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Natamycin, a New Postharvest Biofungicide: Toxicity to Major Decay Fungi, Efficacy, and Optimized Usage Strategies

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by

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ABSTRACT OF THE DISSERTATION

Natamycin, a New Postharvest Biofungicide: Toxicity to Major Decay Fungi, Efficacy, and Optimized Usage Strategies

by

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Doctor of Philosophy, Graduate Program in Plant Pathology
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Dr. James E. Adaskaveg, Chairperson

In California, the main postharvest decay pathogens of citrus are *Penicillium digitatum* causing green mold and *Geotrichum citri-aurantii* causing sour rot. Both pathogens cause significant crop losses if fruit are not treated with fungicides. I sought to determine the efficacy of natamycin against *P. digitatum* and *G. citri-aurantii* in vitro and in fruit efficacy experiments. Natamycin is derived as a fermentation product from *Streptomyces* species. It is incompatible with oxidative sanitizers, thus, organic acids were tested as alternatives.

The results of this dissertation have been obtained from experiments on the 1) in vitro sensitivity of seven postharvest decay fungi; 2) efficacy of natamycin with different application methods in laboratory, experimental packingline and commercial packingline settings; and 3) in vitro sanitation and compatibility of natamycin with organic acids.

In vitro sensitivity studies established ranges of EC_{50} values for *P. digitatum* and *G. citri-aurantii* and other important decay fungi of stone and pome fruits. Distributions

of EC_{50} values were unimodal, and no resistance was identified. Natamycin was effective in inhibiting mycelial growth of all fungi tested at a range of 0.17 to 3.20 µg/ml.

In laboratory and packingline studies, natamycin reduced the incidence of green mold and sour rot of inoculated citrus fruits. Natamycin was compatible in mixtures and in sequential applications with a storage fruit coating, but not in mixtures with a packing fruit coating. Mixtures of natamycin and propiconazole in aqueous solutions or in storage fruit coatings were highly effective against both pathogens.

Citric and lactic acids were tested against *Escherichia coli* and *Pseudomonas syringae* in vitro, with or without the surfactant sodium dodecylbenzenesulfonate (SDBS). *P. syringae* was highly susceptible to acidic conditions. *E. coli* was effectively sanitized by heated organic acid solutions with SDBS. In fruit studies, natamycin was compatible with citric acid and SDBS against green mold. Lower pH values and higher temperatures improved the efficacy of citric acid and SDBS as a sanitizer.

In summary, this research determined baseline sensitivities of decay fungi to natamycin, optimized utilization of natamycin in a disease management program, and demonstrated effective sanitation of natamycin solutions using organic acids and surfactants.

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GENERAL INTRODUCTION

In the United States, the citrus industry produces a crop worth more than $3 billion annually and California accounts for 63% of that amount (USDA 2019). Postharvest decays are among the leading causes of crop loss for citrus fruits. Most decay fungi of citrus cause infection when spores enter wounds created in the field or during harvest and handling. If postharvest fungicides are not applied, 20 to 40% of harvested fruit may develop decay. There is a continuous need to prevent decay because citrus fruit are stored and processed almost year-round in California packinghouses (Eckert and Eaks 1989). Furthermore, a majority of fruit are distributed to distant national and international markets with high quality standards and low tolerances for decay (Adaskaveg et al. 2002).

The major decay pathogens of citrus in California are the Ascomycota fungi *Penicillium digitatum* and *Geotrichum citri-aurantii*. *P. digitatum* causes green mold, a soft rot with mycelium and green-colored conidia covering the surface of the decayed fruit. The pathogen produces large numbers of air-borne conidia that may contaminate the surface of nearly every fruit in citrus orchards and packinghouses (Eckert and Eaks 1989). Depending on market demands, lemon fruit in California are often stored for prolonged periods at 12 to 15°C and 92 to 98% relative humidity. Under these conditions, *G. citri-aurantii* can be economically devastating if not controlled. The fungus causes sour rot, which is a very soft decay that at high relative humidity is covered by creamy-white fungal mycelium and arthroconidia. Pectolytic enzymes excreted by the pathogen lead to complete breakdown of fruit tissues. The resultant liquids carrying conidia and

enzymes can drip onto nearby healthy fruit and rapidly spread the pathogen in storage, leading to more decay (Baudoin and Eckert 1982, 1985; Brown 1979; Davis and Baudoin 1986; Eckert 1959; McKay et al. 2012a; Suprapta et al. 1996).

Synthetic fungicides are used to manage postharvest decays in many countries. A difficult issue facing the citrus industry is the development of resistance in *P. digitatum* to most previously registered postharvest fungicides of citrus (Adaskaveg and Förster 2015b). Thus, long-term use of the fungicides imazalil and thiabendazole resulted in heavy economic losses in many California citrus packinghouses due to development of resistance in *Penicillium* spp. populations (Adaskaveg and Förster 2015; Eckert and Eaks 1989; Harding 1962, 1972). More recently registered alternatives such as azoxystrobin, fludioxonil, and pyrimethanil are very effective against *P. digitatum* but have a high resistance potential due to their single-site modes of action. Propiconazole is very effective against *G. citri-aurantii* and *P. digitatum*, but has the disadvantage of being cross-resistant with imazalil and therefore, does not control imazalil-resistant isolates of *P. digitatum* (McKay et al. 2012b, 2012c). There is a potential for resistance to develop in populations of *G. citri-aurantii* because only one efficacious fungicide is registered for managing this decay. Thus, there is a need to develop new fungicides that are efficacious, have a broad spectrum of activity against decay fungi, and have a low risk of selecting for resistance in targeted pathogen populations.

In recent years, there has been a push to develop new fungicides that are considered safer for workers, consumers, and the environment, and the US Environmental Protection Agency (EPA) established the biopesticide category. One

biofungicide that has been registered is polyoxin-D. This fungicide is a fermentation product of *Streptomyces cacaoi* var. *asoensis* and is formulated as a salt with zinc to stabilize the molecule. It is approved for field use on turf grasses and pre- and postharvest use on selected fruits and it was recently approved for use on organically grown crops.

Another recently registered biofungicide is natamycin (also known as pimaricin), an antifungal polyene macrolide produced by fermentation of selected *Streptomyces* species such as *S. natalensis* and *S. chattanoogensis* (Du et al. 2009). Natamycin has been used as a food preservative in the cheese, meat, and other food industries globally against molds and yeasts for decades. It is touted as a naturally derived mold inhibitor with no documented cases of viable resistance found in filamentous fungi on food products (Delves-Broughton 2014; Stark 1999, 2003). The molecule's solubility in water is low (approximately 40 µg/ml) where it exists as micelles, but the solubility is sufficient to be active against many fungal species found on food products with minimum inhibitory concentrations ranging from 1 to 10 µg/ml (Delves-Broughton 2014). Natamycin is stable at a broad pH range (pH 3 to 9) with an optimum between pH 6 and 7 (Raab 1972; Stark 2000), but is unstable in ultraviolet light which limits its uses in field applications (Gutteridge et al. 1983).

Natamycin has a unique mode of action and was assigned FRAC Code 48 by the Fungicide Resistance Action Committee. The fungicide directly binds to ergosterol in the fungal cell membrane, thereby immobilizing it and preventing the molecule from performing its normal cell functions. Exposure ultimately leads to death of the fungus (Abe and Hiraki 2009; Gray et al. 2012; te Welscher et al. 2008). Resistance has been

induced in species of the yeast genus *Candida*. However, resistance led to fitness penalties and was found to be unstable (Athar and Winner 1971). Natamycin potentially can be utilized as a treatment in postharvest pathosystems where fungicide resistance has occurred.

In previous studies, natamycin was demonstrated to be effective against postharvest decays of cherry, chestnut, citrus, grape, jujube, mulberry, and strawberry fruits (Chen et al. 2016; He et al. 2019; Jiang et al. 2007; Panagou et al. 2005; Saito et al. 2020). Natamycin is currently registered in the United States as a biofungicide for treatment for mushroom beds against bubble disease caused by *Lecanicillium fungicola*, for pre-plant application to strawberry transplants against anthracnose caused by *Colletotrichum fioriniae* and other species of *Colletotrichum*, and for postharvest application to citrus, stone, and some other fruit crops for the management of postharvest decays.

Postharvest fungicides are routinely applied in citrus packinghouses (Adaskaveg and Förster 2015; Eckert and Eaks 1989) using high- or low-volume systems (Adaskaveg et al 2002). Low-volume applications of fungicides in a fruit coating are effective and highly efficient with little run-off or wasted material. Some packinghouses use high-volume recirculating systems whereby the fungicide solution is applied from a reservoir as a spray, drench, or flood treatment to fruit (Adaskaveg et al. 2002). In the United States, as mandated by the Food Safety Modernization Act (FSMA), water in contact with harvested crops must be sanitized to prevent the accumulation and spread of bacterial contaminants, particularly *Escherichia coli*, which may cause disease in humans

(US Congress 2011, US-FDA 2017). *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Salmonella* spp. are other human pathogenic bacteria of concern (Schroth et al. 2018; Strawn et al. 2013).

Two commonly used sanitizers for fungicide solutions in citrus packinghouses are sodium hypochlorite (NaOCl) and peroxyacetic acid, also known as peracetic acid. The anti-microbial action of both is based on oxidative processes. A common issue with oxidative sanitizers is the production of irritating odors impacting packinghouse workers. Thus, it is important to identify alternative modes of sanitation. Natamycin has been shown to be unstable in the presence of oxidizers (Dekker and Ark 1959), and this could prevent the use of natamycin in recirculating fungicide systems that need to be sanitized. Organic acid-based sanitizers are potential alternatives for use with natamycin. Unlike oxidative sanitizers, organic acids produce low or no odor and are not affected by organic load. Organic acids create low pH conditions in solutions that are inhibitory to many genera of bacteria. Low pH causes disruptions in cell regulation processes, increasing the energy usage by bacteria to maintain their internal pH. This in turn leads to the production of alkaline metabolites and free radicals that cause irreversible damage to the bacterial cell components (Boomsma et al. 2015; Fatica and Schneider 2009).

The organic lactic and citric acids were identified as possible sanitizers for natamycin. Both are Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (US-FDA). Several commercial sanitizing products contain citric or lactic acids, and these are currently in use for decontamination of hard surfaces, disinfestation of produce, and sanitation of agricultural water (Boomsma et al. 2015; Fatica and

Schneider 2009). Sodium dodecylbenzenesulfonate (SDBS) is a food-grade surfactant (US-FDA 2012) that can improve the killing potential of citric and lactic acids against *E. coli*. Surfactants improve the action of organic acids by fluidizing bacterial cell membranes, weakening them, and causing increased susceptibility to the action of organic acids (Jackson-Davis et al. 2018; Predmore and Li 2011).

The objectives of the following studies were to: 1) observe spore germination characteristics of selected postharvest decay fungi in the presence of natamycin as compared with a demethylation-inhibiting fungicide and to determine the in vitro sensitivities of seven major postharvest decay fungi (including *P. digitatum* and *G. citri-aurantii*) to natamycin to establish baseline references for future resistance monitoring; 2) elucidate the efficacy of natamycin by itself and in mixtures with other fungicides when applied with or without fruit coatings to citrus fruits using spray or flooder applications in laboratory as well as in experimental and commercial packingline studies; and 3) ascertain the compatibility of sanitizers with natamycin in vitro and in laboratory and experimental packingline studies using spray and recirculating flooder application systems.

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CHAPTER I. Baseline Sensitivities of Major Postharvest Pathogens of Citrus, Pome, and Stone Fruits to Natamycin and Estimation of the Resistance Potential in *Penicillium digitatum*

ABSTRACT

Natamycin is a biofungicide that was recently registered for postharvest use on citrus and stone fruits in the United States. It has been used as a food preservative for many decades, with no resistance ever observed. Baseline sensitivities for mycelial growth of 43 to 72 isolates of seven postharvest pathogens to natamycin were determined using the spiral gradient dilution method. Mean effective concentrations to inhibit mycelial growth by 50% (EC_{50} values) were 0.90 μg/ml for *Alternaria alternata*, 0.76 μg/ml for *Botrytis cinerea*, 3.20 μg/ml for *Geotrichum citri-aurantii*, 0.17 μg/ml for *Monilinia fructicola*, 1.54 μg/ml for *Penicillium digitatum*, 1.14 μg/ml for *P. expansum*, and 0.48 μg/ml for *Rhizopus stolonifer*. Distributions of EC_{50} values for each pathogen were unimodal and mostly normal with no outliers detected. Natamycin was also inhibitory to spore germination with inhibitory values for five of the species similar to those for mycelial growth. Microscopically, natamycin generally arrested spores at the pre-germination swelling stage. Mass platings of conidial mixtures of ten isolates of *P. digitatum* were done onto agar media with 2.5-log radial concentration gradients of natamycin or fludioxonil, and platings of *G. citri-aurantii* were done onto media amended with natamycin or propiconazole. No resistant isolates were observed for both species to natamycin or for *G. citri-aurantii* to propiconazole, whereas a resistance

frequency of 4.5 x 10^{-6} to 3.1 x 10^{-6} was calculated for *P. digitatum* to fludioxonil. The wide spectrum of activity against different fungal pathogens and a low resistance potential support the registration of natamycin as a postharvest treatment and its integration into a management program with other practices including other fungicides.

INTRODUCTION

Postharvest fungal decays are one of the leading causes of crop loss worldwide for tree fruits. Infections by most decay fungi typically occur when spores enter wounds occurring in the field or during harvest and handling. For citrus, 20 to 40% of harvested fruit often develop decay if no postharvest fungicides are applied (Eckert and Eaks 1989). Major decay pathogens of citrus are *Penicillium digitatum* and *Geotrichum citri-aurantii*, those of pome fruits are *P. expansum* and *Alternaria alternata*, and those of stone fruits are *Monilinia fructicola* and *Rhizopus stolonifer*, whereas *Botrytis cinerea* affects numerous crops including stone, pome, and citrus fruits. Synthetic fungicides are used to manage postharvest decays with high efficacy in many countries. In recent years, however, there has been an increasing trend to develop natural products as fruit treatments that are considered safer to packinghouse personnel, consumers, and the environment. One such natural product is the antifungal polyene macrolide natamycin, also known as pimaricin.

Natamycin is a fermentation product of certain species of *Streptomyces* such as *S. natalensis* and *S. chattanoogensis* (Du et al. 2009). Natamycin is a globally permitted food preservative and has been used in the cheese, meat, and other food industries against

molds and yeasts for many years where it is touted as a natural mold inhibitor (Delves-Broughton 2014; Stark 1999, 2003). It was shown to be active against many fungi of food products with minimum inhibitory concentrations from 1 to 10 µg/ml but is non-toxic to humans (Delves-Broughton 2014). Even with its extensive use, there have been no documented cases of viable resistance development to natamycin in filamentous fungi on food products (Delves-Broughton 2014). Resistance has been described in the laboratory for species of the yeast genus *Candida*, but it led to fitness penalties and was characterized as unstable (Athar and Winner 1971). Thus, natamycin potentially can be used as a fungicide in postharvest pathosystems where resistance in fungal pathogens has challenged the management of decays. For example, *P. digitatum* has developed resistance to most previously registered postharvest fungicides of citrus (Adaskaveg and Förster 2015b).

Natamycin has a unique mode of action and immobilizes ergosterol in the fungal cell membrane by direct binding and subsequently prevents normal cell functions, leading to cell death (Abe and Hiraki 2009; Gray et al. 2012; te Welscher et al. 2008). Microscopic evaluations on the effect of natamycin on spore germination of major fruit pathogens in comparison with other fungicides that affect sterols such as the demethylation inhibitors have not been done. This information may provide insights into understanding the direct effects of these postharvest fungicides on fungal spores.

