of the fruit, in order to control for individual fruit response. The fruits were placed in moist chambers and incubated at 25˚C in the dark for 2 to 3 weeks. A digital caliper was used to estimate the

expansion rate of lesions by measuring the horizontal and vertical diameters every other day. Four fruits with signs or symptoms typical of other common postharvest diseases, like blue sporulation or soft rot, were discarded. After two weeks, diseased citrus fruits were surface sterilized using 95% ethanol, and a fungus was isolated from tissue within the lesions of a sampling of three infected individual orange and clementine fruits. All isolates were confirmed to be *P. niveus* by colony and microscopic characteristics including color, quick growth on potato dextrose agar, and abundant production of spherical naked asci (Samson et al., 2009). To complete Koch's postulates, these isolates were used to infect an additional six oranges and six clementines using the same procedures as above.

## Results

All inoculated oranges showed lesion development by day five (average lesion diameter: 10 mm). By six days, over 85% of treated toothpicks on clementines resulted in lesions (average lesion diameter: 9 mm), and at ten days, all treatment toothpicks in clementines resulted in lesions (average lesion diameter: 11.6 mm). 95% of the control toothpicks used in oranges and 97.2% of the control toothpicks used in clementines resulted in no detectable symptom development. 5% of control toothpicks used in oranges and 2.8% in clementines showed small diameter brown lesion development towards the end of the experiment, likely due to cross-contamination with spores from treatment toothpicks. Oranges and clementines infected using the reisolated pathogen to test Koch's postulates displayed identical lesions. As an additional control, flesh from three uninoculated oranges and clementines was plated on potato dextrose agar and yielded no *P. niveus* growth.

Developing lesions were tough, hard, leathery, tan to dark brown circles that radiated from the point of wounding (Fig. 3.1). Occasionally, concentric circles of

varying shades of brown appeared around the point of wounding. Lesion borders were sharply defined. Fruit flesh was found to be partially detached from the pith directly under the lesion. Sectioning infected fruits revealed that lesions extended deeply into the flesh and the necrotic tissue contained mycelium and asci of *P. niveus*. In oranges, the center of the fruit showed signs of rot and the interface between the orange flesh and fungus developed a dark red-orange hue.

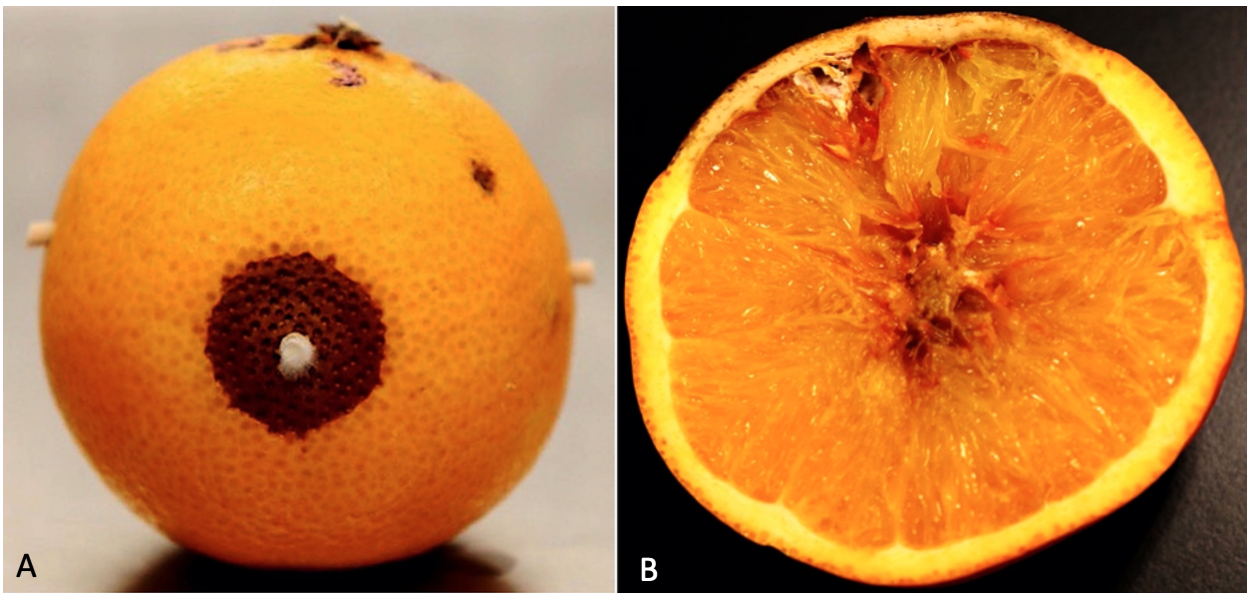


**Figure 3.1. Diseased citrus fruits.** A) Clementine (Citrus x clementina) infected with *Paecilomyces niveus*, 14 days postinoculation, showing a typical lesion. B) Orange (Citrus x sinensis) infected with *P. niveus* 20 days postinoculation, showing dark discoloration of internal flesh.

## Discussion

This is the first report of the ability of the mycotoxigenic mold *Paecilomyces niveus* to grow and sporulate in wounded *Citrus* fruits. Because *P. niveus* ascospores are resistant to heat treatment and can grow in low oxygen environments, understanding the sources of *P. niveus* contamination of orange juice is important to prevent spoilage and improve food safety. Future work should address effective

processing temperatures for control of this spoilage fungus in citrus products. This study confirms our hypothesis that wounded citrus fruits can be infected by *Paecilomyces niveus* and suggests a potential route of *P. niveus* contamination of orange juice: through infected fruits.

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# CHAPTER 4

# EVALUATING FARM-TO-FOOD ROUTES OF ENTRY FOR PAECILOMYCES NIVEUS, A MYCOTOXIGENIC FRUIT PATHOGEN AND FOOD SPOILING MOLD[3]


## Abstract

Ascospores of the mycotoxigenic food spoilage mold *Paecilomyces niveus* can survive some pasteurization temperatures, spoil fruit products, then contaminate them with patulin, an FDA-regulated mycotoxin. The fungus was shown to be a pathogen of apples, revealing a potential new route of spoilage inoculum entry into apple products – through infected fruits. The description of this postharvest disease renews interest and urgency to evaluate potential sources of *P. niveus*. In order to determine spoilage inoculum levels from infected fruits, fruits arising from apple blossoms sprayed with a *P. niveus* spore suspension, soil, and apple cider, we designed a new robust and culture-independent detection method. Species-specific primers were generated (PnPATf/r) based on the *patK* gene, encoding a 6-methylsalicylic acid synthase, in *P. niveus*, for use in a rapid qPCR assay. Primer specificity was validated using 24 diverse isolates of *P. niveus* and 16 fungi, including other important food spoilers and fruit pathogenic fungi. The threshold for detection of qPCR was 14.1 genome equivalents. Our results show that strawberry fruits are susceptible to Paecilomyces rot. In addition, we demonstrate that a pre-treatment of 70˚C with severe bead-beating is a viable strategy for cell lysis and DNA extraction from *P. niveus* ascospores. Preventing *P. niveus* from entering food systems requires


[3] Wang, Tristan W., David A. Strickland, Yasmin Haredy, Kerik D. Cox, and Kathie T. Hodge. 2023. Evaluating farm-to-food routes of entry for Paecilomyces niveus, a mycotoxigenic fruit pathogen and food spoiling mold. Manuscript in Preparation. Preprint: https://doi.org/10.1101/2023.02.18.529073

a robust detection method to effectively determine sources of *P. niveus* spoilage and disease inoculum. Results from this study may help fruit producers better assess risk factors concerning spoilage and patulin contamination by this food spoiling fungus.

## Introduction

*Paecilomyces* species are among the most widely encountered heat resistant molds (HRM) in processed fruit products (Snyder et al., 2019). *Paecilomyces niveus* Stolk & Samson (*Byssochlamys nivea* Westling) in particular can not only produce ascospores that can survive lower temperatures of pasteurization, but can also contaminate food products with patulin, an FDA-regulated mycotoxin (Frisvad 2018; Affairs 2020). This ascomycete fungus has recently been described to cause the fruit disease Paecilomyces rot, and is able to infect and grow in apples (Biango-Daniels & Hodge, 2018; Wang & Hodge, 2022). Processing fruits infected with *P. niveus* can lead to product infested with *P. niveus* ascospores and juice contaminated with patulin (Biango-Daniels et al., 2019). Infected fruits have not generally been considered significant sources of HRM spoilage inoculum, and the description of Paecilomyces rot raises urgent questions about the disease biology of *P. niveus*, and its potential routes into foods.

*Paecilomyces niveus* is an extremotolerant fungus that can grow in environments with low oxygen, high acid levels, and low water activity (Affairs, 2020; Silva & Evelyn, 2020). Spores that persist through processing can germinate and grow, producing a variety of mycotoxins including byssochlamic acid, byssochlamysol, and mycophenolic acid in addition to patulin (Mori et al., 2003, 2003; Puel et al., 2005). Previous work has explored strategies of reducing *P. niveus* spoilage inoculum in fruit products, employing methods including ultraviolet light, hydrostatic pressure, and thermal treatment (Ferreira et al., 2009; Menezes et al.,

2020; Palou et al., 1998). However, intense processing and treatment may degrade food quality and nutrition (Van Boekel, 2008). Detecting and preventing *P. niveus* from entering food systems is a desirable strategy for reducing spoilage and patulin contamination by this fungus while also preserving food quality.

It has been assumed that *P. niveus* spoilage inoculum originates from environmental sources and previous surveys confirm that the fungus is a common soil microbe (Biango-Daniels & Hodge, 2018; Put, 1964; Tournas, 1994). The fungus has been found in various fruit products like apple juices (Santos et al., 2018) and in naturally infected fruits (Khokhar et al., 2019). Traditional culturing and morphological identification are laborious and time-consuming and require taxonomic expertise. In addition, the sporadic nature of food spoilage presents a challenge for spoilage prevention strategies that rely on growing out food spoiling agents. We sought to develop a robust detection protocol to identify and quantify *P. niveus* DNA in industrially relevant environments, and to deploy our protocol to evaluate hypotheses about routes by which P. niveus enters fruits and foods

We developed and validated a highly specific primer pair based on the single copy *patK* gene encoding a 6-methylsalicylic acid synthase, present in only a handful of fungi, to detect *P. niveus* in fruits, soils, and foods (Banani et al., 2016). We then applied the protocol to potential sources of spoilage and disease inoculum including: 1) soil and apple tissue infested with ascospores, and 2) infected apple and orange fruits. We also deployed the protocol in hypothesis testing about disease biology: Completing Koch's postulates to show that *P. niveus* is a pathogen of strawberry fruits, and investigating the potential role of blossom infection in *P. niveus* infection of apple fruits. A reliable detection method for *P. niveus* can contribute to strategies for reducing spoilage and contamination in downstream food processing and help answer questions about disease biology and implications for food safety of

Paecilomyces rot.