Because natamycin is unstable under ultraviolet light **(**Gutteridge et al. 1983**),** its current agricultural uses are limited. In previous studies, aqueous solutions of natamycin were shown to be effective in preventing decay caused by various postharvest pathogens

of cherry, strawberry, chestnut, mulberry, grape, jujube, and mandarin fruits (Jiang et al. 2007, Panagou et al. 2005, He et al. 2019; Saito et al. 2020). Natamycin is registered in the United States as an Environmental Protection Agency (EPA)-approved bio-pesticide for treatment for mushroom beds against bubble disease caused by *Lecanicillium fungicola*, as a pre-plant treatment for strawberry plants against anthracnose caused by *Colletotrichum fioriniae* and other species of *Colletotrichum*, and as a postharvest treatment to manage major decays of citrus and stone fruits, as well as other crops (Environmental Protection Agency 2016).

To evaluate the potential of natamycin as a postharvest treatment against decays of a range of fruit crops, one objective of this study was to determine its toxicity based on effective concentrations to inhibit mycelial growth and spore germination by 50% (EC_{50} values). For this, I used populations of seven species of major decay fungi obtained from tree fruits and soil from numerous geographic locations. The goal was to establish baseline sensitivity ranges that can be used in future monitoring of sensitivities shifts and to determine if isolates that were previously characterized as imazalil- or thiabendazole-resistant are sensitive to natamycin. I also compared spore germination characteristics for *M. fructicola, P. digitatum*, and *R. stolonifer* in the presence of natamycin and the C14-demethylation inhibitor propiconazole that is also registered as a postharvest fungicide. Another objective was to estimate the in vitro potential of resistance development in populations of *P. digitatum* and *G. citri-aurantii* to natamycin.

MATERIALS AND METHODS

Fungicides used. Wettable powder formulations of natamycin (DelvoCid Instant 50WP; DSM Food Specialties B.V., Delft, The Netherlands), fludioxonil (Scholar WP; Syngenta Crop Protection, Greensboro, NC), and propiconazole (Mentor 45WP; Syngenta Crop Protection) were used. Solutions were prepared in sterile deionized water.

Fungal isolates. Fungal isolates used in the study are listed in Table 1.1 and mostly originated from commercial orchards or packinghouses. Selected isolates of *P. digitatum* were previously characterized for their sensitivity to imazalil and thiabendazole (TBZ) and of *P. expansum* for sensitivity to TBZ (Kanetis et al. 2008; Adaskaveg *unpublished*). For spore production, *G. citri-aurantii*, *P. digitatum*, *P. expansum*, and *R. stolonifer* were cultured on potato dextrose agar (PDA; BD Diagnostic Systems, Sparks, MD), *B. cinerea* on King's medium B agar (KMB; King et al. 1954) under laboratory lighting (700 lux; Daylight Full Spectrum, 5,000K, 40W; Osram Sylvania, Danvers, MA), and *M. fructicola* on non-clarified V8 agar (Ribeiro 1978) in the dark. *A. alternata* was cultured on 5% PDA under fluorescent light (2,200 lux; Daylight Full Spectrum). All isolates were maintained in liquid nitrogen for long-term storage. Spore suspensions were prepared from 3- to 5-day-old (*G. citri-aurantii*, *R. stolonifer*), 7-day-old (*P. digitatum*, *P. expansum*, *M. fructicola*), or 10- to 14-day-old (*A. alternata*, *B. cinerea*) cultures.

Determination of EC_{50} values for natamycin for mycelial growth and spore germination using the SGD method. Procedures were followed as published previously (Förster et al. 2004; Kanetis et al. 2008). Natamycin concentration gradients were established by applying stock concentrations of 2000 µg/ml (for *G. citri-aurantii*) or 1000

µg/ml (for the other fungi) to 15-cm diameter PDA plates using a spiral plater (AutoPlate 4000; Advanced Biotech, Norwood, MA) set at the exponential deposition mode. This resulted in radial concentration ranges (from the edge to the center of the plates) from approximately 0.08 µg/ml to 15 µg/ml or 0.04 µg/ml to 7.5 µg/ml for the two stock concentrations, respectively. Water was applied to control plates. After 2 to 4 h, aliquots of 10 µl of conidial suspensions (5×10^5 conidia/ml for *A. alternata, B. cinerea, M. fructicola,* and *R. stolonifer*; 1×10^6 conidia/ml for *G. citri-aurantii, P. digitatum,* and *P. expansum*) were radially streaked along the concentration gradients using a disposable plastic pestle (Fisher Scientific, Pittsburgh, PA). On each plate, four isolates were streaked out with opposing duplicate streaks. Plates were then placed randomly into plastic containers and incubated in the dark at 20°C for 20 h (*R. stolonifer*) or at 25°C for three days (all other species). For each isolate, the radial distance from the center of the plate to where growth was inhibited by 50% as compared to the controls was determined, and local concentrations of natamycin (EC_{50} values) were calculated using the Spiral Gradient Endpoint program (SGE; Advanced Biotech). Each fungal isolate was evaluated in three experiments.

Evaluation of multiple resistance in *P. digitatum* and *P. expansum.* Natamycin EC_{50} values for isolates of *P. digitatum* and *P. expansum* that were previously characterized as either sensitive or resistant to TBZ (both species) and/or imazalil (*P. digitatum*) (Kanetis et al. 2008; McKay et al. 2012) were compared. Fourteen isolates were used for each group except for *P. digitatum* where 8 isolates were sensitive to imazalil and resistant to TBZ, and 13 isolates were sensitive to both fungicides.

Comparison of EC_{50} values for natamycin for inhibition of mycelial growth and spore germination. For three representative isolates of each species, EC_{50} values for spore germination were compared to those for mycelial growth using the same natamycin stock concentrations for preparing SGD plates as indicated above. For determining inhibitory values for spore germination, SGD plates were examined microscopically at 100× magnification after 10 h (*R. stolonifer*) or 15 h (*A. alternata, B. cinerea, G. citri-aurantii, M. fructicola, P. digitatum, P. expansum*) at 25°C as described previously (Förster et al. 2004), and natamycin concentrations were calculated using the SGE program. A spore was considered germinated if the germ tube length was equal to or longer than twice the spore diameter. Each fungal isolate was evaluated in three independent experiments. Mean EC_{50} values for spore germination and mycelial growth for each isolate were then plotted against each other to visualize differences in sensitivity between the two fungal growth stages. In addition, spore germination characteristics of *M. fructicola, P. digitatum,* and *R. stolonifer* on non-amended and amended agar were compared using stock concentrations of 1000 µg/ml natamycin or 50 to 2000 µg/ml propiconazole for generating fungicide concentration gradients. Micrographs were obtained after 10 h (*R. stolonifer*) or 15 h (*M. fructicola, P. digitatum*) using a Zeiss Primo Star microscope (Carl Zeiss AG, Oberkochen, Germany).

Determination of the natural resistance frequencies of *P. digitatum* and *G. citri-aurantii* to selected postharvest fungicides. The stock concentration of natamycin to be used to locate the average EC_{95} value of isolates of *P. digitatum* and *G. citri-aurantii* approximately 15 to 20 mm from the edge of a 15-cm diameter petri dish was

calculated as 20,000 µg/ml using the SGE program. For fludioxonil, a stock concentration of 500 µg/ml was used for *P. digitatum* (Kanetis et al. 2010) and for propiconazole, a stock concentration of 3,000 µg/ml was used for *G. citri-aurantii* (McKay et al. 2012). Fungicides were applied to 15-cm diameter petri dishes containing PDA using a spiral plater in the exponential deposition mode as described above. After 2 to 3 h, conidial suspensions (1×10^8 to 2×10^8/ml) of equal parts of ten randomly selected isolates of *P. digitatum* (four of them resistant to imazalil and all sensitive to TBZ, fludioxonil, and azoxystrobin) or *G. citri-aurantii* (all isolates sensitive to propiconazole) collected from decaying fruit throughout California was prepared, and 60 µl were applied uniformly to each plate using the "lawn" deposition mode of the spiral plater. After 5 to 7 days of incubation in the dark at 25°C, plates were evaluated for the presence of *P. digitatum* or *G. citri-aurantii* colonies developing at concentrations higher than those of the respective average EC_{95} values. Resistance frequencies were calculated based on the number of *P. digitatum* or *G. citri-auantii* colonies growing at concentrations higher than the EC_{95} value of the total number of conidia deposited inside the EC_{95} zone. Three plates were used for each fungicide in each of two experiments.

Statistical analysis of data. Statistical analyses were performed using SAS (ver. 9.4, SAS Institute, Cary, NC) with a significance value of $\alpha = 0.05$. Shapiro-Wilk probability values were calculated for mean EC_{50} values for mycelial growth inhibition for isolates of each pathogen and fungicide to determine if values were normally distributed. Frequencies of mean EC_{50} value categories for each fungicide were then plotted as histograms (i.e., Scott's distributions) with bin widths determined using the

formula $H_n=3.49sn^{-1/3}$ with *s* being an estimate of the standard deviation and *n*, the number of isolates tested (Scott 1979). The number of bins for each fungal species and fungicide was determined based on bin width and EC_{50} range.

For comparing EC_{50} values for natamycin for spore germination and mycelial growth inhibition, as well as for comparing EC_{50} values among isolates of *P. digitatum* or *P. expansum* sensitive or resistant to other postharvest fungicides, one-way analyses were performed using generalized linear mixed models with the GLIMMIX procedure. For this, fungal species or groups of isolates of *P. digitatum* or *P. expansum* characterized for imazalil or TBZ resistance were treated as fixed effects, and replication and the overall error term were treated as random effects. Fixed effects were tested for significance, and least squares treatment means were determined using the lsmeans statement with the Tukey adjustment in PROC GLIMMIX. Contrast estimates for pairs of treatments were used to determine multiple comparison differences among treatment means.

RESULTS

Determination of EC_{50} values for natamycin for mycelial growth of seven postharvest decay pathogens. Fungal species varied in their growth responses to natamycin concentration gradients on SGD plates. For *B. cinerea* (Fig. 1.1.C), *P. expansum* (Fig. 1.2.A), and *G. citri-aurantii* (Fig. 1.2.E) with a blunt-ended growth pattern, there was a very sudden shift from full growth to no growth over a very narrow concentration range. For *A. alternata* (Fig. 1.1.A), *P. digitatum* (Fig. 1.2.C), and *R. stolonifer* (Fig. 1.1.G), this shift occurred over a somewhat wider concentration range,

whereas for *M. fructicola* (Fig.1.1.F) with a pointed growth pattern, growth was more gradually reduced with increasing concentrations of natamycin.

Narrow ranges of EC_{50} values were observed among isolates of each of the seven pathogens with between 2.0-fold (i.e., *P. expansum*) and 6.4-fold (i.e., *B. cinerea*) differences for the most and least sensitive isolates (Table 1.2). The range of EC_{50} values was lowest for *M. fructicola* (i.e., 0.08 µg/ml to 0.37 µg/ml) and highest for *G. citri-aurantii* (i.e., 1.82 µg/ml to 5.60 µg/ml). In the frequency histograms of EC_{50} values for each species (Fig. 1.1.B, D, F, H, Fig. 1.2.B, D), no outliers considered to be resistant to natamycin were present in the higher concentration ranges. Shapiro-Wilk probabilities for log_{10}-transformed EC_{50} values were not significant ($P \geq 0.205$) for *A. alternata, B. cinerea, G. citri-aurantii, M. fructicola,* and *P. expansum* (Table 1.2), indicating that values were normally distributed for each species. For *P. digitatum* and *R. stolonifer, P* values were significant ($P = 0.006$ and $P = 0.0002$, respectively), indicating a deviation from normality.

Evaluation of multiple resistance in *P. digitatum* and *P. expansum.* EC_{50} values for mycelial growth inhibition by natamycin were not significantly different ($P = 0.11$) for *P. digitatum* isolates either sensitive to imazalil and TBZ, resistant to both fungicides, or resistant to one of the two fungicides (Table 1.3). Similarly, EC_{50} values for natamycin for TBZ-sensitive and –resistant isolates of *P. expansum* were similar ($P = 0.19$).

Comparison of EC_{50} values for natamycin for inhibition of mycelial growth and spore germination. The relationship between EC_{50} values for mycelial growth and

spore germination for three representative isolates of each of the seven decay pathogens is shown in Fig. 1.3. There were significant differences between the two values for *R. stolonifer* ($P = 0.0003$) and *M. fructicola* ($P = 0.0142$). Mean EC_{50} values for three isolates of each of the two species of 0.52 and 0.16 µg/ml for mycelial growth were significantly lower than those for spore germination with values of 1.56 and 0.24 µg/ml, respectively. For the other five species, EC_{50} values for the two growth stages were statistically similar ($P \geq 0.12$).

Microscopic observation of spore germination in the presence of natamycin and propiconazole. Spore germination of *R. stolonifer, P. digitatum,* and *M. fructicola* was observed microscopically on non-amended agar and over a wide range of natamycin and propiconazole concentrations that included EC_{50} and EC_{95} inhibitory values (Figs. 1.4,1.5). Spore germination on non-amended agar is shown in Fig. 1.4.A for *R. stolonifer*, in Fig. 1.4.F for *M. fructicola,* and in Fig. 1.5.A for *P. digitatum*. At 50% inhibition ranges of natamycin, spores were swollen and germ tubes of all three fungi were often shorter and became distorted (Figs. 1.4.B, 1.4.G, 1.5.B). Spores of *R. stolonifer* often germinated with multiple germ tubes, and branching was reduced (Fig. 1.4.B). At concentrations higher than the respective EC_{95} values (i.e., 3 µg/ml, 2 µg/ml, and 0.5 µg/ml for *R. stolonifer, P. digitatum,* and *M. fructicola*, respectively), spores generally were arrested at the pre-germination swelling stage (Figs. 1.4.C, 1.4.H, 1.5.C).

In the presence of propiconazole concentrations near the EC_{50} inhibitory values for mycelial growth that were determined to be 0.6 to 1.3 µg/ml for *R. stolonifer*, 0.01 to 0.02 µg/ml for *P. digitatum*, and <0.006 µg/ml for *M. fructicola*, germ tubes were

distorted and sometimes highly branched (Figs. 1.4.D, 1.4.I, 1.5.D). Spores of the three fungi were not completely inhibited in germination even at concentrations well beyond 95% inhibitory concentrations for mycelial growth (Figs. 1.4.E, 1.4.J, 1.5.E) that were determined to be 2.2 to 3.5 µg/ml for *R. stolonifer*, 0.04 µg/ml for *P. digitatum*, and 0.1 µg/ml for *M. fructicola*. Germ tubes lengths often were still twice as long as spore diameters, and therefore, inhibitory concentrations for spore germination could not be determined.

Determination of the natural resistance frequencies of *P. digitatum* and *G. citri-aurantii* to selected postharvest fungicides. In mass platings of conidial mixtures of *P. digitatum* to fungicide-amended SGD plates, approximately 4 x 10^6 to 8 x 10^6 conidia were deposited onto the area of each plate where fungicide concentrations exceeded the average EC_{95} values (i.e., 3.0 µg/ml for natamycin and 0.13 µg/ml for fludioxonil). After 5 to 7 days of incubation, no colonies were present on natamycin-amended plates at concentrations >3.0 µg/ml (Fig. 1.6.A). On fludioxonil-amended plates, between 13 and 23 colonies developed at concentrations >0.13 µg/ml (Fig. 1.6.B), and a natural resistance frequency of 4.5 x 10^{-6} to 3.1 x 10^{-6} was calculated based on the total number of conidia exposed.