## Materials and methods

### Specific primers based on the patulin polyketide synthase gene

Low-coverage genomes of 23 isolates of *P. niveus*, sampled from across New York State, were aligned to the genome of *P. niveus* isolate CO7 for visualization of polymorphic sites within the *patK* gene, a component of the patulin secondary metabolite gene cluster (Biango-Daniels et al., 2018). Primers PnPATf and PnPATr (Table 4.1) were selected from appropriate regions using PrimerQuest software from Integrated DNA Technologies. Primer specificity was validated in silico using Primer-BLAST searches on related fungal species (Fig. 4.1).

**Table 4.1. Primers developed in this study for qPCR assays.**

| PnPATf/r | Primer nucleotide sequences | Tm (˚C) | Product size |
|---|---|---|---|
| Forward primer | 5′-GCAAGCCAAGACCCTTCAGT-3′ | 60.54 | 155 bp |
| Reverse primer | 5′-CGTCGAGTCCCTTTACAGCA-3′ | 59.76 | |

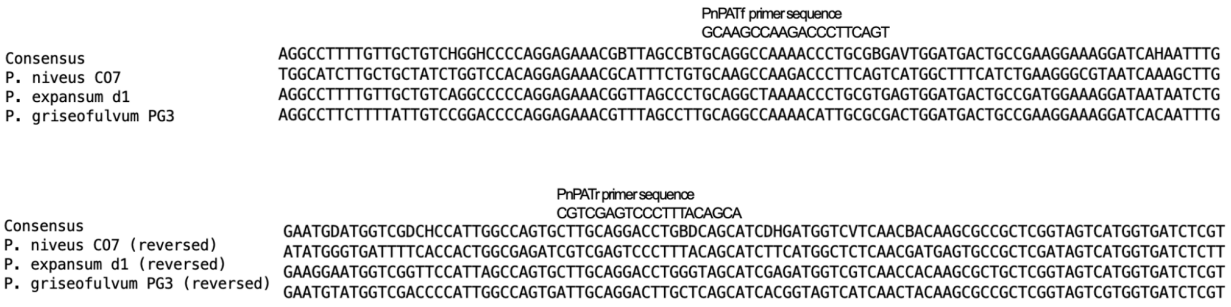


**Figure 4.1. Nucleotide sequence alignment of portion of *patK* gene with corresponding PnPATf/r primer sequences of *P. niveus*, *Penicillium expansum*, and *Pen. griseofulvum*.**

**Culture preparation and DNA extraction**

Fungal isolates used to evaluate primer specificity (Tables 4.1 and 4.2) were obtained from laboratory culture collections of the Hodge Lab at Cornell University and the USDA-Agricultural Research Service (NRRL) culture collection. Fungi were cultured on sterile cellophane overlaid on Potato Dextrose Agar (PDA; Criterion) at 25˚C for 2 weeks in the dark, with the exception of *Aspergillus flavus* AF-36, which was grown at 33˚C. Genomic DNA was isolated using the DNeasy PowerSoil Pro Kit (Qiagen) following the manufacturer's instructions.

**Ascospore extraction and soil and cider infestation**

Spores for the qPCR assay were harvested from sterile cellophane overlaid on PDA. After four weeks, the surface of the colony was covered with 2mL of sterile deionized water and agitated to release asci and ascospores. To remove hyphal fragments, the spore suspension was filtered through eight layers of cheesecloth and Whatman No. 1 filter paper. A haemocytometer was used to quantify spore concentration by counting both free ascospores and 8-spored asci. Spore suspension underwent centrifugation to increase spore concentration if needed.

Soil samples for the qPCR assay were collected from an apple orchard near Ithaca, New York, and apple cider was purchased from a local supermarket. $10^7$ ascospores in 10 µl of water was used to infest 100 mg samples of orchard soil and 100 µl aliquots of apple cider. Negative controls were treated similarly but with 10 µl sterile water. Our DNA extraction method was modified for efficient *P. niveus* ascospore lysis: Microcentrifuge tubes containing soil or cider samples infested with *P. niveus* ascospores underwent a 2-hour heat treatment at 70˚C in a water bath. Heat-treatment was immediately followed by alternating periods of intense bead-

beating using a mini-beadbeater (Biospec Products, Bartlesville, OK) (5 x 60 second intervals at 50 RPMx100) alternating with 2 minutes of rest to prevent overheating prior to DNA extraction with the DNeasy PowerSoil Pro Kit.

**Fruit infection assays and *Paecilomyces niveus* quantification**

For qPCR detection of *P. niveus* in infected fruits, Fuji apples and California Navel oranges purchased from a local supermarket were sanitized and then infected using infested toothpicks according to the method outlined by Biango-Daniels and Hodge (2018). Albion strawberry fruits obtained from Cornell Orchards were sanitized similarly. The strawberry fruits were wounded with a pipette tip about 1 cm deep and $10^5$ ascospores suspended in 10 µl water were pipetted into the wounds. All fruits were kept in moist chambers in the dark at 25˚C. Roughly 400 mg of infected fruit flesh was extracted from the resulting lesions of infected fruits, 2 cm away from the point of inoculation. Diseased flesh was extracted from apple and orange fruits two weeks post-inoculation and from strawberry fruits one-week post-inoculation. Fruit flesh was extracted directly from apple and strawberry lesions, while the peel was removed before fruit flesh extraction of orange fruits. DNA was quantified using qPCR for DNA extracts from three Fuji apples, three California Navel oranges, and four Albion strawberry fruits. This process was repeated for control fruit samples that had been mock-inoculated with sterile toothpicks under the same sanitization, incubation, and extraction protocol (Biango-Daniels & Hodge, 2018). Because this is the first study to investigate strawberry susceptibility to Paecilomyces rot, disease-causing ability was evaluated based on satisfaction of Koch's postulates.

**Apple blossom inoculations**

To investigate the possibility that blossom infection may be one route by which *Paecilomyces niveus* invades developing apples, *P. niveus* ascospore suspension was sprayed onto apple blossoms at various phenological stages: 10% bloom, 100% bloom, and petal fall. Developing fruits were later tested for presence or absence of *P. niveus* using our qPCR assay.

A field trial was conducted at a Cornell AgriTech research orchard in Geneva, NY in 2020 and 2021. The experiment was performed in a high-density orchard of dwarf apple trees ('NY109' ('Firecracker') on G.935 rootstocks) that were established in 2016. Trees were planted at approximately 2 meter in-row spacing and trellised to three wires. The planting received no pesticides throughout the trial years.

Treatments were made on 8 single-tree replications arranged in a randomized complete block design. Treatments were made to runoff, allowing spore solution to drip after application, with a 4-gallon HDPE backpack sprayer (Solo Inc., Newport News, VA), with application focus on the blossoms of each tree. Applications consisted of inoculum suspended in water amended with 0.02% Tween-80 to avoid spore clumping, with a *P. niveus* spore concentration of $1 \times 10^5$ asci plus ascospores per mL. Prior to application, inoculum was produced on PDA as described in Section 2.2. In 2020, ascospore suspensions were applied on 20 May (10% bloom), 27 May (100% bloom), and 2 June (petal fall). On September 28, 2020, half the fruits were harvested and stored at room temperature to allow for rot development, and the other half was frozen for later evaluation. In 2021, ascospore suspensions were applied on 6 May (10% bloom), 10 May (100% bloom), and 17 May (petal fall). On September 14, 2021, apple fruits were harvested and stored under the same conditions as in the previous year.

Three batches of five apples were blended for each set of apples: 10% bloom, 100% bloom, petal fall, and uninoculated apples. This process was repeated for both the 2020 and the 2021 growing season. 400 mg of apple puree of each homogenized batch underwent DNA extraction for a total of 24 samples.

**PCR and qPCR amplification conditions**

End-point PCR reaction mixture contained 5 µl of 5X Q5 reaction buffer, 0.5 µl 10 mM dNTPs, 1.25 µl 10 µM of each primer, 1.25 µl template DNA, 0.25 µl of Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA), and 15.5 µl nuclease-free water. PCR amplification was carried out on a PTC-200 Peltier Thermal Cycler (BioRad, Toronto, Ontario). The initial denaturation occurred at 98˚C for 30s, followed by 26 cycles of denaturation at 98˚C for 10 s, annealing at 64˚C for 30 s, and extension at 72˚C for 45 s. A final extension at 72˚C occurred for 2 minutes. ITS4 and ITS5 Primers amplifying the internal transcribed spacer were used as a positive control (White et al. 1990). PCR products were then electrophoresed with TBE in 1.0% agarose gels (7V/cm, 60min) stained with Gel-Red (Biotium Inc., Hayward, USA).

*P. niveus* DNA from extracted fruit, soil, and food was quantified using the CFX Connect Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA). qPCR reaction mixtures contained 25 µl 2x Power SYBR Green PCR Master Mix (Applied Biosystems, Madrid, Spain), 1 µl 10 µM PnPATf primer, 1 µl 10 µM PnPATr primer, 10 µl of eluted fungal DNA, and 13 µl deionized water (50 µl total). qPCR was performed on the CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA) programmed to hold at 95˚C for 10 minutes and then to complete 40 cycles of 95˚C for 15 s (denaturation), 60˚C for 30 s (annealing), and 72˚C for 45 s (extension).

Reactions were done in triplicate and results were measured by quantification cycle (Cq).

### Standard curve

A ten-fold serial dilution of *P. niveus* CO7 DNA ranging from 5.5 to 5.5 x $10^{-4}$ ng/µl was generated and quantified using a Qubit 4 Fluorometer (Invitrogen; Thermo Fisher). These DNA concentrations were used to generate a standard curve for qPCR by plotting genome equivalents against Cq values.

## Results

### Testing the PnPATf and PnPATr primer pair for species specificity

End-point PCR was used to validate primer specificity in vitro for a set of 19 fungal species and 24 isolates of *Paecilomyces niveus* (Table 4.2). These fungal isolates include apple pathogens (*Botrytis obtusa*, *Mucor piriformis*, *Penicillium* spp., *Venturia inaequalis*) and a citrus pathogen (*Penicillium italicum*). Primers were also tested on a variety of food spoilage fungi (*Aspergillus* spp., *Neosartorya* spp., *Talaromyces* spp.) and three closely related species (*Thermoascus crustaceou*s, *Paecilomyces fulvus*, *Paecilomyces variotii*). The 155 bp PCR product amplified by primers PnPATf and PnPATr was observed only in reactions including any of the 24 *P. niveus* isolates, as confirmed by gel electrophoresis.

**Table 4.2. 16 fungal species and 24 *Paecilomyces niveus* isolates used in validation of primers PnPATf and PnPATr, and presence or absence of PCR product.**

| Species | Strain | Source of isolation | PCR product |
|---|---|---|---|
| *Aspergillus flavus** | AF-36 | Cotton fields, AZ | - |
| *Aspergillus terreus** | NRRL 269 | Food, fruit (dates), CA | - |
| *Botrytis obtusa*** | 97017 | Unknown substrate | - |
| *Mucor piriformis*** | NRRL 3637 | Soil, Austria | - |
| *Neosartorya fisherii** | 84-142 | Unknown substrate | - |
| *Neosartorya glabra** | NRRL 3434 | Soil, Japan | - |
| *Neosartorya lanciniosa** | NRRL 4076 | Hay, England | - |
| *Neosartorya spinosa** | NRRL 32569 | Soil, Brazil | - |
| *Talaromyces bacillisporus** | NRRL 1025 | Begonia leaf, NY | - |
| *Talaromyces islandicus** | NRRL 1036 | Unknown substrate, South Africa | - |
| *Paecilomyces fulvus** | 7 | Spoiled food | - |
| *Paecilomyces niveus* | 109-21 | Orchard soil, NY | + |
| | 107-1 | Orchard soil, NY | + |
| | MC4 | Residential soil, NY | + |
| | CO7 | Culled apples, NY | + |
| | 102-1 | Research station soil, NY | + |
| | 100-10 | Orchard soil, NY | + |
| | CF6 | Corn field soil, NY | + |
| | 108-11 | Orchard soil, NY | + |
| | CFO13 | Corn field soil, NY | + |
| | CC8 | Compost, NY | + |
| | 111-1 | Orchard soil, NY | + |
| | 112-22 | Soil, NY | + |
| | BY11 | Barn yard Soil, NY | + |
| | 146-311 | Residential soil, NY | + |
| | EP1 | Rhizosphere soil, unknown location | + |
| | 141-11 | Farm soil, NY | + |
| | 104-22 | Orchard soil, NY | + |
| | 110-3 | Orchard soil, NY | + |
| | 125-31 | Farm market soil, NY | + |
| | AF01 | Alfalfa field soil, NY | + |
| | 101-3 | Research farm soil, NY | + |
| | 106-3 | Orchard soil, NY | + |
| | KRA4 | Food facility, unknown location | + |
| | 140-11 | Farm soil, NY | + |
| *Paecilomyces variotii** | 103-2 | Soil, NY | - |
| *Penicillium expansum*** | 94222 | Unknown substrate | - |
| *Penicllium griseofulvum*** | NRRL 2159A | Unknown substrate | - |
| *Penicillium carneum*** | NRRL 25170 | Unknown substrate | - |
| *Penicillium italicum*** | KH1 | Diseased clementine, NY | - |

* = Food spoiling agent, ** = Fruit pathogen

**Determining sensitivity and amplification efficiency of SYBR green qPCR assay**

The detection limit of the qPCR method was established by testing a dilution series of *Paecilomyces niveus* isolate CO7 genomic DNA. Concentrations ranging from 5.5 ng/µl to 0.55 pg/µl were tested in triplicate. The qPCR assay was able to detect *P. niveus* DNA when there were only 5.5 pg of *P. niveus* DNA (about 14.1 genome equivalents) in the 50 µl reaction (Cq value of 31.46 ± .41) (Figure 4.2). The amplification efficiency of the qPCR method was 96.48% based on the equation $\varepsilon = 100 \times (10^{-1/slope} - 1)$ (Broeders et al., 2014). There was no detection in any of the negative controls.

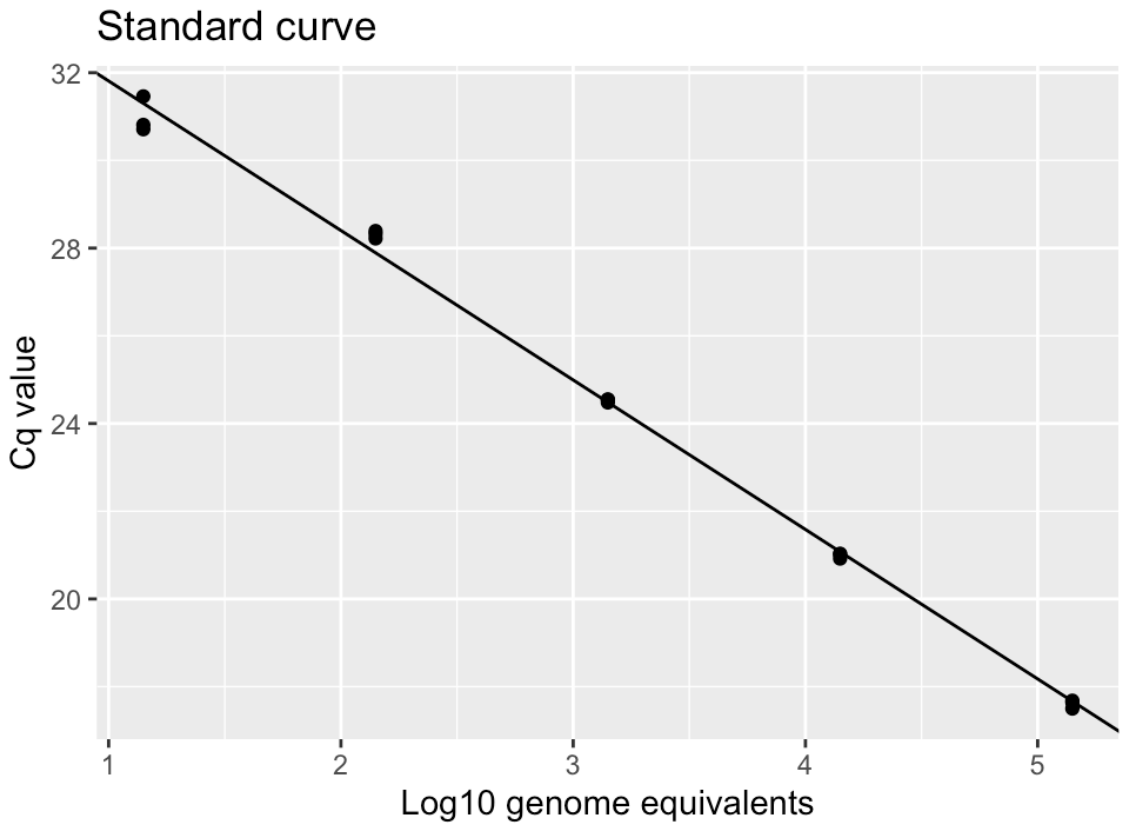


**Figure 4.2. qPCR amplification of *Paecilomyces niveus* DNA.** Concentrations range from 5.5 ng/µl to 0.55 pg/µl, with $\log_{10}$ values of *P. niveus* genome equivalents plotted against quantification cycle (Cq). Standard equation: Cq = -3.4093x + 35.2214, $R^2$ = .9965 (efficiency = 96.48%)

**Quantification of Paecilomyces niveus DNA in fruit, soil, and cider**

The qPCR assay was applied to eluted DNA extracted from developing lesions of fruits infected with *P. niveus*. DNA was extracted from 3 infected apple fruits, 3 infected orange fruits, and 4 infected strawberry fruits. Control fruits were treated similarly but without the presence of *P. niveus*. *Paecilomyces niveus* DNA was detected in all infected apple and orange fruits and in three of the four strawberry fruits (Table 4.3). No evidence of *P. niveus* was detected in the control fruits. The SYBR green assay was then used to quantify *P. niveus* CO7 DNA from 3 soil and 3 apple cider samples infested with *P. niveus* ascospores. The assay was then used to quantify *P. niveus* DNA from DNA extract obtained from puree of apple fruits that developed from infested blossoms. Cq values for the apple puree made from fruits that developed from infested blossoms were negative for the 10% bloom, 100% bloom, and petal fall trials.

*P. niveus* DNA was not detected in one sample from an infected strawberry. DNA was extracted from fruit flesh of infected apple and orange fruits two weeks post-inoculation, from ciders and soils infested with *P. niveus* hyphae, and cider and soils infested with $10^7$ *P. niveus* ascospores.