In mass platings of conidial mixtures of *G. citri-aurantii*, a similar number of conidia as indicated above were deposited onto the area of each plate where natamycin or propiconazole concentrations exceeded the average EC_{95} values of 3.98 µg/ml or 0.78 µg/ml, respectively. After 5 to 7 days of incubation, no colonies were present on plates amended with either fungicide (Fig. 1.6.C,D).

DISCUSSION

In this study, I demonstrate the in vitro activity of natamycin against important postharvest fungal pathogens. Among these, *A. alternata, B. cinerea,* and *R. stolonifer* have a wide host range and commonly cause postharvest decays on many different fruit crops, whereas *P. digitatum* and *G. citri-aurantii* only infect citrus fruits, and *P. expansum* mostly causes losses in pome fruits. Inhibition of mycelial growth and spore germination of these fungi support the registration of natamycin as a postharvest fungicide treatment with a wide spectrum of activity. Furthermore, it is only the second single-site mode of action postharvest fungicide registered that is very efficacious against both Penicillium decays and sour rot. In this study, isolates of each fungal species originated mostly from fruit production areas throughout California, and they were collected before the commercial use of natamycin on fruits. Therefore, sensitivity ranges represent a baseline distribution that can be used in resistance monitoring in the future.

EC_{50} values that I determined for natamycin for the seven species of postharvest pathogens were within similar ranges as reported previously for a variety of fungi. For example, EC_{50} values for mycelial growth of *B. cinerea* isolates from mandarin fruit ranged from 1.021 to 2.007 µg/ml (Saito et al. 2020) and for *Colletotrichum acutatum* from strawberry from 0.526 to 1.996 µg/ml (Haack 2018), whereas minimum inhibitory concentrations for forty fungal species were between 1 and 10 µg/ml (Delves-Broughton 2014). In comparison with some of the newer, conventional postharvest fungicides evaluated previously, the in vitro toxicity of natamycin is lower (i.e., higher EC_{50} values). For example, EC_{50} values for fludioxonil and azoxystrobin against *P. digitatum* ranged

from 0.007 to 0.028 µg/ml and from 0.009 to 0.072 µg/ml, respectively (Kanetis et al. 2008), and for fludioxonil against *B. cinerea* from 0.003 to 0.038 µg/ml (Zhao et al. 2010). For pyrimethanil, EC_{50} values for *B. cinerea* isolates were between 0.034 and 0.049 µg/ml (Sholberg et al. 2005). With EC_{50} values for natamycin between 1.82 and 5.60 µg/ml, *G. citri-aurantii* was the least sensitive of the seven fungal species evaluated in my study. This compares to a range of 0.10 to 0.83 µg/ml for propiconazole in a study where many of the same isolates of *G. citri-aurantii* were used (McKay et al. 2012) as in my current evaluations. The lower in vitro toxicity of natamycin as compared to many conventional fungicides is reflected by its sometimes reduced efficacy in managing postharvest decays irrespective of rate or application method used (Adaskaveg et al. 2019; Chen et al. 2020). Still, as an EPA-approved bio-pesticide, natamycin shows unprecedented efficacy against numerous postharvest diseases (Adaskaveg and Förster 2015a; Adaskaveg et al. 2019; Chen et al. 2016; Saito et al. 2020). Furthermore, natamycin was similarly toxic to isolates of *P. digitatum* and *P. expansum* sensitive or resistant to TBZ and to isolates of *P. digitatum* sensitive or resistant to imazalil or resistant to both fungicides (i.e., imazalil and TBZ). Thus, isolates resistant to other postharvest fungicides were sensitive to natamycin and there was no multiple resistance to this fungicide. TBZ and imazalil have been widely used as postharvest treatments since the 1970s and 1980s, respectively, and resistance is widespread in *Penicillium* spp. populations especially in California citrus packinghouses.

The SGD method proved to be valuable in the efficient evaluation of many isolates of each species. In contrast to the original description of the method (Förster et

al. 2004), I streaked spore suspensions along the natamycin concentration gradients, and mycelial growth was measured following spore germination. This was done because some fungi are difficult to grow on cellophane strips, but it also made the method more time-efficient. This strategy is justified because toxicity of natamycin to spore germination was similar to that of mycelial growth for most of the species evaluated. Additionally, mycelial growth was evaluated after longer incubation periods when growth responses of the fungus to the fungicide had developed.

Spore germination of *R. stolonifer, P. digitatum,* and *M. fructicola* was observed microscopically on natamycin- and propiconazole-amended media. In the presence of natamycin that directly binds to ergosterol and inactivates its functions in the fungal cell (te Welscher et al. 2008), germination was completely inhibited at higher concentrations. This agrees with studies by van Leeuwen et al. (2012) on the transcriptome of conidia of *Aspergillus niger*, where natamycin at 2 µg/ml inhibited spore germination. In contrast, in the presence of propiconazole, a fungicide that is used in the management of decays caused by these fungi on citrus and stone fruits and that inhibits the C-14 demethylation step in ergosterol biosynthesis (Ragsdale 1975), germination still occurred even at concentrations well beyond EC_{95} inhibitory values for mycelial growth. Therefore, in the presence of propiconazole, preformed sterols inside the spores apparently allow germination and growth until they are exhausted, whereas in the presence of natamycin, the normal functioning of these preformed sterols seems to be blocked immediately. These two modes of action targeting sterols and subsequent membrane function in fungi were clearly differentiated in microscopic observations.

EC_{50} values for natamycin of each of the fungi evaluated exhibited unimodal distributions, and no outliers with greatly reduced sensitivity were detected. This is an indication of a lower resistance potential in the pathogen populations (Gray et al. 2018). A low risk for resistance development was also demonstrated in my studies with mass platings of conidia of *P. digitatum* and *G. citri-aurantii* onto agar media amended with a natamycin concentration gradient where no colonies developed at concentrations higher than the EC_{95} values. In contrast, a natural resistance frequency of 4.5×10^{-6} to 3.1×10^{-6} for *P. digitatum* was calculated for fludioxonil, and this agrees with previous studies (Kanetis et al. 2010). No isolates of *G. citri-aurantii* resistant to propiconazole were detected similar to research done by McKay et al. (2012). There have been no reports of viable resistance in filamentous fungi to natamycin (Delves-Broughton 2014; Stark and Tan 2003). Furthermore, in transfers of 20 isolates of *Aspergillus* and *Fusarium* spp. to increasing concentrations of natamycin for prolonged time, inhibitory concentrations for four isolates increased only by approximately 2-fold (Streekstra et al. 2016). Therefore, natamycin has the potential to be used to manage fungicide resistance in problematic postharvest pathosystems such as decays of citrus (Adaskaveg and Förster 2015b), stone fruits, and other crops (Adaskaveg and Förster 2019) by integration with other fungicides.

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Table 1.1. Isolates of postharvest decay pathogens used in this study

Fungal species	Source	Collection date	No. of isolates
Alternaria alternata [a]	*Malus sylvestris*	2011	18
	Pyrus pyrifolia	1999-2009	7
	Pyrus communis	2005	18
Botrytis cinerea [b]	*Actinidia deliciosa*	1993	1
	Fragaria x ananassa	1993	1
	Malus sylvestris	2012	7
	Prunus armeniaca	2010	2
	Prunus avium	1992-1996	12
	Prunus domestica	1992-1993	2
	Prunus persica	1994-2000	3
	P. persica var. *nucipersica*	2005	1
	Prunus salicina	2000	2
	Pyrus communis	1993-2006	17
	Pyrus pyrifolia	1999-2001	12
Geotrichum citri-aurantii [c]	*Citrus limon*	2006-2008	28
	Citrus x *paradisi*	2007	1
	C. sinensis	2006-2007	5
	Citrus soil	1963-2008	38
Monilinia fructicola [d]	*Malus sylvestris*	1981	1
	Prunus avium	1980, 1996	2
	Prunus dulcis	1999-2013	7
	Prunus persica	1977-2010	25
	P. persica var. *nucipersica*	1980-2000	11
	Prunus salicina	1997-2010	11
Penicillium digitatum [c]	*Citrus limon*	2003-2004	45
	Citrus x *paradisi*	2003	2
	C. sinensis	2003-2004	12
Penicillium expansum [a]	*Malus sylvestris*	2008	32
	Pyrus communis	2001-2006	31
Rhizopus stolonifer [d]	*Fragaria x ananassa*	2015	2
	Prunus dulcis	2014-2015	51
	Soil	2015	10

[a] Isolates of *A. alternata* from apple, European pear, and Asian pear were collected in Fresno and Yuba Co., CA. Isolates of *P. expansum* came from European pear (several counties in CA and WA), and apple (several counties in WA).

[b] Isolates of *B. cinerea* from kiwifruit were collected in Sutter Co., CA, from apple in Yakima Co., WA, from nectarine in Fresno Co., CA, from apricot in Yolo Co., CA, from cherry in San Joaquin Co., CA, from nectarine, plum, peach in Fresno Co., CA, from prune in Tehama Co., CA, from European pear in Yuba Co., CA, from Asian pear in Fresno Co., CA, and from strawberry in Ventura Co., CA.

[c] Isolates of *G. citri-aurantii* and *P. digitatum* from grapefruit, lemon, and orange were collected in Fresno, Kern, Riverside, San Bernardino, Tulare, and Ventura Co., CA; and Maricopa and Yuma Co., AZ. 28 isolates of G. citri-aurantii were obtained from soil in California (Kern, Tulare, and Ventura Co.), and 11 isolates came from Florida, Argentina, China, Israel, Mexico, Nepal, and Trinidad.

[d] Isolates of *M. fructicola* from *Prunus* spp. were collected in California (Butte, Fresno, Kern, Kings, Riverside, San Joaquin, Stanislaus, Sutter, Tulare, and Yuba Co.) and from AZ (one isolate); one isolate from apple originated from Placer Co., CA. Isolates of *R. stolonifer* were collected from almond (Colusa, Fresno, Kern, Stanislaus Co., CA), strawberry (Riverside, Co., CA), and from soil (Fresno, Co., CA).

Table 1.2. In vitro sensitivity ranges and mean EC_{50} values for inhibition of mycelial growth of seven postharvest decay pathogens for natamycin

Fungal species	No. of isolates (n)	Mycelial EC_{50} values (µg/ml)[a]		
		Range	Mean	*P* values[b]
Alternaria alternata	43	0.57 - 1.34	0.90	0.205
Botrytis cinerea	60	0.25 - 1.54	0.76	0.634
Geotrichum citri-aurantii	72	1.82 - 5.60	3.20	0.670
Monilinia fructicola	57	0.08 - 0.37	0.17	0.805
Penicillium digitatum	59	0.88 - 2.20	1.54	0.006
Penicillium expansum	63	0.78 - 1.55	1.14	0.453
Rhizopus stolonifer	63	0.18 - 0.70	0.48	0.0002

[a] EC_{50} values were determined using the spiral gradient dilution method for all isolates of each species.

[b] Shapiro-Wilk probabilities for estimating normality of each distribution were calculated from log_{10}-transformed inhibitory values, and *P* values are shown.

Table 1.3. Comparison of natamycin EC_{50} values for isolates of *Penicillium digitatum* and *P. expansum* sensitive (S) or resistant (R) to imazalil and/or thiabendazole[a]

Pathogen	IMZ	TBZ	n	Natamycin EC_{50}	Lsmeans Tukey[b]
Penicillium digitatum	R	R	14	1.65	A
	R	S	14	1.48	A
	S	R	8	1.74	A
	S	S	13	1.45	A
Penicillium expansum	---[c]	R	14	1.25	A
	---	S	14	1.17	A

[a] EC_{50} values for imazalil (IMZ)- and thiabendazole (TBZ)-resistant isolates were >1.5 and >25 µg/ml, respectively.

[b] Statistical comparisons were made separately for each of the two fungal pathogens.

[c] *P. expansum* was not tested against imazalil because this fungicide is not used on pome fruit crops.

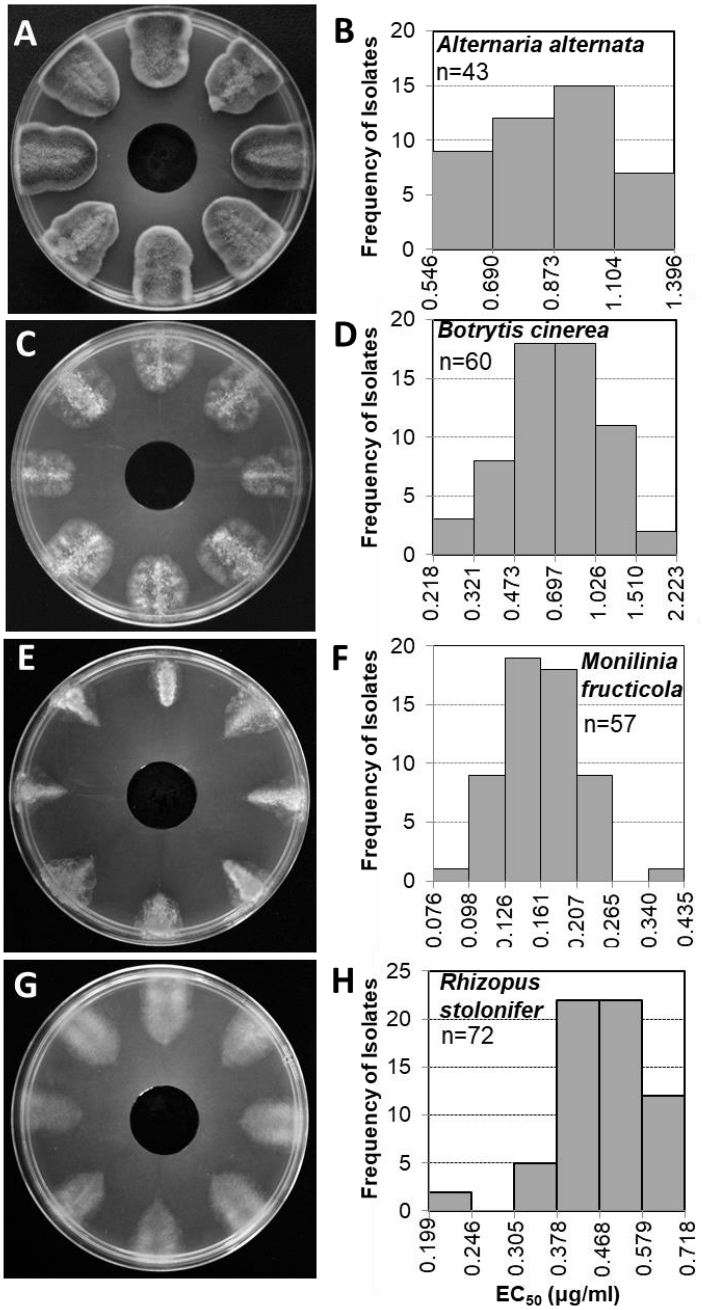

Fig. 1.1. Spiral gradient dilution plates with exponential gradients of natamycin concentrations (the highest concentration is in the center of the plate) to determine effective concentrations to inhibit mycelial growth by 50% (EC_{50} values) of four representative isolates (replicated oppositely on each plate) of each pathogen and frequency histograms of EC_{50} values of **A,B,** *Alternaria alternata*, **C,D,** *B. cinerea,* **E,F,** *M. fructicola,* and **G,H,** *R. stolonifer,* respectively. Bar height indicates the number of isolates within each bin, and bin width was calculated based on Scott (1979).