**Table 4.3. Average Cq values from qPCR amplification of *Paecilomyces niveus* CO7 DNA from apple and orange fruits, food, and soil (n = 3) and from strawberries (n = 4).**

| Substrate | *Paecilomyces niveus* treatment | Cq value (average ± sd) |
|---|---|---|
| Infected apple fruit | Hyphae | 20.80 ± 1.27 |
| Infected orange fruit | Hyphae | 17.78 ± 2.26 |
| Infected strawberry fruit | $10^5$ ascospores | 22.49 ± 10.37 |
| Infested cider | $10^7$ ascospores | 19.41 ± 1.44 |
| Infested soil | $10^7$ ascospores | 27.34 ± .13 |

## Discussion

*P. niveus* has several potential routes into food: through environmental sources like air and water, and by infection of fruits with Paecilomyces rot (Biango-Daniels et al., 2019). In this study we deployed our qPCR detection method to evaluate whether Paecilomyces rot can develop in apples after ascospore infestation of apple blossoms, and after ascospore infection of strawberry fruits. The fungus has been shown to be a wound-infecting pathogen of several rosaceous fruits, and to colonize the apple core during infection (Biango-Daniels & Hodge, 2018; Wang & Hodge, 2022). In several other postharvest fruit pathogen systems like moldy-core caused by *Alternaria alternata* and gray mold of strawberries caused by *Botrytis cinerea*, disease inoculum is presumed to enter fruits before fruit maturation and cause latent infections (Bristow et al., 1986; Reuveni et al., 2002). A previous report of natural Paecilomyces rot infections in apple fruits showed diffuse brown lesions suggesting the presence of internal rot (Khokhar et al., 2019). Contrary to our hypothesis that Paecilomyces rot can result from blossom infestation, our qPCR results did not detect significant *P. niveus* DNA from apples that developed from a small sample of previously infested blossoms. It is unclear whether blossoms do not present a viable entry for *P. niveus* into apple fruits, or whether not enough *P. niveus* biomass had accumulated for detection. We did observe lesion development in strawberry fruits after *P. niveus* ascospore inoculation, thus this the first study to demonstrate the susceptibility of strawberry fruits to Paecilomyces rot.

This study presents a robust qPCR approach for detecting *Paecilomyces niveus* spores or mycelium in fruit, soil, and fruit matrices. We developed a straightforward DNA extraction protocol to break tough-to-lyse *P. niveus* ascospores to improve detection at lower levels through a pre-treatment of 70˚C for two hours

and a severe bead-beating. We found that the tough ascospore walls, coupled with their small size (ascus diameter < 10 µm) presented a challenge for lysis. Various spore pre-treatments were evaluated to optimize DNA yield, including the use of NaOH, phenol-chloroform, and cell wall digesting enzymes. However, using our bead-beating and heat treatment protocol, we were able to detect *P. niveus* in all samples of infested soil and cider.

Our designed primer pair (PnPATf and PnPATr) is specific to a 155-bp locus within the *patK* gene. The primer pair was validated in silico and in vitro through end-point PCR on a sample of 24 *P. niveus* isolates and 16 food spoilage fungi, fruit pathogens, and close relatives. PCR products were obtained only from the 24 *P. niveus* isolates. From qPCR amplification of pure *P. niveus* CO7 DNA, a standard curve ($R^2$ = .99) was established, showing that this method was able to detect and quantify *P. niveus* DNA across a range of conditions including low concentrations of DNA, on par with previous qPCR work on food spoiling fungi (Gil-Serna et al., 2009; Panek & Frąc, 2018). Importantly, our newly developed primers did not detect the close relatives *P. fulvus* and *P. variotii*, nor various patulin-producing members of the Eurotiales including the *Penicillium expansum*, *P. carneum*, and *P. griseofulvum* due to an absent or divergent *patK* (Puel et al., 2007; Urquhart et al., 2018).

We deployed the primer pair PnPATf/PnPATr in our newly developed qPCR assay for detection of *P. niveus* DNA in infected fruits, and in infested soil and cider. Higher Cq values from cider and soil samples infested with $1x10^7$ ascospores, compared to Cq values from pure *P. niveus* DNA extract suggest some level of inhibition from the food and soil matrices during DNA extraction and PCR. Compared to a growing array of methods for detection of food spoilage fungi including the use of biosensors, metabolic profiles, phenotypic assays, and other molecular techniques like loop-mediated isothermal amplification, our method is promising in that it is both

robust and accessible in not relying on highly sophisticated equipment (Panek & Frąc, 2019; Pertile et al., 2020; Santana Oliveira et al., 2019). Future research may focus on detecting multiple thermotolerant food spoiling or patulin-producing fungi via a multiplexing approach, for use in industrially and agriculturally relevant environments.

Our qPCR assay for detection and quantification of *P. niveus* DNA allowed us to ask questions and test hypotheses regarding *Paecilomyces niveus* disease biology. This study provides a tool to help determine sources of *P. niveus* spoilage and disease inoculum, an important first step to combating spoilage and patulin contamination by this notorious fungus. Incorporating results from this study can aid the development of comprehensive preventative strategies for spoilage and mycotoxin contamination in foods.

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# CHAPTER 5

## PATULIN CONTAMINATION OF HARD APPLE CIDER BY PAECILOMYCES NIVEUS AND OTHER POSTHARVEST APPLE PATHOGENS: ASSESSING RISK FACTORS[4]


### Abstract

Hard apple cider is considered to be a low-risk product for food spoilage and mycotoxin contamination due to its alcoholic nature and associated food sanitation measures. However, the thermotolerant mycotoxin-producing fungus *Paecilomyces niveus* may pose a significant threat to hard cider producers. *P. niveus* is known to infect apples (*Malus* x*domestica*), and previous research indicates that it can survive thermal processing and contaminate finished apple juice with the mycotoxin patulin. To determine if hard apple cider is susceptible to a similar spoilage phenomenon, cider apples were infected with *P. niveus* or one of three patulin-producing Penicillium species and the infected fruits underwent benchtop fermentation. Cider was made with lab inoculated Dabinett and Medaille d'Or apple cultivars, and patulin was quantified before and after fermentation. Results show that all four fungi can infect cider apples and produce patulin, some of which is lost during fermentation. Only *P. niveus* was able to actively grow throughout the fermentation process. To determine if apple cider can be treated to hinder *P. niveus* growth, selected industry-grade sanitation measures were tested, including chemical preservatives and pasteurization. High concentrations of preservatives inhibited *P. niveus* growth, but apple cider flash pasteurization was not found to significantly impact spore


[4] Wang, Tristan W., Amanda G. Wilson, Gregory M. Peck, Patrick A. Gibney, and Kathie T. Hodge. 2023. Patulin contamination of hard apple cider by Paecilomyces niveus and other postharvest apple pathogens: assessing risk factors. Submitted to Int. J. Food Microbiol.

germination. This study confirms that hard apple cider is susceptible to fungal-mediated spoilage and patulin contamination. *P. niveus* should be of great concern to hard apple cider producers due to its demonstrated thermotolerance, survival in fermentative environments, and resistance to sanitation measures.

## Introduction

The mycotoxin patulin is an important apple-associated mycotoxin produced by a handful of fungi and noted for its genotoxic, immunosuppressant, and cytotoxic properties (Ülger et al., 2020). Patulin contamination in apple (*Malus* x*domestica*) juices and ciders, subject to regulation in many parts of the world, is a long-standing issue that has been reported in various countries (Affairs, 2020; Commission, 2003; Commission Regulation, 2006; Harris et al., 2009; Spadaro et al., 2007; Yuan et al., 2010). It has been long assumed that patulin contamination of fruits and fruit products occurs through fruit infection by patulin-producing Penicillium species, particularly the notorious postharvest pathogen *Penicillium expansum*.

While patulin has been detected in a variety of fruit products (Spadaro et al., 2007; Zouaoui et al., 2015), the mycotoxin is not traditionally thought to be an issue for their fermented counterparts. This is in part due to research that has shown that fermentation reduces patulin levels significantly (Erdoğan et al., 2018; Stinson et al., 1978; Zhang et al., 2019).

An alternative route of patulin entry into fruits and fruit products is through the notorious food-spoiling mold *Paecilomyces niveus*. This ascomycete produces heat-resistant ascospores that can survive lower temperatures of pasteurization and grow in acidic and low-oxygen conditions (Biango-Daniels et al., 2019; Biango-Daniels and Hodge, 2018; Taniwaki et al., 2009). A common soil fungus, *P. niveus* is particularly problematic for finished fruit products, and has been reported in apple puree, orange

juice, and strawberry puree, raising concerns that it may be a source of patulin contamination in other juices like lemonade and orange juice (*Citrus* spp.) (Santos et al., 2018).

New research shows that *P. niveus* can grow as a postharvest pathogen in various fruits, including apples (Biango-Daniels and Hodge, 2018; Wang and Hodge, 2022, 2020). Juice from infected fruits can become infested with living *P. niveus* mycelium and spores and contaminated with patulin (Biango-Daniels et al., 2019). Furthermore, this fungus can resist high temperatures, grow in low-oxygen environments, and resist fairly high concentrations of alcohol (Brown and Smith, 1957). The connection between patulin and post-fermented products is understudied (Al Riachy et al., 2021). But if *P. niveus* can survive and grow during fermentation, it may continuously produce patulin, contaminating the finished cider.

In this study, we investigate how each step of hard cider production impacts patulin-producing fungi introduced through infected fruit. We also evaluate the ability of common sanitation methods to decrease risks of spoilage and patulin contamination.

Cider apples are known for their higher tannin and phenol levels which have been hypothesized to be inhibitory to pathogens (Serrano et al., 2009). However, *P. niveus* has recently been found to be able to infect a variety of fruits beyond apples, so we hypothesize that cider apples too are susceptible to Paecilomyces rot (Wang and Hodge, 2022). Understanding how the different steps of hard cider production impact fungal growth and patulin production will help apple producers and hard cider makers better assess the risk of mycotoxin contamination.

Our objectives are to: 1. Test the susceptibility of traditional cider apple cultivars to four known patulin-producing strains of apple pathogens: *Penicillium expansum*, *Penicillium griseofulvum*, *Penicillium carneum*, and *Paecilomyces niveus*;

2. Assess the ability of each fungus to produce patulin in cider apple fruits; 3. Test the ability of *P. niveus* to produce patulin in lemonade and orange juice; 4. Test potential inhibitory effects of fermentation on the growth of the four fungi in small-scale bench-top fermentation by evaluating growth; 5. Develop and validate primers based on the RPBII gene for quantification of *Paecilomyces* sp. DNA; 6. Quantify the effects of three different preservative treatments and flash pasteurization on *P. niveus* growth and viability.

## Materials and methods

To evaluate the impact of infected apples on patulin content of finished cider, we first prepared lab-infected apples of two traditional cider apple cultivars (Dabinett and Medaille d'Or) using four patulin-producing fungi. Two additional cider apple cultivars, Harry Masters Jersey and Chisel Jersey, were tested for *P. niveus* susceptibility. We performed bench-scale cider fermentation, then evaluated two important attributes of the finished fermented cider: 1) patulin content, and 2) evidence of viable fungus. Any visible hyphae was grown and identified. To test the impact of preservatives on *P. niveus* spore germination and growth, ascospores underwent three preservative treatments in apple cider, each at three concentrations. Fungal growth of *P. niveus* was measured using qPCR, applying our newly designed PCR primers. *Paecilomyces niveus* spores in apple cider were additionally treated by capillary tube flash pasteurization, and spore viability was measured as colony forming units after plating on PDA.

### Fungal cultures and apple infection

*Penicillium expansum* isolate 94222 was obtained from a department fungal collection. *Penicillium griseofulvum* NRRL 2159A and *Penicillium carneum* NRRL

25170 were obtained from the ARS NRRL fungal culture collection, and *Paecilomyces niveus* CO7 from the Hodge lab culture collection (Biango-Daniels et al., 2018). Fungi were grown in the dark at 25˚C. To prepare toothpick inoculum, toothpick halves were tyndallized by autoclaving first in water, then in potato dextrose broth. They were placed on potato dextrose agar along with three to four plugs from the growing edges of each of the four fungal isolates. Control toothpicks were treated similarly but without the presence of fungi.

Once the toothpicks were fully colonized after two weeks, they were used to inoculate apples for cultivar trials and patulin experiments. Four cider apple cultivars were sourced from The Cornell Agricultural Experiment Station research orchard in Ithaca, NY(Chisel Jersey, Dabinett, Harry Masters Jersey, and Medaille d'Or) and inoculated following methods of Biango-Daniels and Hodge (2018). Briefly, apple fruits were first sanitized in 70% ethanol for 30 seconds, then two minutes in 1% sodium hypochlorite, and finally in 70% ethanol for 15 seconds. Fruits were numbered and left to dry in a class IIB biological safety cabinet. Infested toothpicks were inserted 1 cm deep into the fruit.

**Bench-top fermentation**

Toothpicks used in inoculation were removed from roughly 4.5 kg of Dabinett and Medaille d'Or cider apples infected 4 weeks prior with *Pe. expansum*. The apples were chopped and homogenized with a blender, and the puree was strained through 4 layers of cheesecloth. Samples of 150mL of juice were aliquoted in three separate 500mL flasks. The remaining cider was frozen for brix and patulin quantification. One mL (1.13g of yeast and emulsifier) of activated *Saccharomyces cerevisiae* strain EC1118 (Lalvin) prepared under manufacturer's instructions was added to each of the three flasks. This process was repeated for fruits infected with *Pe. griseofulvum*,

*Pe. carneum*, and *P. niveus*. Flasks were sealed with autoclaved rubber bungs that were fitted with air locks. The flasks of cider were left to ferment at room temperature for four weeks and $CO_2$ bubbling was monitored daily. Preservative treatments were not used during fermentation but were tested later. At four weeks, fermented product was checked for fungal growth and any present hyphae was grown and identified. As a baseline, uninfected Dabinett and Medaille d'Or apples stored at 25˚C in the dark for four weeks were processed and fermented in the same way.

**Patulin quantification**

Lemonade, orange juice, and apple cider used in the pasteurization and preservative treatment protocols were free from preservatives and purchased from a local supermarket in New York. For patulin analysis of infested lemonade, orange juice and apple cider, 50mL aliquots were submitted to Trilogy Analytical Laboratory (Washington, MO). Patulin was quantified by high-performance liquid chromatography following official AOAC International methods for apple juice (Brause et al., 1996). In addition, patulin was quantified from cider extracted from infected cider apples both before fermentation (n=1) and after fermentation (n=1). Yeast cells were removed (racked) by decanting clear cider from the sediment of the *P. niveus* infected cider apple sample. Both racked and unracked samples were quantified for patulin. 200 mL store-bought lemonade and orange juice were infested with 1x 10^7 *P. niveus* asci/ascospores and patulin was quantified (n=1) after two weeks. The limit for patulin contamination in the United States and Europe for fruit juices is 50 µg/kg (Affairs, 2020; Commission, 2003; Commission Regulation, 2006).

**Impact of common preservatives on Paecilomyces niveus spore germination and growth**

*Paecilomyces niveus* ascospore viability was tested using three food-relevant treatment conditions designed to limit microbial growth: potassium sorbate, sulfur dioxide, and sodium benzoate. Food-grade apple cider was aliquoted into 2-mL tubes infested with 2 x $10^5$ *P. niveus* spores harvested from 4-week-old cultures on PDA. For each of the three preservatives, three different concentrations were tested. Tubes were then capped and air sealed. Acidity of the cider was measured with a digital pH meter and found to be 3.6. Food grade potassium sorbate was weighed to create three concentrations of potassium sorbate at .02%, .06%, and .1%. Campden tablets were crushed and weighed to create .05%, .1% and .15% treatments of sulfur dioxide in apple cider. Food grade sodium benzoate was weighed to create .05%, .1%, and .15% concentrations. Positive controls lacked any preservative treatment, and negative controls had neither spore treatment nor preservative treatment. Each treatment was performed in triplicate and kept in the dark at 25˚C. After 2 weeks, presence or absence of hyphal growth in each sample was visually assessed. Present hyphae was extracted, grown on PDA, and identified morphologically. Samples were then centrifuged at 16,000 xg for 5 minutes and DNA extraction was performed on the resulting pellet.

**RPBII primer design and qPCR validation**

Primers and a qPCR system were designed for accurate quantification of *Paecilomyces niveus* DNA after exposure to various treatments. Low-coverage genomes of 23 *P. niveus* isolates were visualized using NCBI Genome Workbench and compared to the high-coverage genome of *P. niveus* isolate CO7 (Biango-Daniels et al., 2018). Primers were designed using the PrimerQuest Tool (https://www.idtdna.com/primerquest/Home/Index) and candidate sequences within the RNA polymerase II gene were compared to sequences of *P. fulvus* and *P. variotii*

aligned using MUSCLE (Edgar, 2004) and visualized using Snapgene software (https://snapgene.com/). Primer specificity was additionally verified through BLAST. Primers were tested using PCR on DNA extracts of 9 food-spoiling and postharvest fruit pathogens, 3 isolates each of *P. fulvus* and *P. variotii*, and 24 isolates of *P. niveus*. Fungal cultures were obtained from Hodge lab collections at Cornell University and from the NRRL Culture Collection.

Genomic DNA was extracted from fungal isolates using the DNeasy PowerSoil Pro Kit (Qiagen) following manufacturer instructions. Q5 High Fidelity DNA Polymerase (New England Biolabs, United States) was used for end point PCR to determine specificity of the PaeRPB2f/r primers (Table 2) under the following PCR cycles: 98˚C for 30s and 26 cycles of 98˚C for 10s (denaturation), 60˚C for 30s (annealing), and 72˚C for 45s (extension). For *P. niveus* DNA quantification, the CFX Connect Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA) was programmed to run at 95˚C for 10 minutes and then to complete 40 cycles of 95˚C for 15s (denaturation), 60˚C for 30s (annealing), and 72˚C for 45s (extension). All reactions were done in triplicate and results were measured by quantification cycle (Cq).

qPCR reaction mixtures contained 25µl 2x Power SYBR Green PCR Master Mix (Applied Biosystems, Madrid, Spain), 1µl 10µM PaeRPB2f primer, 1µl 10µM PaeRPB2r primer, 10µl of eluted fungal DNA, and 13 µl deionized water (50µl total). Real time qPCR was performed on the CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA) programmed to hold at 95˚C for 10 minutes followed by 40 cycles of 95˚C for 15s (denaturation), 60˚C for 30s (annealing), and 72˚C for 45s (extension).

**Heat-treatment**

*Paecilomyces niveus* asci and ascospores were extracted from 4-week-old plates on PDA using water and autoclaved pipette tips to agitate the surface of the mycelia. The spore solution was filtered through 8 layers of cheesecloth and one of Whatman No. 1 filter paper to remove hyphae. A haemocytometer was used to determine spore concentration and to confirm absence of hyphal fragments. Apple cider free from preservatives was purchased from a local supermarket, autoclaved and spiked with *P. niveus* asci and ascospores. 20µl samples containing roughly 200 *P. niveus* colony forming units were aliquoted into 75mm long thin walled (.2 ± .02 mm thick) soda glass capillary tubes (Kimble Chase). The empty ends of the capillary tubes were sealed using fire. Each was exposed to either a light heat-treatment of 71.1˚C for 6 seconds (n=14), a heavy treatment of 71.7˚C for 15 seconds (n=14) or no treatment (n=14). The light treatment is based on the United States Food and Drug Administration (FDA) minimum pasteurization process for non-shelf stable juices, and the heavy treatment is based on the US protocol for flash pasteurization of milk, both of which are used for log reduction of harmful pathogens. After heat-treatment, the capillary tubes were cooled in an ice bath for 3 seconds, broken, and spread plated on Rose Bengal Agar. Colony formation was observed and quantified over the next two weeks.

**Statistical analysis**

Significance of cider apple lesion development was tested by comparing lesion sizes via two separate linear mixed-effects models at day 2 and day 8 for both apple cultivars infected by *Pe. griseofulvum* and *P. niveus* (cider apples infected with *Pe. expansum* and *Pe. carneum* were tested at days 3 and 8) and by comparing lesion sizes from both the control and treatments on day 8. Apple identification

number was accounted for as a fixed effect. Variation in pathogenicity by *P. niveus* infection in apple cultivars was evaluated by one-way analysis of variance using diameter of lesions on day 20. Differences between heat-treatments on *P. niveus* spore viability and colony forming units were tested using the Kruskal-Wallis rank sum test. Statistical analysis was followed by a post-hoc Tukey Honest Significant Difference Test. Statistical models were executed in R statistical programming software using the emmeans, lme4, lmerTest, and stats packages. Data was visualized using the ggpubr and ggplot2 packages.

## Results

### Characterization of Paecilomyces niveus and three Penicillium sp. infections on cider apples

In cider apples infected by *Pe. expansum*, dark red-brown circular lesions in Dabinett (Fig. 1B) or brown lesions in Medaille d'Or apples (Fig. 1C) rapidly developed at the site of the inoculation. Apple flesh quickly lost integrity as soft rot developed, particularly with infected Dabinett apples. In Medaille d'Or apples, concentrated tufts of sporulating hyphae appeared on the lesion surface by 20 days post-inoculation.