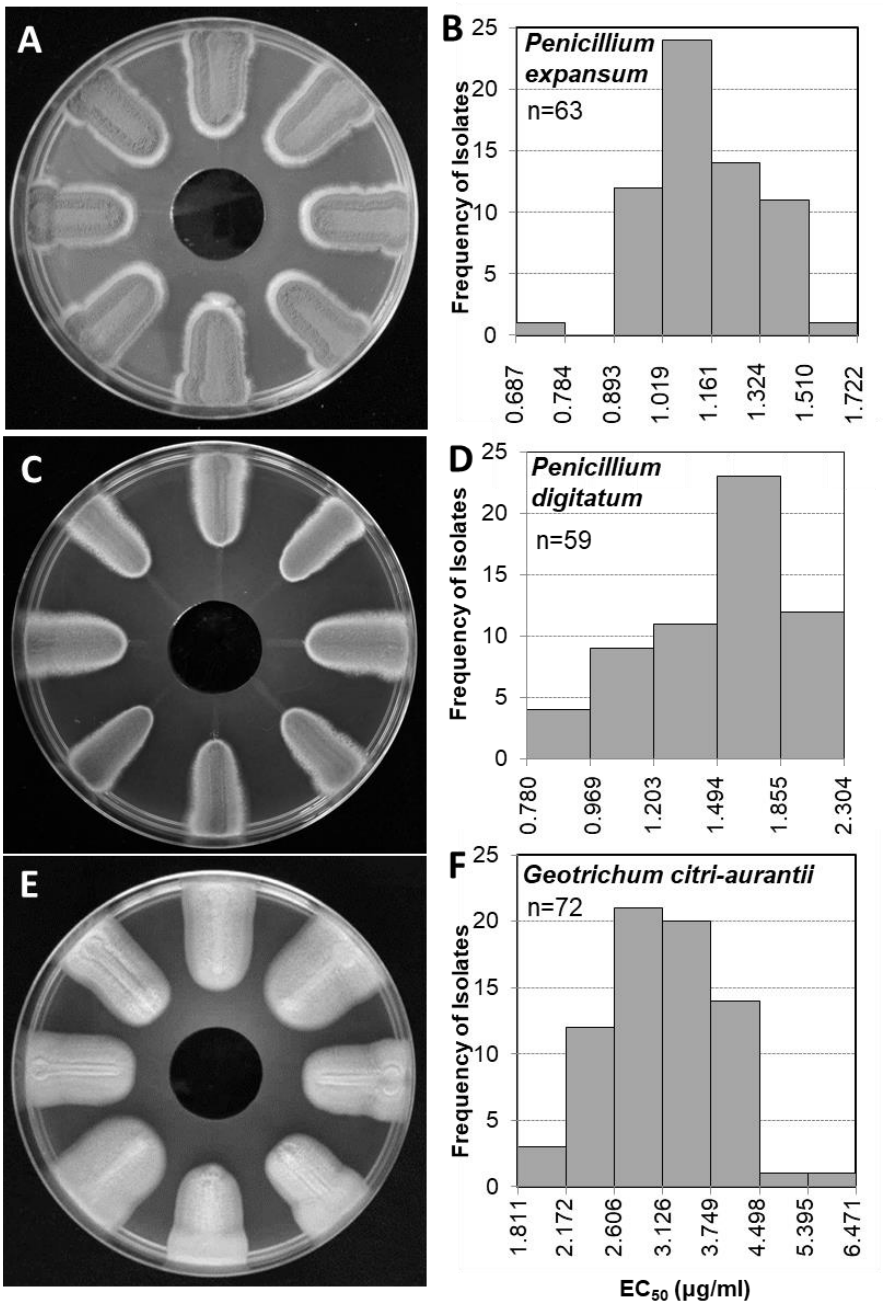

Fig. 1.2. Spiral gradient dilution plates with exponential gradients of natamycin concentrations (the highest concentration is in the center of the plate) to determine effective concentrations to inhibit mycelial growth by 50% (EC_{50} values) of four representative isolates (replicated oppositely on each plate) of each pathogen and frequency histograms of EC_{50} values of **A,B,** *Penicillium expansum*, **C,D,** *P. digitatum,* and **E,F,** *Geotrichum citri-aurantii,* respectively. Bar height indicates the number of isolates within each bin, and bin width was calculated based on Scott (1979).

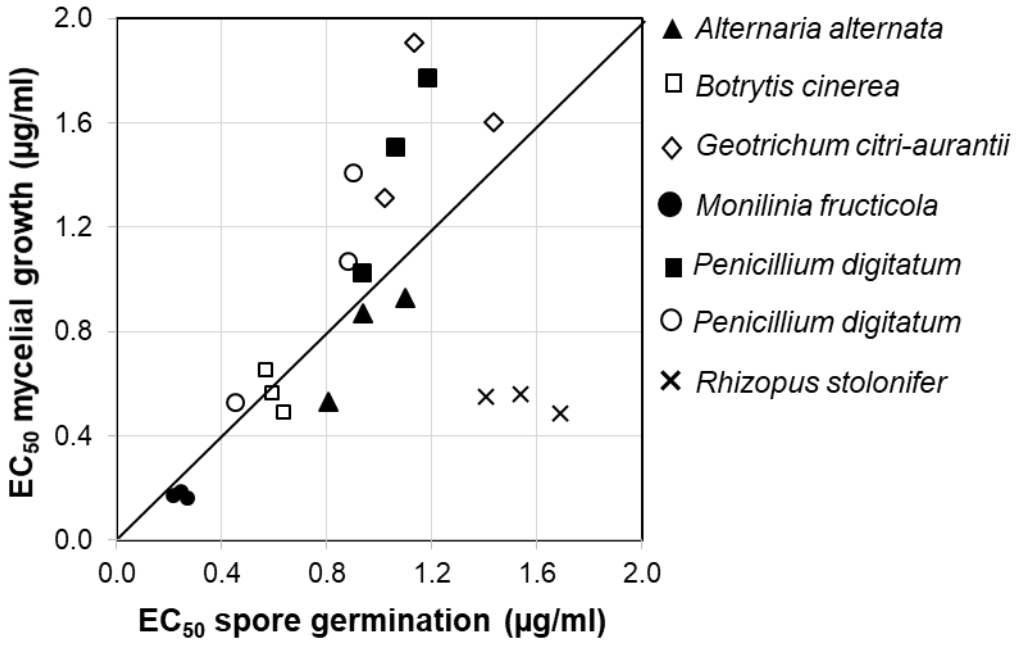

Fig. 1.3. Relationships between 50% inhibitory values of natamycin for spore germination and those for mycelial growth for three representative isolates each of seven postharvest decay fungi. The diagonal line represents a 1:1 relationship between inhibitory values for the two growth stages.

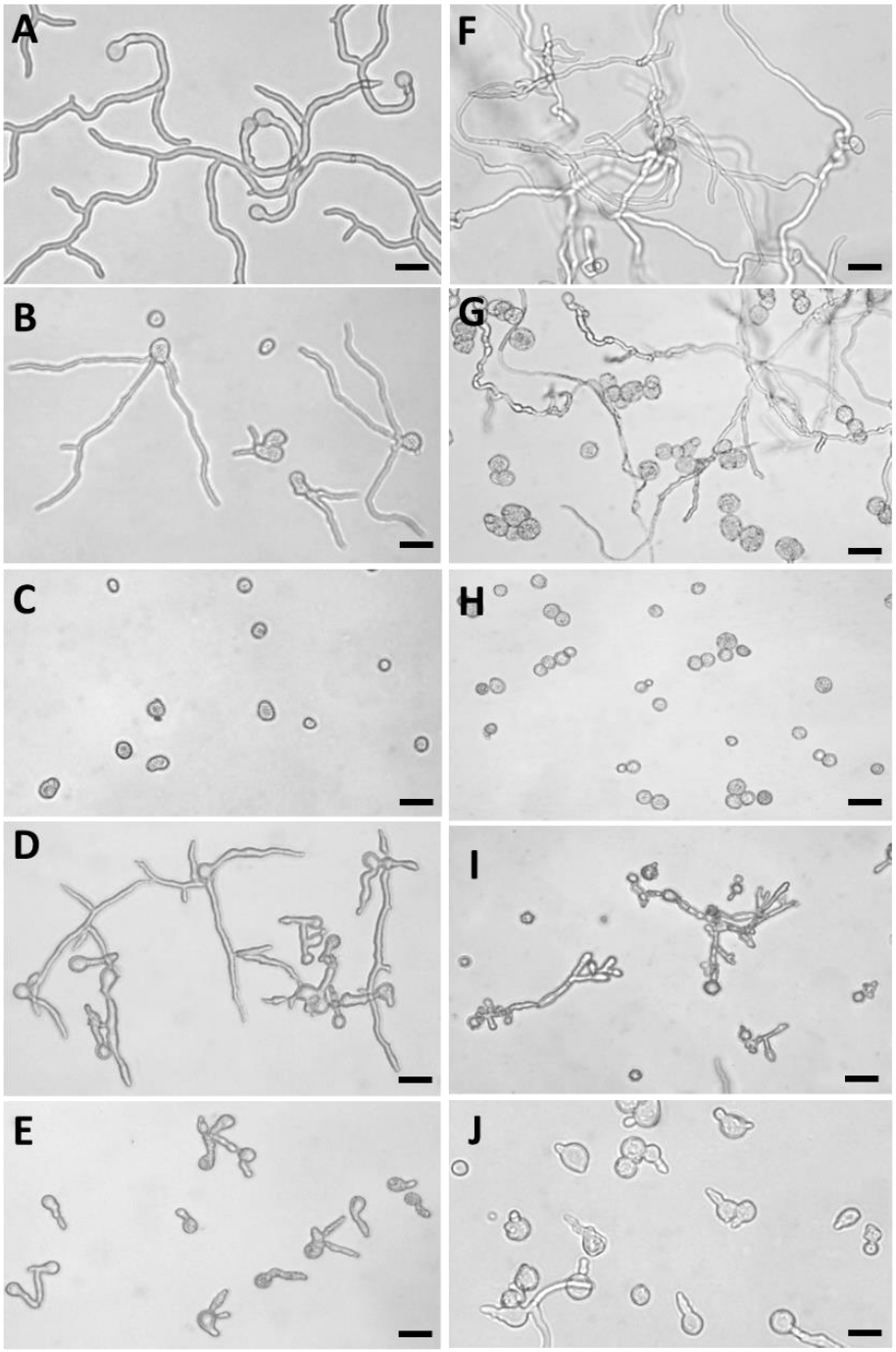

Fig. 1.4. Germination of sporangiospores of **A-E,** *Rhizopus stolonifer* after 10 h at 25°C and of **F-J,** conidia of *Penicillium digitatum* after 20 h at 25°C on potato dextrose agar. **A, F,** non-amended agar; and agar amended with **B, C, G, H**, 1.5 µg/ml, 3 µg/ml, 1 µg/ml, and 2 µg/ml natamycin, respectively; or **D, E, I, J,** with 1 µg/ml, 8 µg/ml, 0.02 µg/ml, and 2 µg/ml propiconazole, respectively. Bars for **A-F, H, I** = 10 µm; bars for **G, J** = 5 µm.

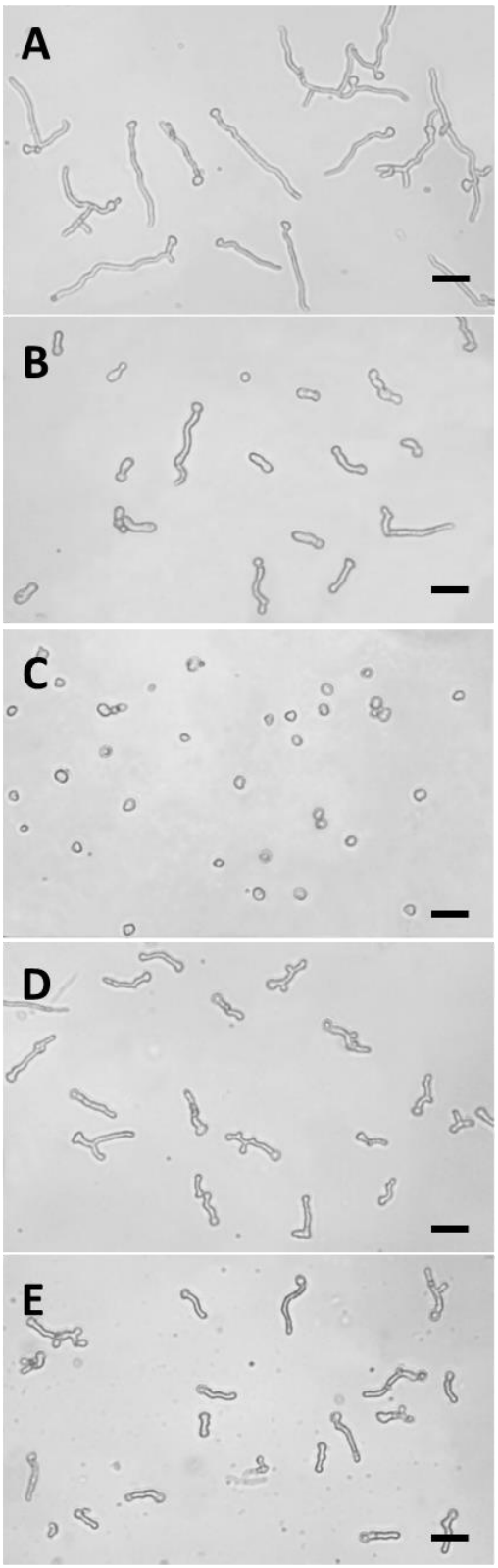

Fig. 1.5. Germination of conidia of *Monilinia fructicola* after 15 h at 25°C on potato dextrose agar. **A,** non-amended agar; and agar amended with **B, C,** 0.25 µg/ml and 0.5 µg/ml natamycin, respectively; or **D, E,** with 0.4 µg/ml and 15 µg/ml propiconazole, respectively. Bars = 10 µm.