Both cultivars (Fig. 1E, 1F) of cider apples infected with *Pe. griseofulvum* developed expanding brown lesions centered at the point of inoculation that were sometimes lighter brown close to the point of inoculation. Lesion borders were distinct and pronounced in both cultivars. Rot within the apple is light brown and unlike the soft rot infections by *Penicillium expansum*, apple flesh infected with *Pe. griseofulvum* was firm and spongy to the touch.

Cider apples infected with *Pe. carneum* developed brown to light brown

lesions and soft rot in both Dabinett and Medaille d'Or cultivars (Fig. 1H, 1I). Lesion borders were often diffuse in Dabinett apples. Lesions sometimes displayed concentric circles with varying shades of brown in Medaille d'Or apples. At 20 days post-inoculation, a faint blue ring of spores was seen developing at the border of the endocarp within infected Dabinett apples.

All four cider apple cultivars (Chisel Jersey, Dabinett, Harry Masters Jersey, and Medaille d'Or) tested for susceptibility to *P. niveus* infection developed circular dark brown lesions at the point of inoculation (Fig. 2A). Lesion borders were generally sharp and distinct in Dabinett and Chisel Jersey apple fruits. Chlorosis sometimes developed near the edges of the lesions in Medaille d'Or fruits. Brown concentric circles sometimes developed, most clearly in Medaille d'Or and Harry Masters Jersey apples. Apple rot in all cultivars was firm to the touch and resulted in brown to light-brown internal rot.

Our results show that each cider apple cultivar tested was susceptible to each of the four patulin-producing fungi, and that apple fruits responded differently to each infection. Infections by *Pe. expansum* and *Pe. carneum* elicited brown lesions and soft rot, symptoms consistent with Blue Mold infections. Infection by *Pe. griseofulvum* was unusual as it resulted in rot with a spongy texture, not a soft rot. This may be a point of interest for apple producers who may look specifically for Blue Mold infections in apple fruits. Additionally, because we used *Pe. griseofulvum* isolate NRRL 2159A, a white mutant, we did not see blue sporulation as a sign of the fungus. In *P. niveus* infections, symptoms of hard rot with occasional concentric circles and slowly spreading lesions were consistent with Paecilomyces rot symptoms in dessert apples (Biango-Daniels and Hodge, 2018). Follow-up studies may consider testing additional isolates of *P. niveus* and each *Penicillium* species for infection assays.

**Susceptibility to patulin-producing apple pathogens**

Inoculations by *P. niveus* and all three *Penicillium* species (*Pe. expansum*, *Pe. griseofulvum*, and *Pe. carneum*) resulted in clear lesion development in each cider apple cultivar tested within 3 to 6 days post-inoculation. The average of horizontal and vertical lesion diameters of each apple fruit's two lesions was measured every 2 to 3 days. Lesion diameters (± standard error) grew rapidly over the course of the next 10 to 20 days. Between 3- and 8-days post-inoculation in apples infected with *Penicillium expansum*, average lesion diameter increased by 21.66 ± 1.95 mm ($n$ = 21) in Dabinett apples, and 22 ± .83 mm ($n$ = 29) in Medaille d'Or apples (Fig. 5.1A). In apples infected with *Pe. griseofulvum*, average lesion diameter increased by 12.06 ± .91 mm ($n$ = 22) and 7.61 ± .48 mm ($n$ = 28) in Dabinett and Medaille d'Or apples, respectively, between 2- and 8-days post-inoculation (Fig. 5.1D). Average lesion diameters in apples infected with *Pe. carneum* increased by 5.81 ± 1.41 mm ($n$ = 22) and 1.17 ± .50 mm ($n$ = 30) in Dabinett and Medaille d'Or apples, respectively, between 3- and 8-days post-inoculation (Fig. 5.1I). The average change in lesion diameter for cider apples infected with *P. niveus* (± standard error) was 5.09 ± .28 mm ($n$ = 27) in Chisel Jersey apples; 3.67 ± .16 mm ($n$ = 38) in Medaille d'Or apples; 6.12 ± .23 mm ($n$ = 27) in Harry Masters Jersey apples; and 6.09 ± .57 mm ($n$ = 40) in Dabinett apples between 2- and 8-days post-inoculation (Fig. 5.1G). Apples observed to be colonized by other fungi were removed from the experiment and from statistical analysis. On day 8, lesion diameters were significantly larger than both control lesions ($P$ < .01), and treatment lesions on day 2 (*Pe. griseofulvum* and *P. niveus*) and day 3 (*Pe. expansum* and *Pe. carneum*) ($P$ < .01). In addition, by one-way analysis of variance of lesion diameters of *P. niveus*-infected apples on day 20, apple cultivar was found to be significant ($P$ < .01).

Neither Dabinett nor Medaille d'Or cider apple cultivars have been previously

evaluated for susceptibility to *P. niveus* infection, nor to Blue Mold infection by *Pe. expansum*, *Pe. griseofulvum*, and *Pe. carneum*. Despite cider apple cultivars being known for their high tannin and phenol content, which have been proposed to confer some resistance to fungal infection (Serrano et al., 2009), all four cultivars were susceptible to infections by the four patulin-producing fungi. Our data suggest there may be some cultivar-based resistance to infections by *P. niveus* and raise the possibility of using more resistant cultivars as a strategy to combat patulin contamination arising from Paecilomyces rot. Previous research has demonstrated that patulin functions as a pathogenicity agent in *Pe. expansum*, facilitating partial lesion formation and breaking down fruit tissue (Bartholomew et al., 2022; Snini et al., 2016). While it is unclear whether patulin functions similarly for other postharvest pathogens, future studies may consider expanding on the scope of this work by exploring additional cultivars for resistance to Blue Mold and Paecilomyces rot and comparing pathogenicity of patulin producing and non-patulin producing isolates.

**Koch's postulates**

For each of the four fungal pathogens, Koch's postulates were satisfied using four-week post-inoculation Medaille d'Or and Dabinett apples. Diseased apple surfaces were sanitized with 70% ethanol, and diseased interior tissue extracted from apple lesions was plated on PDA. Fungal isolates were confirmed morphologically and through Sanger sequencing, and an isolate of each species was used to reinfect healthy apples. The same symptoms were observed, thereby satisfying Koch's postulates and confirming the causal agent of the observed disease.

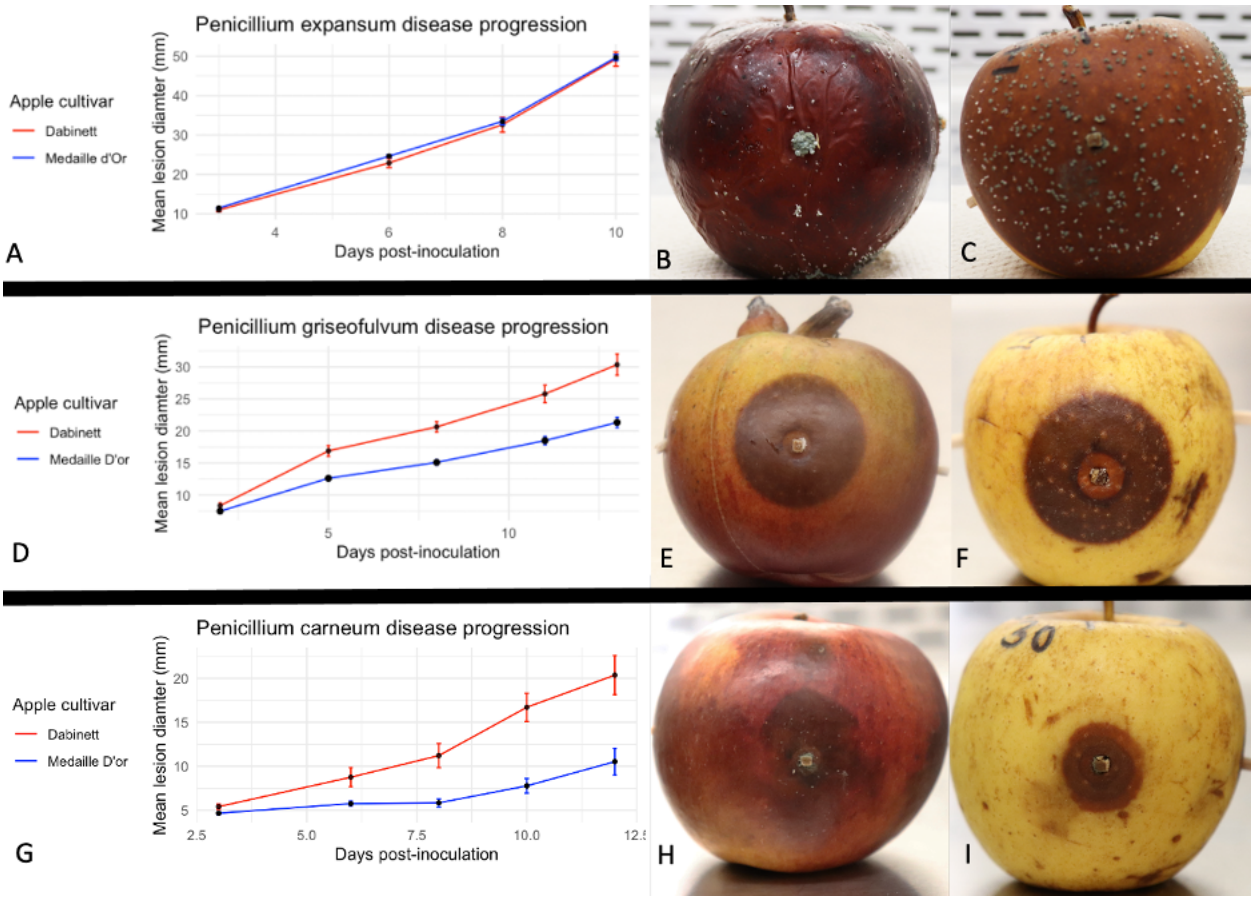


**Figure 5.1. Disease progress and external symptoms of two cider apple cultivars 3 weeks after inoculation with three *Penicillium* spp. and incubation in dark, moist chambers (25˚C, ≥95% humidity).** Top row shows *Penicillium expansum* lesion diameters (A) and infections of Dabinett (B) and Medaille d'Or (C). Middle row shows *Penicillium griseofulvum* lesion diameters (D) and infections of Dabinett (E) and Medaille d'Or (F). Bottom row shows *Penicillium carneum* lesion diameters (G) and infections of Dabinett (H) and Medaille d'Or (I).