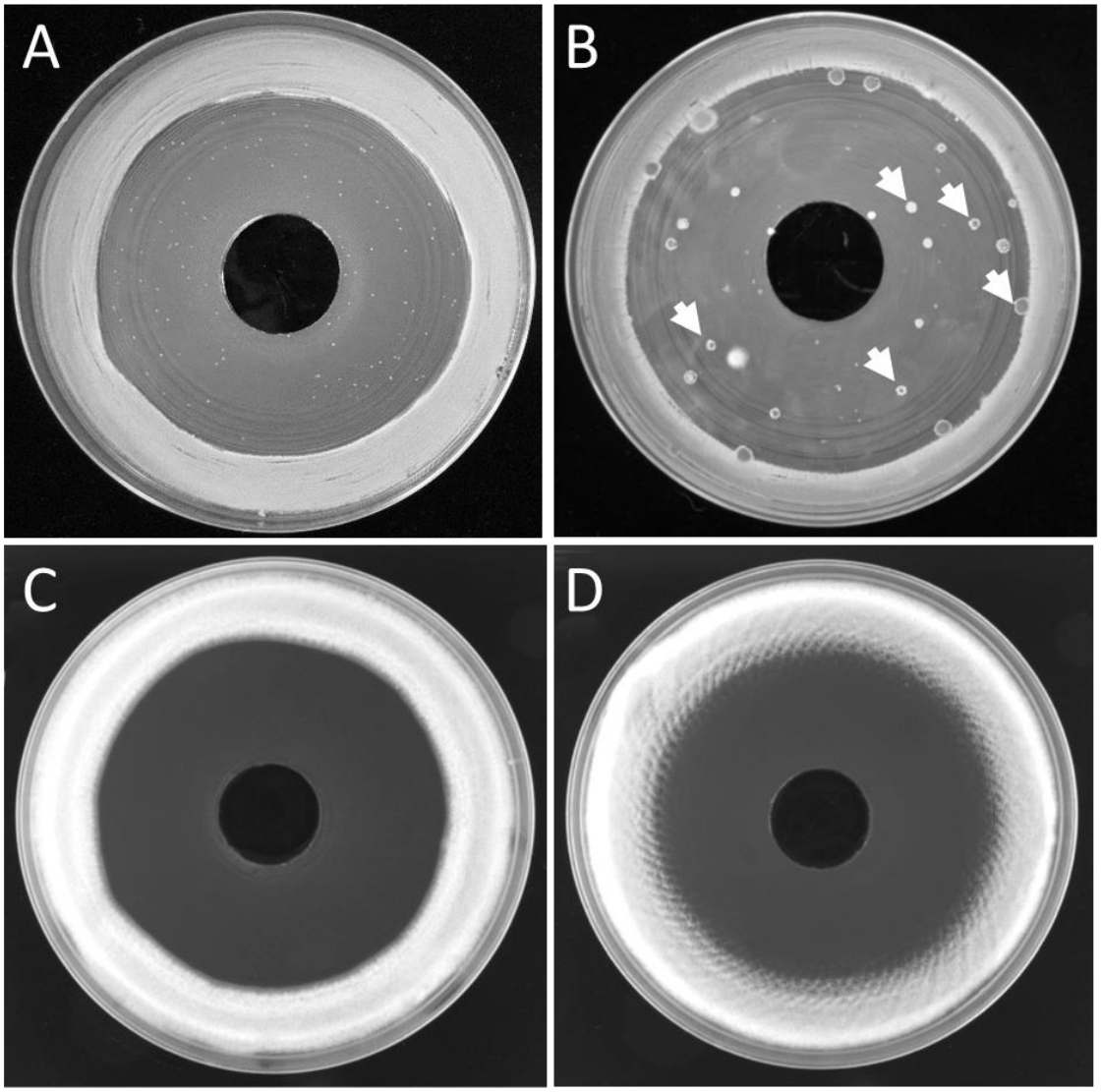

Fig. 1.6. Spiral gradient dilution plates with exponential concentration gradients of **A,C** natamycin, **B,** fludioxonil, and **C,** propiconazole (EC_{95} concentrations were positioned 20 mm from the edge of the petri dish) for **A,B** *Penicillium digitatum* and **C,D** *Geotrichum citri-aurantii*. A spore overlay of equal mixtures of 10 isolates each of *Penicillium digitatum* or *G. citri-aurantii* (1×10^8 conidia/ml) was evenly applied to amended plates. No colonies of *P. digitatum* **(A)** (small spots in the clear area of the agar plate are bacterial contaminants) or *G. citri-aurantii* **(C)** formed at concentrations >EC_{95} of natamycin or for *G. citri-aurantii* **(D)** at >EC_{95} of propiconazole. Several putative fludioxonil-resistant colonies (arrows) of *P. digitatum* **(B)** are found in the clear area of the agar plate treated with fludioxonil.

CHAPTER II. Natamycin, a Biofungicide for Managing Major Postharvest Fruit Decays of Citrus

ABSTRACT

The antifungal polyene macrolide natamycin was evaluated as a postharvest biopesticide for citrus fruits. Aqueous spray applications with 1000 µg/ml were moderately to highly effective against green mold after inoculation but did not reduce sporulation of *Penicillium digitatum* on infected fruits. Treatments with natamycin were significantly more effective against green mold on grapefruit and lemons than on oranges and mandarins with 92.9%, 88.5%, 57.5%, and 60.9% reductions in decay as compared with the control, respectively. The biofungicide was compatible with a storage fruit coating but was less effective when applied in a packing coating. However, when either fruit coating was applied following an aqueous natamycin treatment (i.e., staged applications) the incidence of decay was reduced to ≤10.7% as compared to the untreated control with 81.9%. The incidence of sour rot of lemons and mandarins was also significantly reduced from the untreated control by natamycin (1000 µg/ml), but propiconazole (540 µg/ml) and propiconazole + natamycin (540 + 500 µg/ml) mixtures generally were significantly more effective than natamycin alone when using a severe inoculation procedure. Experimental and commercial packingline studies demonstrated that natamycin-fludioxonil or -propiconazole mixtures applied in a storage fruit coating or as an aqueous flooder treatment were highly effective and typically resulted in a >85% reduction of green mold and sour rot. Resistance to natamycin has never been documented in

filamentous fungi. Thus, the use of natamycin, in contrast to other registered postharvest fungicides for citrus, can be an anti-resistance strategy and an effective treatment in mixtures with other fungicides for the management of major postharvest decays of citrus.

INTRODUCTION

Decays of citrus fruits caused by species of *Penicillium* are an ongoing problem worldwide in the commercial production and distribution of the crop. Green mold caused by *P. digitatum* (Pers.: Fr.) Sacc. is the most important postharvest disease of citrus in semi-arid climates like California (Eckert and Eaks 1989). The pathogen produces very large numbers of aerially dispersed conidia that may contaminate virtually every fruit in citrus orchards and packinghouses (Eckert and Eaks 1989). Fruit injuries are required for infection by *P. digitatum*, and these occur commonly during harvest and postharvest handling (Eckert 1959). Thus, practices that minimize fruit injuries are important to decrease the incidence of decay.

Geotrichum citri-aurantii (Ferraris) E.E. Butler causes sour rot of citrus and is a yeast-like fungus that commonly occurs in soils (Butler and Petersen 1972; Butler et al. 1988). Conidia are disseminated by water splash from irrigation or rain, by wind in dust particles, or by insects to the surface of fruit where they can cause infections at sites of injury (Brown 1979; Domsch et al. 1993; Eckert 1959; Hershenhorn et al. 1992). Although sour rot can occur on fruit in the field, it is most economically important as a postharvest decay. On citrus in California, the disease is particularly destructive on lemon, grapefruit, and mandarin that, depending on market demands, are often stored for

prolonged periods at 12 to 15°C and 92 to 98% relative humidity (Baudoin and Eckert 1982, 1985; Brown 1979; Eckert 1959; Suprapta et al. 1996). Endo-polygalacturonases excreted by the pathogen rapidly macerate the fruit tissue. Decayed fruit leak juices containing enzymes, and spores of the pathogen drip onto healthy fruit, and this can effectively spread the disease in storage (Davis and Baudoin 1986).

In California packinghouses, citrus fruit are processed and stored almost year-round, creating a near constant need to protect fruit from decay and reduce inoculum levels in the packinghouse environment (Eckert and Eaks 1989). Postharvest fungicide treatments that prevent decay and sporulation of the pathogen in storage, transit, and marketing, in addition to various sanitation practices that reduce inoculum levels, are essential components of an integrated decay management program (Eckert and Eaks 1989). Several compounds with different modes of action are currently registered for postharvest use in the United States. Sodium ortho-phenylphenate (SOPP) has some efficacy against sour rot and other decays, but concentrations required to effectively manage the decay can be phytotoxic (Brown 1979) and use has declined due to disposal costs and human safety concerns (Smilanick and Sorenson 2001). Sodium carbonate and bicarbonate salts are used to reduce Penicillium decays and sour rot by shifting the pH of the fruit wound microenvironment to an alkaline condition that is much less favorable for fungal infection. This protective effect, however, only lasts for a few weeks because natural fruit acids eventually neutralize the alkalinity of the fungistatic salts. If excess salts are not rinsed off immediately after application, fruit dehydration and phytotoxicity may occur that results in shorter storage times. Similar to SOPP, sodium

carbonate/bicarbonate salts only have slight to moderate activity against Penicillium decays and sour rot (Eckert and Eaks 1989). The methyl benzimidazole carbamate thiabendazole (TBZ) and the demethylation inhibitor (DMI)-imidazole imazalil have been used for more than 45 and 35 years, respectively. The anilinopyrimidine pyrimethanil, the quinone outside inhibitor (QoI) azoxystrobin, the phenylpyrrole fludioxonil, and the DMI-triazole propiconazole were more recently (after 2006) registered. Propiconazole is the only highly effective fungicide for the management of sour rot and is also effective against Penicillium decays caused by imazalil-sensitive isolates of the pathogen (McKay et al. 2012a, 2012b).

Demand for efficacious postharvest fungicides that are considered safe for consumers, packinghouse workers, and the environment led to the evaluation of antifungal food preservatives. Natamycin is a biofermentation product of *Streptomyces natalensis* and other species of *Streptomyces* and has been in used as a food preservative in the cheese and meat industries against molds and yeasts for more than 40 years (Delves-Broughton 2014; Stark and Tan 2003). Natamycin is a polyene macrolide with no antibacterial properties. Its antifungal properties are due to a unique interaction with ergosterol in the fungal cell membrane. Ergosterol is a conserved sterol in fungi and protozoans and is similar to cholesterol in animals; it stabilizes the fluid membrane environment of a fungal cell and also aids in cellular transport of materials. Natamycin binds directly to ergosterol with its mycosamine functional group. This immobilizes ergosterol and prevents its normal cell functions, thus leading to cell death (Abe and Hiraki 2009; Gray et al. 2012; te Welscher et al. 2008).

As a food preservative, natamycin is classified as 'generally regarded as safe' (GRAS) by the United States (US) Food and Drug Administration (FDA). Recently, the US Environmental Protection Agency (EPA) has listed it as a biopesticide that is 'exempt' from residue tolerances on registered agricultural commodities. Natamycin can be used in the United States in mushroom production against bubble disease caused by *Verticillium fungicola* and as a pre-plant dip of strawberry plants against anthracnose caused by *Colletotrichum* spp. Its recent registration as a postharvest fungicide treatment for citrus and stone fruits was based in part on the studies described herein. Natamycin has been shown to be effective against a broad range of fungal species with typical minimum inhibitory concentrations (MICs) of 1 to 10 μg/ml. Its solubility in water is low (~40 μg/ml) but is sufficient to be active against many fungi (Delves-Broughton 2014). The molecule is stable over a broad pH range (pH 3 to 9) with an optimum between pH 6 and 7 (Raab 1972, Stark 2003) but is unstable under ultraviolet light, thus limiting its uses under field conditions (Gutteridge et al. 1983).

The prolonged use of the citrus postharvest fungicides imazalil and thiabendazole resulted in the development of resistance in *Penicillium* spp. populations and economic losses in many California citrus packinghouses due to insufficient decay control (Adaskaveg and Förster 2015; Eckert and Eaks 1989; Harding 1962, 1972). More recently, highly effective alternatives such as azoxystrobin, fludioxonil, pyrimethanil, and propiconazole have been registered but their resistance potential is high due to their single-site mode of action. Furthermore, imazalil-resistant isolates are cross-resistant to propiconazole. With no documented resistance in filamentous fungi (Delves-Broughton

2014), the introduction of natamycin has the potential to control resistant isolates of decay pathogens.

Objectives of this research focused on the use of natamycin as a biofungicide for the management of postharvest decays of citrus. Specific goals included: i) evaluate the efficacy of natamycin against green mold and sour rot of selected citrus species in laboratory studies; ii) determine the compatibility of natamycin with postharvest fruit coatings and other postharvest fungicides; and iii) assess the performance of natamycin in experimental and commercial packingline studies in comparison with other registered fungicides.

MATERIALS AND METHODS

Fungicides used. Formulated products of natamycin [DelvoCid Instant – a wettable powder (WP) and BioSpectra – a suspension concentrate (SC); DSM Food Specialties, Heerlen, The Netherlands], fludioxonil (Scholar SC), propiconazole (Mentor WP and Mentor EC – an emulsifable concentrate), and a pre-mixture of fludioxonil and azoxystrobin (Graduate A+) (the latter three fungicides from Syngenta Crop Protection LLC, Greensboro, NC) were used at rates recommended by the respective manufacturers in efficacy studies with citrus fruits. Fungicide solutions were prepared in deionized (laboratory studies) or tap (packingline studies) water, in a diluted (1 part plus 15 parts of water) paraffinic oil-based storage fruit coating (LUSTR 202), or in a non-diluted carnauba-based packing coating (Citrus Brite 330; both coatings from Decco US Post-Harvest Inc., Monrovia, CA).

Fungal isolates used. For fruit efficacy studies, *P. digitatum* isolates "Pd" (sensitive to all registered citrus postharvest fungicides) and "5161" (moderately resistant to imazalil, EC_{50} = 0.4 µg/ml; resistant to thiabendazole, EC_{50} >10 µg/ml), as well as *G. citri-aurantii* isolate "4088" (sensitive to propiconazole and sodium ortho-phenylphenate, naturally insensitive to other registered citrus postharvest fungicides) were used. Long-term storage of cultures was in liquid nitrogen. For short-term storage, mycelium-covered agar plugs were kept in sterile water at 4ºC. Cultures were grown on potato dextrose agar (PDA; Difco Laboratories, Becton, Dickinson and Company, Sparks, MD) at 25ºC.

Inoculation of citrus fruits for laboratory and packingline efficacy studies. **'**Eureka' lemon [*Citrus limon* (L.) Burm.], 'Improved Meyer' lemon (*C.* × *meyeri* Y. Tan.), Marsh grapefruit (*C.* × *paradisi* Macfad.), Dancy and Tango mandarin (*C. reticulata* Blanco), as well as Navel and Valencia orange [*C. sinensis* (L.) Osbeck] fruit were obtained from orchards where no preharvest fungicides were applied. Fruit were washed with water or 100 µg/ml sodium hypochlorite (diluted commercial household bleach) and placed into plastic trays inside commercial cardboard fruit boxes (for *P. digitatum*) or inside covered plastic boxes (27 liters volume) with a thin layer of water added to the bottom of the boxes without fruit contacting the water to increase humidity (for *G. citri-aurantii*). The flavedo (exocarp) and albedo (mesocarp) of each fruit were wounded (2 mm long, 0.5 mm wide, 2 mm deep) using a stainless-steel wounding tool without penetrating the juice sacks. Inoculum of *P. digitatum* (1×10^6 conidia/ml) was prepared in sterile water amended with 0.01% Triton X-100 (Sigma-Aldrich, St. Louis, MO). Inoculum of *G. citri-aurantii* was prepared in sterile water (1×10^7 conidia/ml) or

in non-sterile citrus (using the same species that was used for inoculation) juice filtered through two layers of cheesecloth and amended with 2 μg/ml cycloheximide (5×10^5 conidia/ml). For inoculation, 20-μl of conidial suspension was pipetted into each wound. Fruit were incubated for 12 to 14 h at 20°C before treatment.

Laboratory studies on the efficacy of natamycin against postharvest decays of citrus fruits. Treatments were applied to run-off using a hand-operated atomizer (Model 15-RD; DeVilbiss Health Care, Somerset, PA) or by dipping fruit for 15 s in a fungicide solution in a bucket. For some treatments, fruit spray-treated with an aqueous fungicide solution were allowed to air-dry for 5 min and then sprayed with a diluted storage or undiluted packing fruit coating. Control fruit were treated with water. After treatment, fruit were stored for seven days at 20ºC. For evaluation of green mold, each fruit was inspected carefully for decay development that was either easily visible as mycelium- or conidia/mycelium-covered decay or present as soft, often watery lesions around the inoculation site. For evaluation of sour rot, fruit were examined for very soft decay lesions around the inoculation area that at later stages were covered by creamy-white fungal mycelium and conidia. Decay incidence was based on the number of decayed fruit of the total number of fruit inoculated. For each treatment and experiment, 24 fruit were used in each of 3 or 4 replications, and each experiment was done twice.

Experimental and commercial packingline studies on the efficacy of natamycin against postharvest decays. In high-volume, recirculating flooder treatments on the experimental line at the University of California Kearney Agricultural Research and Extension Center in Parlier, CA, the aqueous fungicide solution was pumped from a 50-

liter reservoir into a perforated stainless steel distribution pan (91-cm × 91 cm area with 127 evenly distributed 5-mm-diameter holes) positioned in the center of the treatment area and about 20 cm above a moving roller bed. Treatment durations (i.e., the time that fruit on the roller bed were exposed to the flooding fungicide solutions) were between 12 and 15 s. Fruit then moved underneath a controlled droplet applicator (CDA) that delivered a fruit storage coating at 8.34 liters per 10,000 kg of fruit. Treatment volumes per fruit weight were calibrated by adjusting CDA output volumes and speed of fruit movement through the treatment area. In one study, the flooder fungicide solution was heated to 48ºC before use, and the solution was re-heated for each experimental replicate. Control fruit received a flooder treatment with water at 48ºC that was followed by the fruit coating application. Between treatments, the fungicide reservoir, flooder equipment, and the treatment bed were cleaned with a commercial alkaline detergent (PacFoam Plus; Pace International, Seattle) and then thoroughly rinsed with water.

High-volume aqueous flooder treatments in a commercial packinghouse were applied to inoculated fruit together with commercial fruit. For this, experimental fruit were marked with a marker pen so they could be identified and retrieved after the treatment. The flooder was a cascading system over a moving brush bed with fruit exposure times of approximately 15 s. In one study, fungicides were applied as a solution in diluted storage fruit coating using a calibrated CDA application system that was similar to the one described above for the experimental line studies. Decayed fruit inoculated with *P. digitatum* in this study were also rated for the degree of sporulation using a scale from 0 to 4 where 0 = no or negligible sporulation and 4 = fruit completely green, and fungal

sporulation over the entire surface. Control fruit were left untreated. Between treatments, fungicide reservoirs were rinsed with water, and commercial fruit were treated with a new fungicide solution for approximately 45 min before experimental fruit were treated.

For each packingline treatment, three or four replications of 24 fruit were used, and each study was done twice. Treated fruit were stored at 20ºC for 5 to 7 days or at 12ºC for 4 weeks and evaluated for the incidence of decay as described above for the laboratory studies.

Statistical analysis of data. Statistical analyses were performed using SAS (ver. 9.4, SAS Institute, Cary, NC) with a significance value of $\alpha = 0.05$. Percent incidence data was subjected to arcsine square-root transformation prior to analysis. One-way analyses were performed for treatments in most fruit efficacy experiments using generalized linear mixed models with the GLIMMIX procedure under a binomial distribution. For these, treatment was considered a fixed effect, and trial, replication (block), and the overall error term were considered random effects. Two-way analyses were performed on the effect of fungicide treatment and citrus species or inoculation method on disease incidence using generalized linear mixed models with the GLIMMIX procedure. For this, treatment and citrus species or inoculation method, and their interaction were treated as fixed effects, and trial, replication (block), and the overall error term were treated as random effects. Fixed effects were tested for significance, and least squares treatment means were determined using the lsmeans statement with the Tukey adjustment in PROC GLIMMIX. Contrast estimates for pairs of treatments were used to determine multiple comparison differences among treatment means.

Sporulation data were not transformed and were analyzed using the nonparametric Kruskal-Wallis test as a rank-based method to test the hypothesis that the distribution of sporulation ratings is the same in multiple independently sampled populations (i.e., fungicide treatments). The Kruskal-Wallis statistic was calculated, and *p*-values were determined. The NPAR1WAY procedure was then used to determine mean rank scores of the treatments, Chi-square, and p-values.

RESULTS

Laboratory studies on the efficacy of natamycin against postharvest decays of citrus fruits. Treatments of lemon fruit with natamycin (500 µg/ml) prepared either in water, in a storage fruit coating, or a packing fruit coating and staged applications (aqueous application of natamycin followed by storage or packing fruit coating treatments) all significantly (P <0.0001) reduced the incidence of green mold from that of the water control where 81.9% of the fruit showed decay (Fig. 2.1). Natamycin applied in a mixture with carnauba wax-based packing fruit coating was the least effective (i.e., 54.4% incidence) and was significantly less effective than the other treatments. All other application methods, including an aqueous treatment that was followed by a packing fruit coating, reduced the incidence of green mold to between 5% and 10.7% (Fig. 2.1). In addition, aqueous solutions of two rates of natamycin (500 or 1000 µg/ml) were similarly effective with 5.0% and 5.1% incidence, respectively. In comparison, no decay developed on fruit treated with fludioxonil at 600 µg/ml in diluted storage coating.

Results for comparative efficacy studies on the management of green mold of four citrus species are shown in Table 2.1. Citrus species (P<0.0001), fungicide treatment (P<0.0001), and their interaction (P=0.0162) were significant factors. Therefore, results are presented separately for each species. Natamycin at 1000 μg/ml significantly reduced the incidence of decay on grapefruit from 51.9% in the control to 7.4% (P<0.0001), on lemon from 85.3% to 14.8% (P <0.0001), on mandarin from 84.5% to 44.7% (P<0.0001), and on orange from 74.6% to 40.4% (P=0.0006). The natamycin-fludioxonil mixture was significantly more effective than natamycin by itself on grapefruit and mandarin but the two treatments were similar in performance on lemon and orange. In comparing natamycin efficacy among citrus species, the independent variables fungicide treatment and species were significant (P<0.0001) but their interaction was not (P=0.9266). Natamycin treatments resulted in significantly (P< 0.0001) higher percent reductions of green mold on grapefruit and lemon than on orange and mandarin (Table 2.1).

In studies on sour rot of lemons and mandarins, incidences of decay in the untreated controls were 99.3% and 65.9%, respectively, when inoculum was prepared in fruit juice and 2 μg/ml cycloheximide and were 73.7% and 32.6%, respectively, when inoculum was prepared in water (Fig. 2.2). Inoculation method, treatment, and their interaction were significant with P values of <0.0001, <0.0001, and 0.0024 for lemon (Fig. 2.2.A,C), and <0.0001, and <0.0001, and 0.0004 for mandarin (Fig. 2.2B,D), respectively. Because of the significant interaction between inoculation method and treatment, treatment effects were further evaluated for each citrus species. For this, citrus species, treatment, and interaction were analyzed, and P values were <0.0001, <0.0001,

and 0.0027 when conidial inoculum was prepared in juice-cycloheximide (Fig. 2.2.A,B), and were 0.4273, <0.0001, and <0.0001, when conidial inoculum was prepared in water, respectively (Fig. 2.2.C,D). Because of significant interactions in these statistical comparisons, data in Fig. 2.2 are presented separately for each inoculation method for the two citrus species. All aqueous natamycin treatments significantly reduced the incidence of decay on lemon (P<0.0001 for both inoculation methods) (Fig. 2.2.A,C) and mandarin (P<0.0001 for the juice-cycloheximide inoculum and P=0.002 for aqueous inoculum) (Fig. 2.2.B,D) as compared to the control. Decay incidences ranged from 35.4% to 42.4% for lemons and 24.3% to 31.1% for mandarins for fruit juice-cycloheximide inoculum, and from 0.7% to 4.9% for lemons and 7.6% and 13.7% for mandarins for aqueous inoculum.

There was no significant difference between the 500- and 1000-µg/ml rates of the natamycin SC formulation or between the 1000-µg/ml rates of the SC and WP formulations on lemon and mandarin regardless of inoculation method. Propiconazole at 540 µg/ml by itself or in mixture with 500 µg/ml natamycin in most cases was significantly more effective than natamycin used by itself when inoculum was prepared in fruit juice-cycloheximide (Fig. 2.2.A, B). When using aqueous inoculum suspensions, however, there was no significant difference between any of the natamycin and the propiconazole treatments (Fig. 2.2.C, D).

Experimental and commercial packingline studies on the efficacy of natamycin against postharvest decays of citrus fruits. In a flooder application study on an experimental packingline, *P. digitatum*-inoculated lemon and orange control fruit

(treated with water) developed green mold incidences of 51.3% and 49.4%, respectively (Fig. 2.3.A,B). In comparisons of citrus species, treatment, and their interaction, *P*-values were 0.95, <0.0001, and 0.5412, respectively. On both citrus species, all treatments evaluated significantly reduced levels of decay. Treatments with natamycin at 1000 µg/ml were not significantly different, irrespective when using aqueous applications of the SC or WP formulations or using an aqueous WP application that was followed by a carnauba-based fruit coating. Decay incidences were reduced to between 12.0% and 16.7% on lemon (Fig. 2.3.A) and to between 9.3% and 21.3% on orange (Fig. 2.3.B). Treatments with fludioxonil by itself or mixed with natamycin (the latter treatment either as an aqueous application or as a sequential aqueous application followed by packing fruit coating) in some cases performed significantly better than treatments using natamycin by itself. For these treatments containing fludioxonil, incidences ranged from 5.4% to 6% on lemons and from 6.0% to 11.4% on oranges.

Results of experimental and commercial packingline studies with heated flooder treatments for the management of sour rot after fruit inoculation and of naturally occurring green mold of lemon fruit are shown in Fig. 2.4. Treatment *P*-values for both decays were <0.0001. After four weeks of storage at 12°C, 86.6% of control fruit developed sour rot. In comparison, incidences of 27% developed for natamycin at 1000 µg/ml, 2.8% for propiconazole at 250 µg/ml, and 2.9% for the mixture of 500 µg/ml natamycin and 250 µg/ml propiconazole. Penicillium decays caused by *P. digitatum* and *P. italicum* developed naturally at an incidence of 55.1% on untreated fruit, and this was reduced to less than 3% by the three treatments.

CDA applications with natamycin by itself or selected mixtures were evaluated in additional commercial packingline studies for the management of sour rot and green mold of inoculated lemon fruit. Treatment *P*-values for both decays were <0.0001. After four weeks of storage at 12°C, sour rot incidence of control fruit was 95.3% (Fig. 2.5.A). Natamycin at 1000 µg/ml showed moderate efficacy with 57.7% incidence, whereas the mixture of 500 µg/ml natamycin with 1000 µg/ml propiconazole was the best treatment with 9.4% incidence. The natamycin-propiconazole mixture (750 µg/ml each) and the azoxystrobin-fludioxonil-propiconazole triple mixture (1000 µg/ml each) showed intermediate efficacy. For green mold, the three treatments were all highly and statistically similarly effective and reduced the incidence from 86.5% in the control to between 7.1% (i.e., the triple mixture) and 17.2% (i.e., natamycin by itself at 1000 µg/ml) (Fig.2.5.B). For sporulation ratings, the Kruskal-Wallis statistic was equal to 22.9 with 4 degrees of freedom corresponding to a p-value of 0.0001 indicating that sporulation levels differed among treatments. The probability > Chi-Square was 0.0001. Thus, natamycin and natamycin-propiconazole treatments were not effective in reducing sporulation of *P. digitatum* on decaying fruit as compared to the control, whereas the fludioxonil-azoxystrobin-propiconazole mixture significantly reduced the rating from 4.0 in the control to 0.9 (Fig. 2.5.C).

DISCUSSION

In this research, I demonstrate that natamycin is an effective treatment for the management of green mold and sour rot of citrus fruits. This research builds upon our

previous efforts in developing a new approach using a US EPA-designated biopesticide for postharvest decay management of fruit crops that is also used as a food preservative and approved by the US-FDA (Adaskaveg and Forster 2015; Chen et al. 2016). Others reported that natamycin was moderately effective in laboratory evaluations in reducing green mold (*P. digitatum*) on lemon and blue mold (*P. italicum*) on orange (Yiğiter et al. 2014). In studies on gray mold (*Botrytis cinerea*) of mandarin fruit, the efficacy was highly dependent on the time when treatments were applied after application and was only moderate for treatments done 12 h after inoculation (Saito et al. 2020).

In experiments conducted in the laboratory or on experimental and commercial packinglines, moderate to very high efficacy of natamycin was obtained. Applications methods ranged from high-volume sprays and flooders to low-volume CDA treatments. The latter two methods are currently widely used in commercial settings in California. Furthermore, green mold and sour rot decay after inoculation as well as Penicillium decays from natural infections were successfully managed. Penicillium decays are ubiquitous in arid citrus production areas such as California, but sour rot occurs more sporadically, and thus, data on managing the natural incidence of this decay could not be obtained. Because harvested, treated lemon fruit in California are often stored for one to four months before marketing, I incubated treated fruit for four weeks at 12°C in some of our experiments.

The high activity of natamycin against citrus sour rot is noteworthy because before the introduction of propiconazole in 2014, no highly effective treatment was available in the United States. Two inoculum preparation methods were used to evaluate

sour rot efficacy, an aqueous and a fruit juice-cycloheximide preparation of conidia. This was done because the development of sour rot highly depends on maturity and likely other physiological fruit characteristics. Inoculation with aqueous conidial suspensions is often not successful and strategies have been developed to improve inoculations. Fruit juice-cycloheximide preparations of conidia have been shown to consistently result in a high incidence of decay (McKay et al. 2012a). Cycloheximide prevents wound healing, favoring colonization by the pathogen (Baudoin and Eckert 1985). In previous studies, this resulted in more conducive conditions for disease development and generally reduced efficacy of treatments including natamycin as compared when inoculations were made using aqueous suspensions.

The efficacy of natamycin used by itself often did not reach the high levels that were obtained using fludioxonil, fludioxonil-azoxystrobin, or propiconazole for Penicillium decays or propiconazole for sour rot, fungicides that are currently chemical standards in citrus postharvest decay management. Furthermore, there were significant differences in managing green mold of grapefruit, lemon, mandarin, and orange using laboratory spray applications. The reduction in decay as compared with the control was the lowest when using inoculated mandarin and orange fruit. Furthermore, the efficacy of a natamycin-fludioxonil mixture was also reduced on the latter two citrus species. In experimental packingline studies using flooder applications, however, treatments with natamycin performed similarly on lemon and orange fruit with moderate to high efficacy. High-volume flooder applications were previously shown to be superior to other application methods because of improved coverage (Kanetis et al. 2008). In all studies,

fruit were treated 12 to 14 h after inoculation. This is a recommended time limit for treating citrus fruit in the packinghouse after harvest when most infections occur. Infections at this time are thought to be at an early stage of development when treatment efficacy can be maximized. In the laboratory study comparing fruit species, it is possible that *P. digitatum* advanced more rapidly into mandarin and orange fruit making it more difficult for a contact fungicide like natamycin to inactivate the infection. Histological studies on fruit infection may help to substantiate this explanation. As demonstrated, an optimized application method such as a flooder treatment can compensate for differences in fruit susceptibility to fungal colonization.

In experiments to optimize the efficacy of natamycin, I evaluated several strategies. Increasing the rate from 500 µg/ml to 1000 µg/ml did not significantly increase efficacy. In other studies not presented here, rates of 2000 µg/ml also did not result in higher performance, whereas those lower than 500 µg/ml were inconsistent (Adaskaveg *unpublished*). In contrast, others previously used much higher rates of 4500 µg/ml and 5000 µg/ml to control green mold and blue mold of inoculated citrus fruit (Yiğiter et al. 2014). Still, in the latter study, decay reductions in comparison to the controls of only 20% to 50% were obtained even when treatments were done one to two hours after inoculation. Perhaps differences in natamycin formulations (e.g., particle size, adjuvants) and suppliers of materials used in my studies could explain the differences in performance. Trends for increased efficacy of the SC as compared with the WP formulation were observed in my investigations, but statistically, both performed similarly. Heated treatments have been shown to improve efficacy of some citrus

postharvest fungicides (Schirra et al. 2011; Smilanick et al. 2008). In my studies, however, I consistently obtained approximately 80% control whether the natamycin solutions were heated to 48°C or used at ambient temperature (25°C).