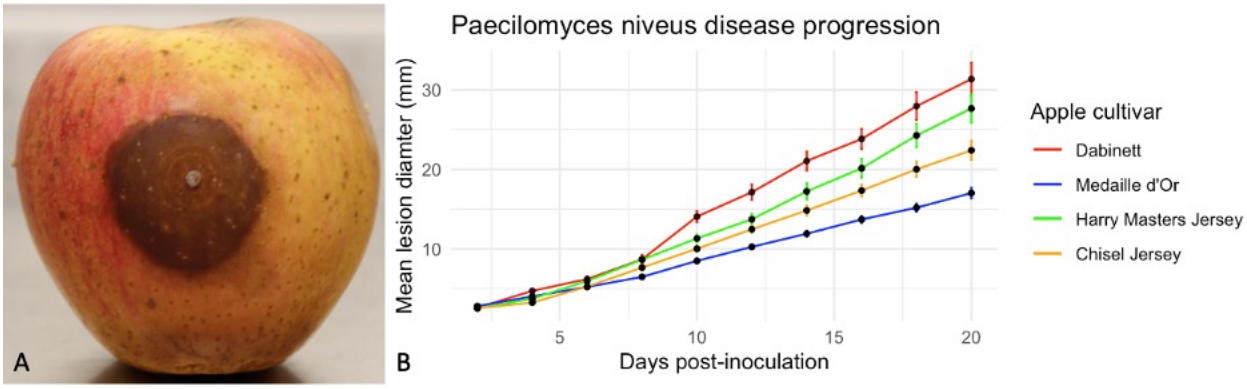


**Figure 5.2. Paecilomyces rot in four cider apple cultivars.** A, Harry Masters Jersey apple infected by *P. niveus*, 3-weeks post-inoculation. B, Line plot shows increase in mean lesion diameter over 3 weeks in four cider apple cultivars.

### Benchtop fermentation and patulin quantification

After four weeks of fermentation, no mycelial growth was observed in the control flasks, nor in fermented cider extracted from apples infected with *Pe. expansum*, *Pe. griseofulvum*, or *Pe. carneum*. However, mycelial growth was present in all three replicates of cider extracted from apples infected with *P. niveus*. Mycelium was extracted and grown on PDA and confirmed morphologically to be *P. niveus*. Soluble solid concentration values were 10.13, 9.77, 10.63, 11.67, and 10.6 ˚Brix before fermentation and 2.9, 3.5, 4, 3.4, and 4.5 ˚Brix after four weeks of fermentation for cider samples made from uninfected apples and apples infected with *Pe. expansum*, *Pe. griseofulvum*, *Pe. carneum*, *P. niveus* respectively.

After four weeks of fermentation, cider made from apples infected with *Pe. expansum* exhibited the highest patulin concentration at 26,942.8 µg/kg, while cider from apples infected with *P. niveus* had the lowest concentration of patulin at 2,055.1 µg/kg (Table 5.1). We observed post-fermentation reduction in patulin levels in all four cider samples extracted from infected apples. The largest reduction in patulin was observed in cider extracted from apples infected with *Pe. griseofulvum* (90.6%) while

the smallest reduction occurred with cider extracted from apples infected with *P. niveus* (80.0%). In all cider samples before and after fermentation, patulin levels far exceeded the United States FDA and Europe limit of 50 µg/kg (Affairs, 2020; Commission, 2003; Commission Regulation, 2006).

Store bought lemonade and orange juice, in 200 mL aliquots, were each spiked with *P. niveus* 10^7 asci and ascospores and left to sit at room temperature. A surface layer of white mycelium was observed developing in the treated samples. Patulin was quantified after two weeks at 22,884.8 µg/kg and 49,302.8 µg/kg for lemonade and orange juice respectively.

In this study, we observed that fermentation does not completely inhibit the growth of *P. niveus* in cider, which raises the possibility of spoilage of finished hard ciders by the fungus. Patulin reduction after fermentation was smallest in cider samples infested with *P. niveus*. Our results suggest this may be due to growth and production of patulin by *P. niveus*. Our data suggest *P. niveus* is also able to produce patulin in lemonade and orange juice. Although our study did not include a control for the confounding variable of natural patulin loss in the absence of yeast, previous work has shown this form of reduction is minimal (Stinson et al., 1978).

**Table 5.1. Patulin concentrations (µg/kg) in cider and juices.** Quantified patulin levels of apple cider extracted from diseased apple fruits, fermented product, and infested orange juice and lemonade.

| Treatment | Patulin level (µg/kg) |
|---|---|
| Juice, *Pe. expansum* infected apples | 175,253.3 |
| Juice, *Pe. griseofulvum* infected apples | 14,441.4 |
| Juice, *Pe. carneum* infected apples | 33,253.4 |
| Juice, *P. niveus* infected apples | 12,960.9 |
| Juice, untreated control apples | 0 |
| Fermented cider, *Pe. expansum* infected apples | 26,942.8 |
| Fermented cider, *Pe. griseofulvum* infected apples | 1,355.3 |
| Fermented cider, *Pe. carneum* infected apples | 5,636.7 |
| Fermented cider, *P. niveus* infected apples | 2,586.4 |
| Fermented cider, *P. niveus* infected apples (racked) | 2,055.1 |
| Fermented cider, untreated control apples | 0 |
| Lemonade infested with *P. niveus* | 22,884.8 |
| Orange Juice infested with *P. niveus* | 49,302.8 |
| Control lemonade | 0 |
| Control orange juice | 0 |

**RPBII primer design for detection of Paecilomyces spp.**

Primers based on the RNA polymerase II gene were designed and tested on multiple strains of three different *Paecilomyces* species: *P. niveus* (n=24), *P. fulvus* (n=3), and *P. variotii* (n=3), in addition to nine close relatives, including postharvest pathogens and food spoiling molds in the order Eurotiales (Table 5.2). PCR products were obtained from each of the three *Paecilomyces* species, but not from any of the other Eurotiales fungi tested.

**Table 5.2. Sequences and specificity of RPBII primers designed in this study for quantification of *P. niveus* growth using real-time PCR.** End-point PCR was performed to test the specificity of the primers to *Paecilomyces* species.

| PaeRPB2f/r | Primer nucleotide sequences | Tm (˚C) | Product size |
|---|---|---|---|
| Forward primer | 5'-ATGGGCCATTGATGGGTATC-3' | 54.9 | 412 bp |
| Reverse primer | 5'-GCACCATTGAAGAAAGCCA-3' | 53.6 | |

| Species | Strain | Source of isolation | PCR product |
|---|---|---|---|
| *Aspergillus flavus* | AF-36 | Cotton fields, AZ | - |
| *Aspergillus terreus* | NRRL 269 | Food, fruit (dates), CA | - |
| *Botrytis obtusa* | 97017 | Unknown substrate | - |
| *Talaromyces bacillisporus* | NRRL 1025 | Begonia leaf, NY | - |
| *Talaromyces islandicus* | NRRL 1036 | Unknown substrate, South Africa | - |
| *Thermoascus crustaceous* | NRRL 1536 | Dung, Java | - |
| *Paecilomyces fulvus* | 7 | Spoiled food | + |
| | Glov | Unknown substrate | + |
| | Gat8 | Unknown substrate | + |
| *Paecilomyces niveus* | 109-21 | Orchard soil, NY | + |
| | 107-1 | Orchard soil, NY | + |
| | MC4 | Residential soil, NY | + |
| | CO7 | Culled apples, NY | + |
| | 102-1 | Research station soil, NY | + |
| | 100-10 | Orchard soil, NY | + |
| | CF6 | Corn field soil, NY | + |
| | 108-11 | Orchard soil, NY | + |
| | CFO13 | Corn field soil, NY | + |
| | CC8 | Compost, NY | + |
| | 111-1 | Orchard soil, NY | + |
| | 112-22 | Soil, NY | + |
| | BY11 | Barn yard Soil, NY | + |
| | 146-311 | Residential soil, NY | + |
| | EP1 | Rhizosphere soil, unknown location | + |
| | 141-11 | Farm soil, NY | + |
| | 104-22 | Orchard soil, NY | + |
| | 110-3 | Orchard soil, NY | + |
| | 125-31 | Farm market soil, NY | + |
| | AF01 | Alfalfa field soil, NY | + |
| | 101-3 | Research farm soil, NY | + |
| | 106-3 | Orchard soil, NY | + |
| | KRA4 | Food facility, unknown location | + |
| | 140-11 | Farm soil, NY | + |
| *Paecilomyces variotii** | 103-2 | Soil, NY | + |
| | 3001 | Unknown substrate | + |
| | 3016 | Unknown substrate | + |
| *Penicillium expansum* | 94222 | Unknown substrate | - |
| *Penicllium griseofulvum* | NRRL 2159A | Unknown substrate | - |
| *Penicillium carneum* | NRRL 25170 | Unknown substrate | - |
| *Penicillium italicum* | KH1 | Diseased clementine, NY | - |

**Effects of industrial preservative treatments on Paecilomyces niveus spore survival and growth**

To determine the effects of industry standard food treatment on *Paecilomyces niveus* spore survival and growth, food-grade apple cider infested with *P. niveus* CO7 spores was subjected to treatments commonly used to control human disease and food spoilage agents including three preservative treatments and two flash pasteurization protocols.

Treatments of potassium sorbate, sodium benzoate, and sulfur dioxide were applied in three concentrations, and fungal growth was assessed visually for presence of hyphae. As a proxy for *P. niveus* biomass, DNA was quantified by qPCR using primers developed in this study. After two weeks of exposure, *P. niveus* hyphal growth was observed in 2mL tubes containing food-grade apple cider, as well as in low concentrations of each of the three preservatives (.02% potassium sorbate, .05% sodium benzoate, and .05% sulfur dioxide). Hyphae were also observed at .1% concentration of sulfur dioxide, but growth was not detected at higher concentrations of the three preservative treatments (Table 5.3). Mean Cq values (n=3) estimating *P. niveus* DNA in the 2mL tubes were obtained for each treatment. Higher Cq values were generally observed for tubes treated with high preservative concentrations, suggesting less *P. niveus* biomass (Table 5.3).