Coatings are commonly applied in the packinghouse to fruit in a spray treatment either before storage as a storage coating to reduce water loss while still allowing gas exchange (i.e., fruit respiration) or before marketing as a packing coating to increase shine and overall appearance of fruit. If fungicides are applied, they may be prepared in the coating, but this requires that their activity is not negatively affected. In my studies a diluted paraffinic oil-based storage coating was compatible with natamycin, and there was no significant difference in comparison with an aqueous treatment. When natamycin was prepared in a carnauba-based packing coating, however, it was no longer very effective. This is important information for the commercial use of this fungicide, and as I demonstrated, a packing coating can be used without loss of efficacy when it is applied sequentially after an aqueous natamycin treatment. Most packinghouses are equipped to do this type of application, and in fact, it is also used for some other citrus postharvest fungicides (Kanetis et al. 2008).

Because natamycin does not reduce sporulation on decaying fruit, its suggested use should be positioned strategically in the postharvest handling and treating process for citrus fruits. Preventing sporulation of *Penicillium* spp. is very important during storage of fruit where the pathogen is exposed for extended times to the fungicide, and resistance may be selected, but also for fruit going to market where sporulation causes soilage and off-grading of fruit lots. To improve its performance and overcome its lack of sporulation

control, natamycin is best used in mixtures with fludioxonil, fludioxonil-azoxystrobin, imazalil, or imazalil-TBZ. A key role of natamycin is its ability to reduce total populations of targeted pathogens, minimizing the risk for selecting sub-populations resistant to single-site mode of action fungicides registered on citrus that are much more prone to resistance development. Similarly, for sour rot, mixtures of natamycin and propiconazole with their unique and complementary modes of action (inactivation or inhibition of production of ergosterol, respectively) are also a strategy for maximizing efficacy while minimizing the risk for resistance development in *G. citri-aurantii* (Adaskaveg et al. 2019).

Prolonged use of a single chemical mode of action to manage postharvest diseases of citrus has led to the development of resistance in citrus pathogens, namely *Penicillium* spp. (Adaskaveg and Förster 2015; Eckert and Eaks 1989). The lack of resistance development against natamycin in filamentous fungal populations over many years of usage has been demonstrated (de Boer and Stolk-Horsthius 1977; Stark 2003), and only an up to three-fold reduction in sensitivity was obtained for some fungi after repeated exposures to natamycin (Streekstra et al. 2016). The introduction of natamycin as a postharvest fungicide for managing green mold and sour rot of citrus is important because it has a new mode of action and a low potential for resistance development. With these characteristics, it may prevent resistance development to other fungicides registered one citrus when used in mixtures or rotations.

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Table 2.1. Efficacy of natamycin and natamycin-fludioxonil mixtures for management of green mold of different citrus species in laboratory studies

	Marsh Grapefruit				Eureka Lemon				Navel Orange				Dancy Mandarin			
Treatment[a]	Incid. (%)[b]	LSCE[c]	Red. (%)	LSCE	Incid. (%)	LSCE	Red. (%)	LSCE	Incid. (%)	LSCE	Red. (%)	LSCE	Incid. (%)	LSCE	Red. (%)	LSCE
Water Control	52	a	---		85.3	a			74.6	a			84.5	a		
Natamycin 1000 µg/ml	7.4	b	85.7	A	14.8	b	82.6	A	40.4	b	45.5	B	44.7	b	47.2	B
Natamycin 1000 µg/ml + Fludioxonil 125 µg/ml	0	c	100	A	4.8	b	94.3	A	22.6	b	69.5	B	21.4	c	74.6	B
Citrus species			92.9	A			88.5	A			57.5	B			60.9	B

[a] Fruit were inoculated with Penicillium digitatum and dip-treated in aqueous fungicide solutions after 12 to 13 h of incubation at 20°C.

[b] The incidence of green mold was assessed after 7 days at 20°C based on the number of fruit with decay of the total number of fruit evaluated.

[c] Values followed by the same letter are not significantly different as denoted by the lsmeans statement and contrast estimates (LSCE) of the GLIMMIX procedure in SAS v9.4 ($P < 0.05$). Statistical comparisons within columns are in lower case letters, and those by row are in upper case letters.

[d] Percent reduction in decay as compared to the untreated control.

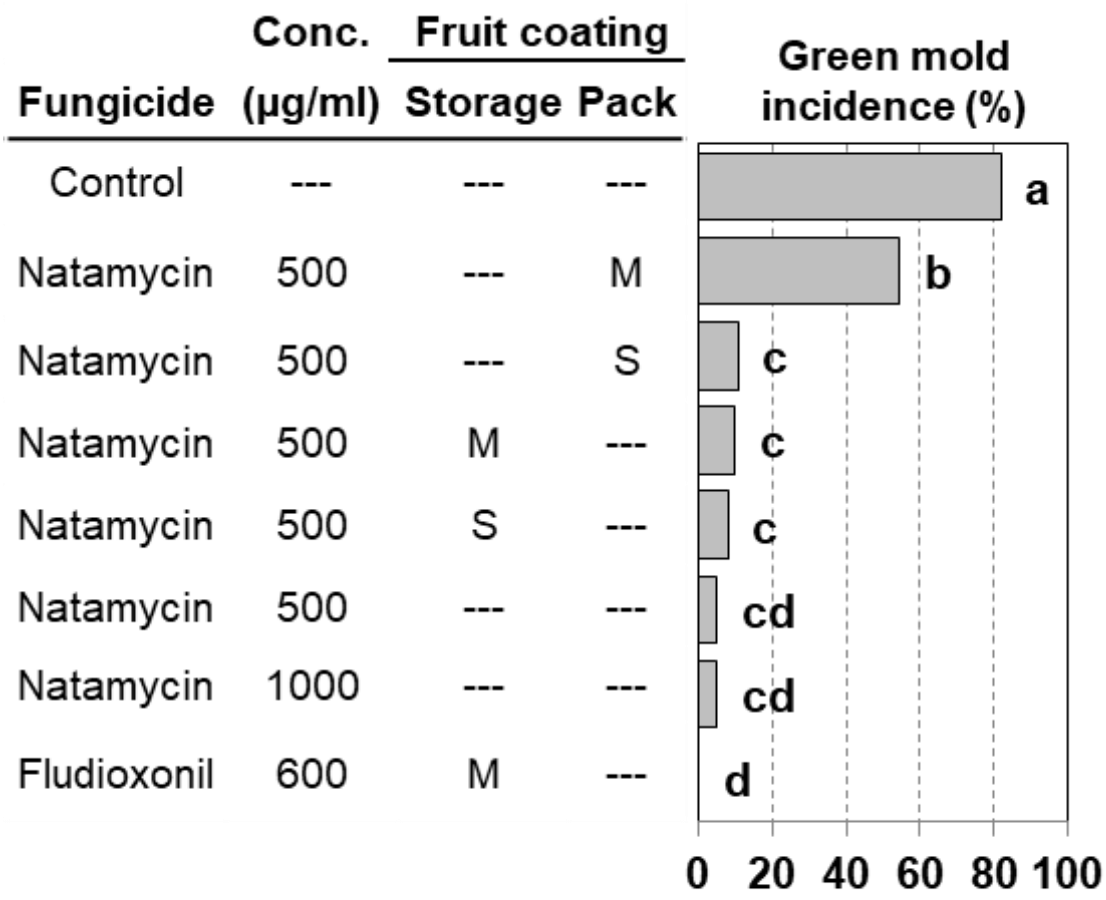

Fig. 2.1. Laboratory studies on the efficacy of natamycin and fludioxonil (both used as soluble concentrate formulations) for managing green mold of 'Eureka' lemon caused by *Penicillium digitatum*. Fruit were wound-inoculated (1 x 10^6 conidia/ml), treated after 12 to 14 h using a hand sprayer, and incubated for 7 days at 20ºC. Natamycin was applied in a mixture (M) with a storage or a pack fruit coating, or sequentially (S) as an aqueous solution that was followed by either fruit coating. Values are the mean of two experiments. Horizontal bars followed by the same letter are not significantly different ($P > 0.05$) as determined by using 'lsmeans' with the Tukey adjustment in the GLIMMIX procedure in SAS v9.4.

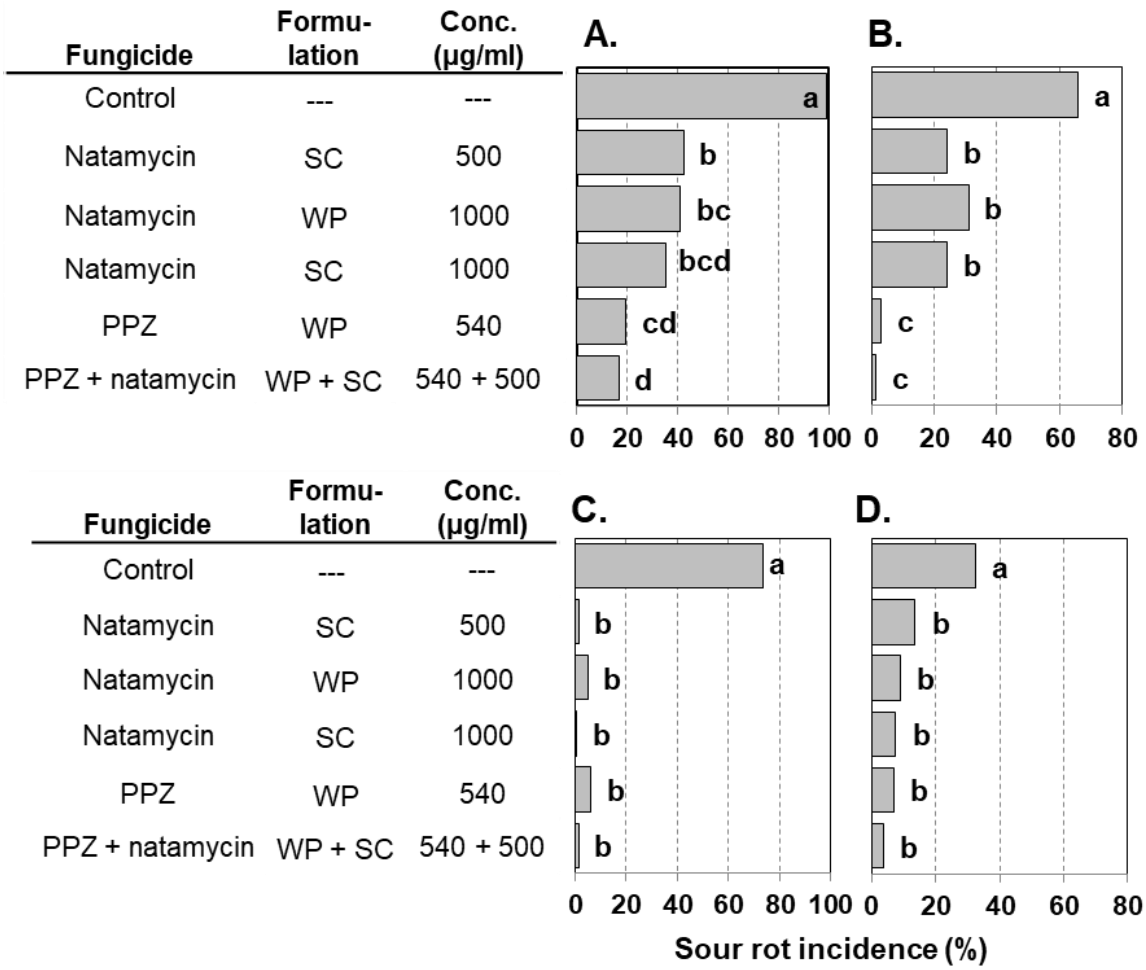

Fig. 2.2. Laboratory studies on the efficacy of natamycin and propiconazole (PPZ) for managing sour rot caused by *Geotrichum citri-aurantii* of **A**, **C,** 'Eureka' lemon and **B**, **D,** 'Tango' mandarin. Fruit were wound-inoculated with **A, B,** conidia ($5x10^5$/ml) prepared in citrus juice with 2 µg/ml cycloheximide or **C**, **D**, with conidia ($1x10^7$/ml) prepared in water. Fruit were treated after 12 to 14 h with aqueous solutions using a hand sprayer and incubated for 5 to 7 days at 20ºC. Values are the mean of two experiments. Horizontal bars followed by the same letter are not significantly different ($P > 0.05$) as determined by using 'lsmeans' with the Tukey adjustment in the GLIMMIX procedure in SAS v9.4. SC = soluble concentrate, WP = wettable powder.

Fungicide	Formu-lation	Conc. (µg/ml)	Fruit coating
Control	---	---	---
Natamycin	WP	1000	---
Natamycin	WP	1000	S
Natamycin	SC	1000	---
Fludioxonil	SC	125	---
Fludioxonil + natamycin	SC + WP	125 + 1000	S
Fludioxonil + natamycin	SC + WP	125 + 1000	---

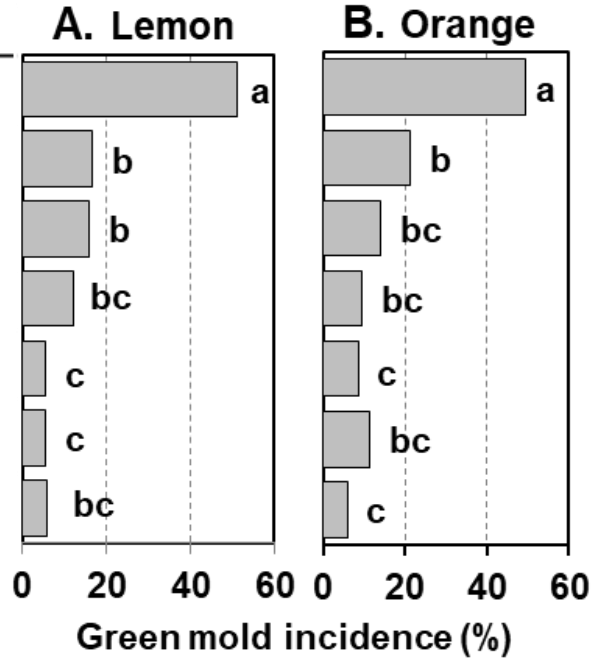

Fig. 2.3. Re-cycling flooder treatments with natamycin and fludioxonil on an experimental packingline for managing green mold caused by *Penicillium digitatum* of **A**, 'Improved Meyer' lemon and **B**, Valencia orange. Fruit were wound-inoculated (1 x 10^6 conidia/ml), treated after 12 to 14 h, and incubated for 5 to 7 days at 20ºC. A carnauba-based fruit coating was sequentially (S) applied after selected treatments using a control droplet applicator. Values are the mean of two experiments. Horizontal bars followed by the same letter are not significantly different ($P > 0.05$) as determined by using 'lsmeans' with the Tukey adjustment in the GLIMMIX procedure in SAS v9.4. SC = soluble concentrate, WP = wettable powder.