**Table 5.3. *P. niveus* germination and growth in apple cider.** Cider was treated with potassium sorbate, sodium benzoate, and sulfur dioxide and were observed for visible hyphal growth after two weeks. Cq values were generated as a proxy for fungal biomass. Mean Cq and standard deviation values from real-time qPCR assays on 2mL aliquots of apple cider infested with *P. niveus* spores after a 2-week exposure to industry standard preservative treatments.

| Treatment | Concentration (%) | Observed Hyphal Growth | Mean Cq values |
|---|---|---|---|
| Cider negative control | N/A | - | N/A |
| Cider positive control | N/A | + | 22.59 ± .82 |
| Potassium sorbate (Low) | .02 | + | 21.53 ± 1.94 |
| Potassium sorbate (Medium) | .06 | - | 28.83 ± 4.50 |
| Potassium sorbate (High) | .1 | - | 30.87 ± 5.99 |
| Sodium benzoate (Low) | .05 | + | 22.33 ± 1.77 |
| Sodium benzoate (Medium) | .1 | - | 25.48 ± 1.11 |
| Sodium benzoate (High) | .15 | - | 26.80 ± 1.41 |
| Sulfur dioxide (Low) | .05 | + | 24.40 ± 3.86 |
| Sulfur dioxide (Medium) | .1 | + | 20.94 ± 1.37 |
| Sulfur dioxide (High) | .15 | - | 25.64 ± 1.93 |

To determine the effects of standard pasteurization protocols on *Paecilomyces niveus* ascospore viability, spores that underwent two flash pasteurization protocols in soda glass capillary tubes were assessed for colony forming units. In both treatments, a significant number of *P. niveus* spores remained viable (Figure 3). There was no significant difference between the 71.1˚C 6 second treatment and the 71.7˚C 15 second treatment under the Kruskal-Wallis rank sum test ($\chi 2 = 5.405$, df = 2, p=.067).

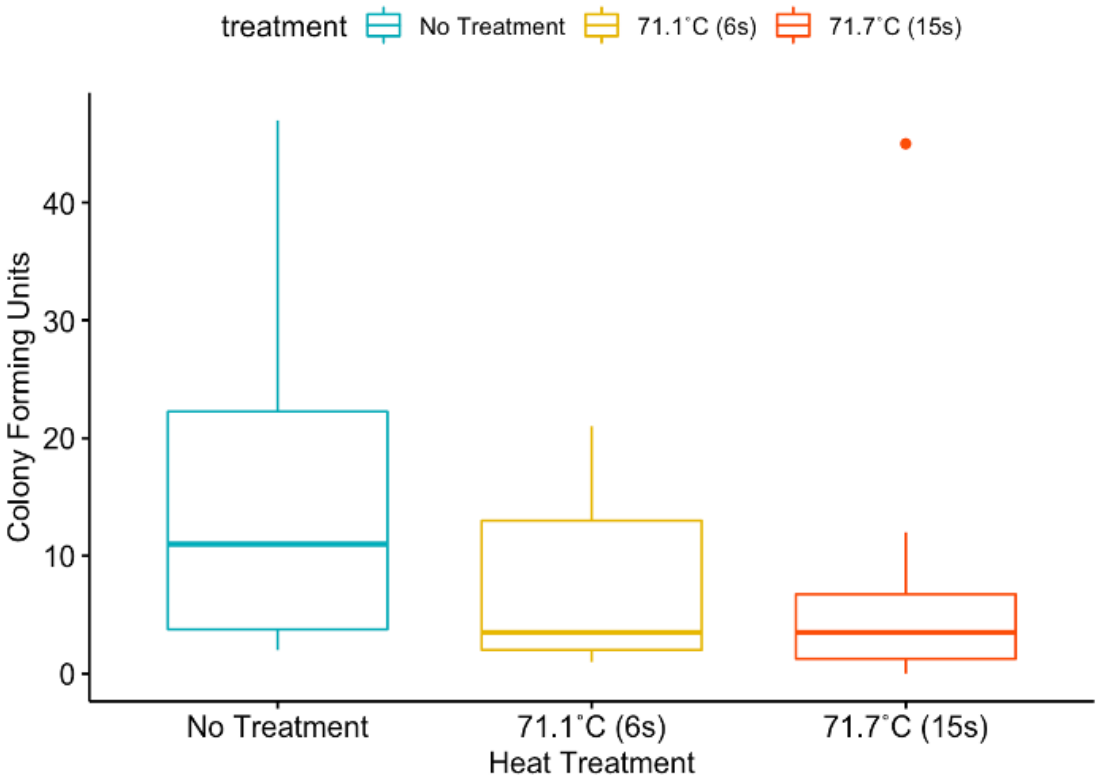


**Figure 5.3. Box plots showing the impact of heat treatment on spore viability.** The number of colony-forming units is shown for three treatments. Roughly 200 asci/ascospores in 20µl glass capillary tube aliquots of food-grade apple cider were exposed to 6s at 71.1˚C, 15s at 71.7˚C (n=14), or no treatment.

## Conclusions and discussion

This study is the first to report on the susceptibility of cider apples to Paecilomyces rot. In addition, we also report on the susceptibility of two cider apple cultivars Dabinett and Medaille d'Or to *Penicillium expansum*, *Penicillium griseofulvum*, and *Penicillium carneum* infections. Cider apple cultivars typically contain higher levels of polyphenolic compounds, which are known to have antimicrobial ability, yet each of the four fungi were able to infect and grow in all cider cultivars tested (Marks et al., 2007; Serrano et al., 2009). These results are consistent with previous pathogenicity assays of *Pe. expansum* with traditional apple cultivars

(Lončarić et al., 2021). Patulin has been previously reported as a cultivar-dependent virulence agent for *Pe. expansum* infections of apples and future studies could investigate if patulin serves a similar role in infection for other patulin-producing pathogens (Snini et al., 2016).

Hard cider makes up a significant portion of the beverages in several countries and is a growing industry in the United States (Ewing and Rasco, 2018). Traditional cider production and apple harvesting, especially in European countries, may employ machinery to shake trees before sweeping up “drop” fruits that fall to the ground, a practice referred to as “shake and sweep” (Karl et al., 2022; Miles et al., 2020). As drop fruits may be both wounded and exposed to soil, they may introduce spoilage inoculum and harmful foodborne disease causing agents (Ewing and Rasco, 2018; Guo et al., 2002), including the patulin-producing fungi highlighted here. Future research could investigate risk factors for introducing patulin-producing spoilage inoculum in hard ciders that originate from practices related to fruit harvesting and processing.

Our results demonstrate that *P. niveus*, unlike the other three tested *Penicillium spp.*, can not only survive apple processing and bench-top fermentation, but can also grow in low-oxygen, finished hard cider product. Food spoilage by *P. niveus* is a long-standing problem for fruit juices and products and has been found in apple juice production facilities and in various fruit products (Salomão et al., 2014; Santos et al., 2018). *Paecilomyces niveus* ascospores can also survive high temperatures, making this food-spoiling agent troublesome for fruit processing facilities (Biango-Daniels et al., 2019; Taniwaki et al., 2009).

Previous studies have explored strategies for patulin mitigation in solid foods and fruit juices (Ioi et al., 2017; Moake et al., 2005). Alcoholic fruit products are however not traditionally considered high-risk for patulin contamination, in part

because fermentation has been shown to significantly reduce patulin levels (Erdoğan et al., 2018; Stinson et al., 1978; Zhang et al., 2019). Our results raise the possibility that *P. niveus* spoilage inoculum remains a human health hazard in hard ciders, in that it may survive fermentation and grow in the finished fermented product. This concern extends to other fruit juice products as we observed patulin levels far above the 50 µg/kg in lemonade and orange juice inoculated with *P. niveus* spores. To help quantify the risk of *P. niveus* spoilage in hard cider products, next steps should include surveying patulin contamination and the incidence of *P. niveus* spoilage in finished ciders.

Bioassays testing the effectiveness of sulfur dioxide, potassium sorbate, and sodium benzoate showed variable effectiveness at restricting *P. niveus* growth after two weeks. We observed that under low (.05%) and moderate (.1%) concentrations of sulfur dioxide, significant *P. niveus* growth was noted as the presence of fungal hyphae. However, while sulfur dioxide is commonly used as preservatives to control fungal and bacterial growth in fruit juices, its effectiveness is highly pH dependent due to the mode of action of sulfurous acid (Silva and Lidon, 2016). It is possible that the pH of the apple cider we used (pH = 3.6) would need to be lowered for effective sulfur dioxide treatment. Both sodium benzoate and potassium sorbate, the salts of organic acids (benzoic acid and sorbic acid respectively), are also extensively used as preservatives in low pH foods (Silva and Lidon, 2016), and potassium sorbate is additionally used to help stop post-fermentation of unfermented sugars, especially in wines (Lück, 1990; Silva and Lidon, 2016). Moderate levels of both organic acids (.1% sodium benzoate and .06% potassium sorbate) in food-grade apple cider were observed to effectively restrict *P. niveus* hyphal growth over two weeks, compared to the control.

Results from this study help assess potential patulin contamination of apple

cider and hard cider by four patulin-producing apple pathogens, especially *P. niveus*. Our work also introduces a primer pair specific to the RPBII region of *Paecilomyces spp.* and a qPCR protocol to detect and quantify three troublesome food spoilage fungi: *P. niveus*, *P. fulvus*, and *P. variotii*. qPCR methods introduced here can be applied to future bioassays involving these three *Paecilomyces spp.* Patulin is a regulated mycotoxin by various regulatory agencies and risk of contamination is both an issue for consumer health and food production concern (Affairs, 2020; Commission, 2003; Commission Regulation, 2006). These insights raise a concerning potential connection between patulin and hard cider and will aid in optimizing apple fruit and cider production with respect to food safety and consumer health.

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