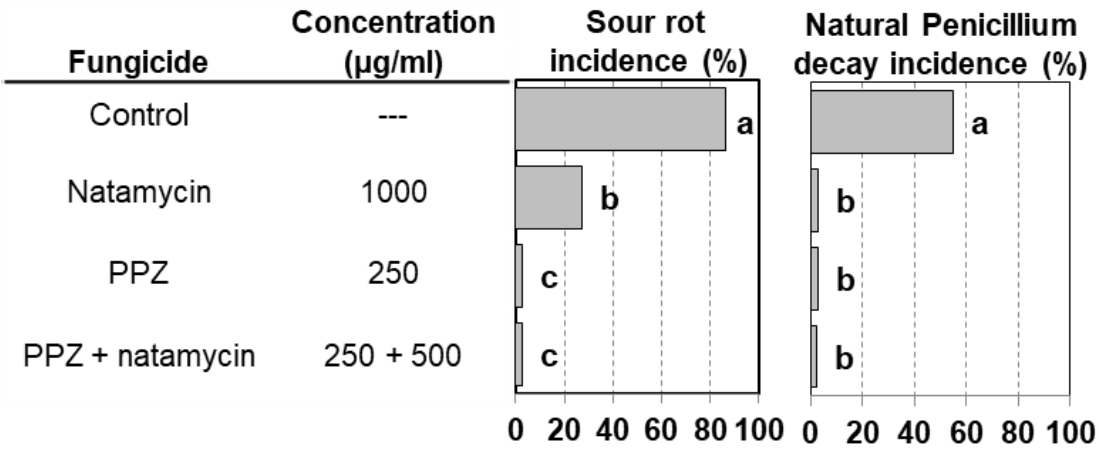

Fig. 2.4. Experimental and commercial packingline studies on the efficacy of heated (48°C) applications with natamycin (soluble concentrate) and propiconazole (PPZ; wettable powder) for managing sour rot of 'Eureka' lemon fruit caused by *Geotrichum citri-aurantii* and Penicillium decays. Sour rot was evaluated after wound-inoculation (5×10^5 conidia/ml lemon juice and 2 µg/ml cycloheximide), whereas Penicillium decays occurred naturally. After 12 to 14 h of incubation at 20ºC, fruit were treated using a re-cycling flooder. Fruit were evaluated for decay after 4 weeks of storage at 12ºC. Values are the mean of two experiments. Horizontal bars followed by the same letter are not significantly different ($P > 0.05$) as determined by using 'lsmeans' with the Tukey adjustment in the GLIMMIX procedure in SAS v9.4.

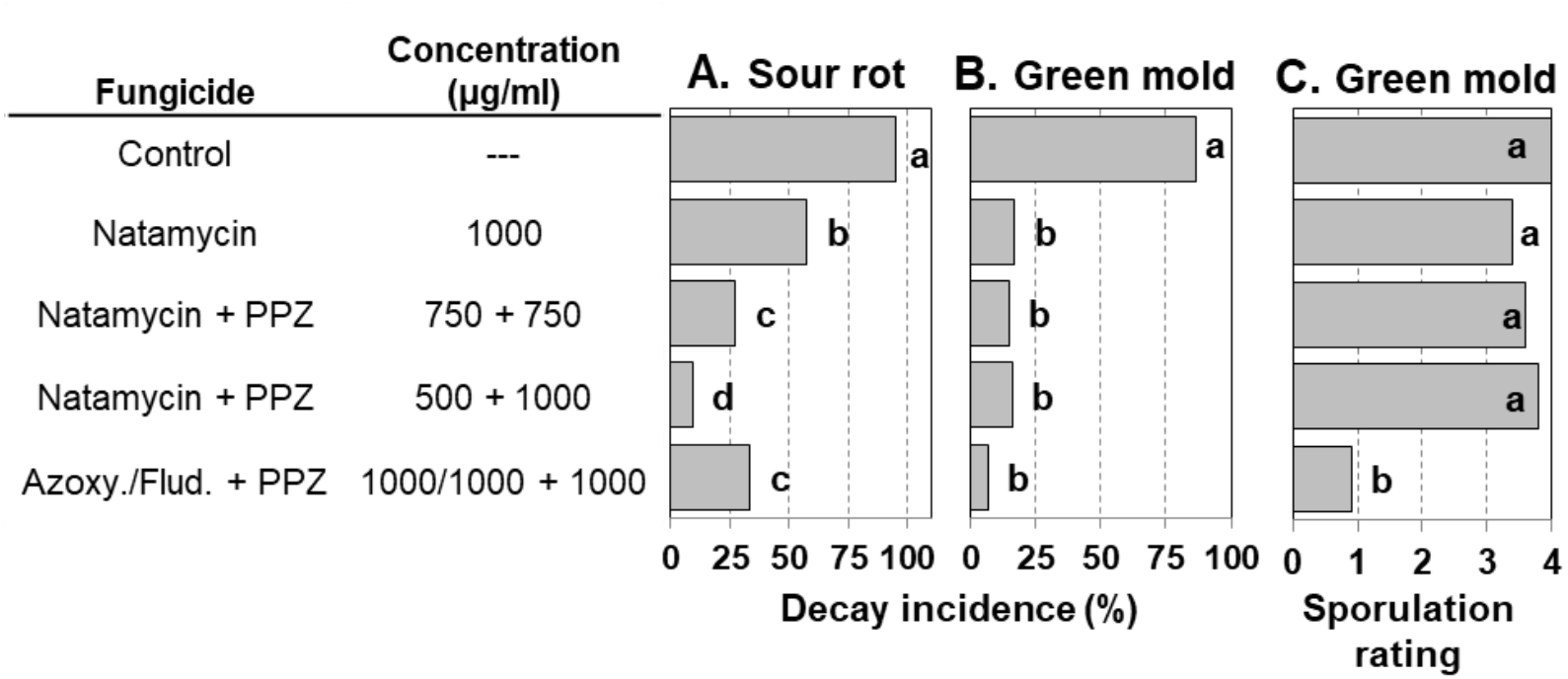

Fig. 2.5. Commercial packingline studies on the efficacy of natamycin (soluble concentrate) and propiconazole (PPZ, emulsifable concentrate) for management of **A,** sour rot, **B,** green mold of lemon, or **C,** green mold sporulation on lemon. Fruit were wound-inoculated with *Geotrichum citri-aurantii* (5 x 10^5 conidia/ml in lemon juice with 2 µg/ml cycloheximide) or *Penicillium digitatum* (1 x 10^6 conidia/ml in water). After 12 to 17 h of incubation at 20ºC, fruit were treated with fungicides prepared in a diluted storage fruit coating by controlled droplet application. Fruit were then stored for 4 weeks at 12ºC. Sporulation was rated using a scale from 0 (no sporulation) to 4 (fruit completely covered by sporulating mycelium). Values are the mean of two experiments. For decay incidence, horizontal bars followed by the same letter are not significantly different ($P > 0.05$) as determined by using 'lsmeans' with the Tukey adjustment in the GLIMMIX procedure in SAS v9.4. For sporulation ratings, the Kruskal-Wallis (K-W) test was used to determine frequency differences in ratings among treatments. The p-values for the K-W and Chi-Square tests were significant (P = 0.0001) indicating that the treatments differed in sporulation ratings. PPZ = propiconazole, Azoxy./Flud. = azoxystrobin-fludioxonil soluble concentrate pre-mixture.

CHAPTER III. Sanitizer Compatibility with Natamycin in Recirculating Fungicide Treatment Systems for Use in Citrus Packinghouses

ABSTRACT

Natamycin is a new postharvest biofungicide for citrus and some other fruit crops in the United States that can be effectively used in recycling drench or flooder treatments. This necessitates sanitation of the fungicide solution to ensure that it remains free of bacterial contamination. Natamycin was incompatible with peroxyacetic acid resulting in reduced efficacy of the fungicide against green mold, whereas sodium hypochlorite lost its toxicity to a non-pathogenic *Escherichia coli* strain (a surrogate for human pathogenic strains). In in vitro experiments, heated (48°C) citric acid (1,100 μg/ml or 2,200 μg/ml) amended with sodium dodcylbenzensulfonate (SDBS; 55 μg/ml or 110 μg/ml, respectively) significantly reduced viability of *E. coli* in natamycin solutions by >5 log. In laboratory studies with *Penicillium digitatum*-inoculated lemon fruit, 1,000 μg/ml natamycin with lactic or citric acids (1,000 μg/ml each), with or without SDBS (55 μg/ml) was effective in significantly reducing green mold. Natamycin mixed with lactic acid at 2000 μg/ml or higher, however, caused fruit injury resulting in browning and pitting of the rind. In heated (average 49°C) drench treatments on an experimental packingline, natamycin (1,000 μg/ml), fludioxonil (300 μg/ml), or azoxystrobin (300 μg/ml) mixed with citric acid (1,000 μg/ml)-SDBS (55 μg/ml) were effective against green mold without fruit injury. At a pH between 3.6 and 3.8, citric acid-SDBS significantly reduced the viability of *E. coli* in fludioxonil and azoxystrobin, but not in

natamycin mixtures. Natamycin (1,000 μg/ml) mixed with 2000 μg/ml citric acid and SDBS (55 μg/ml), however, significantly reduced *E. coli* counts within 4 min when the pH was maintained between 3.0 and 3.3, while retaining the efficacy of the fungicide. The use of citric acid with a surfactant can be a viable alternative sanitation method for natamycin in citrus packinghouses utilizing heated recirculating fungicide systems.

INTRODUCTION

Citrus is a major fruit crop in the United States, and in 2019, it was valued at more than $3 billion (USDA 2019). To reduce postharvest crop losses from fungal decays during storage and long distance transportation to domestic and international markets, fungicides are routinely applied in citrus packinghouses (Adaskaveg and Förster 2015; Eckert and Eaks 1989) using high- or low-volume systems (Adaskaveg et al 2002). Low-volume applications of fungicides in a fruit coating are effective and highly efficient with little run-off or wasted material. Some packinghouses use high-volume aqueous applications in a recirculating drench or flooder system that are followed by a sequential low-volume spray with a fruit coating. These high-volume treatments have been demonstrated to be superior in performance (Kanetis et al. 2008), although they not highly efficient because fungicide concentrations need to be continuously monitored, and periodic disposal of used solutions is required (Adaskaveg et al. 2002).

The postharvest biofungicide natamycin, a fermentation product of certain *Streptomyces* species (te Welscher et al. 2008), was previously identified as an effective treatment and mixture partner with other fungicides for reducing postharvest decays

(Adaskaveg et al. 2019; Chen et al. *submitted for publication*). It is currently registered in the United States for citrus and some other fruit crops. Because resistance to natamycin in filamentous fungi was never described in its use as a food preservative over many years (Delves-Broughton 2014) and resistance in major citrus postharvest pathogens could not be detected in laboratory studies, it has great potential to be utilized in anti-resistance strategies (Adaskaveg et al. 2019; Chen et al. *submitted for publication*). To facilitate the adoption of natamycin into current packinghouse decay management practices, a goal of our research was to identify a sanitizing agent that does not negatively impact decay control.

In the United States, the Food Safety Modernization Act (FSMA) of 2011, agricultural recycled treatments used on harvested crops are required to be sanitized to prevent the build-up of bacterial contaminants that may be pathogenic to humans. A sanitizer should be effective in inactivating bacterial contamination in recirculating fungicide solutions with a targeted greater than 4-$\log_{10}$ reduction in population size as mandated by the FSMA (US Congress 2011). According to federal regulations, human pathogenic bacteria of special concern are *Escherichia coli, Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Salmonella* spp. (Schroth et al. 2018; Strawn et al. 2013; US-FDA 2017). Potential sources of these bacterial contaminants of agricultural crops in the field include manure, contaminated irrigation water/soil, as well as human activity, and many factors influence their incidence, dissemination, survival, and proliferation (Alegbeleye et al 2018; Strawn et al. 2013). Industry standard practice is to sanitize fresh fruit upon arrival in the packinghouse. However, there is no treatment that will eliminate

all microbial contamination, and microbes residing inside fruit injuries are protected from sanitizing action. Additionally, there may be new introductions of contamination either by handling equipment that is often difficult to clean or by packinghouse workers. In the packinghouse, these microbes may disseminate and proliferate in water handling systems and in recirculating fungicide solutions that are sometimes being used for several days.

Sanitation treatments of postharvest fungicide solutions have been investigated previously (Kanetis et al. 2008; McKay et al. 2012). These studies indicated that imazalil and pyrimethanil are not stable in the presence of NaOCl at recommended concentrations, but both can be used without loss of efficacy in combination with peroxyacetic acid (PAA). In contrast, azoxystrobin, fludioxonil, and thiabendazole were found to be stable with both sanitizers. Because the oxidizing sanitizers NaOCl and peroxyacetic acid are commonly considered irritants for packinghouse workers, organic acid sanitizers were evaluated as alternatives. Organic acids are currently used for disinfecting food and food-contact surfaces. Several commercial products are available, and the most common ones are based on citric and lactic acids (Boomsma et al. 2015; Fatica and Schneider 2009). Preliminary studies indicated that organic acids may be compatible with fungicide solutions (Adaskaveg et al. 2017). I previously identified the high potential of natamycin as an effective postharvest treatment for citrus fruits in recirculating and non-recovery fungicide application systems for control of sour rot caused by *Geotrichum candidum* and green mold caused by *Penicillium digitatum* (Chen et al. 2016). Natamycin was recently registered as a biofungicide for postharvest use on

citrus and stone fruits, but sanitizing methods for recirculating solutions of the fungicide have not been evaluated.

The goals of this study were to: i) evaluate the efficacy and stability of natamycin as compared to other citrus postharvest fungicides (i.e., azoxystrobin and fludioxonil) against green mold in the presence of common sanitizers, ii) determine the antimicrobial activity of the sanitizers in the presence of natamycin, and iii) identify best usage strategies for the fungicide to maximize decay control while keeping recirculating treatment solutions sanitized. The organic acids I used are lactic and citric acids, both considered Generally Recognized as Safe (GRAS) by the United States Food and Drug Administration (US-FDA), and some treatments were done in combination with the food grade surfactant sodium dodecylbenzenesulfonate (SDBS; US-FDA 2012). In vitro sanitizing studies were conducted using a non-human-pathogenic strain of *E. coli* and the plant pathogen *Pseudomonas syringae* as surrogates for human pathogens. Studies on treatment efficacy and sanitation activity with lemon fruit inoculated with *Penicillium digitatum* were done in the laboratory and on an experimental packingline.

MATERIALS AND METHODS

Chemicals used. Formulated fungicide products of natamycin, (BioSpectra 100SC – a suspension concentrate - SC; DSM Food Specialties, Heerlen, The Netherlands), fludioxonil (Scholar SC), and azoxystrobin (Diploma 2F) (the latter two fungicides from Syngenta Crop Protection LLC, Greensboro, NC) were used at rates recommended by the respective manufacturers for commercial application on citrus

fruits. Citric acid (Mallinckrodt Baker, Inc., Phillipsburg, NJ), lactic acid (Sigma-Aldrich, St. Louis, MO), and SDBS (Sigma-Aldrich) were used in sanitizer solutions. Other sanitizers included a pre-mixed formulation of citric acid and SDBS (Pro-San; 66% citric acid, 3.6% SDBS; Microcide, Troy, MI), NaOCl (household bleach 6.25%), and peroxyacetic acid (PAA; Perasan A; EnviroTech Chemical Services, Inc., Modesto, CA). Treatment solutions were prepared in sterile deionized (laboratory studies) or tap (packingline studies) water.

Microorganisms used. *P. digitatum* (isolate "Pd"; obtained from a packinghouse in California and sensitive to all registered citrus postharvest fungicides) was grown on potato dextrose agar (PDA; Difco Laboratories, Becton, Dickinson and Co., Sparks, MD) at 25ºC. For inoculum production, 7- to 10-day-old cultures were used. A non-pathogenic *E. coli* strain (K-12; obtained from C. Roper, University of California, Riverside) was used as a surrogate for a human pathogenic strain of the species, and a strain of *P. syringae* (obtained from blasted sweet cherry flowers) was used as a surrogate for *P. aeruginosa*. Bacteria were preserved in 20% glycerol at -80 ºC. *E. coli* was grown on nutrient agar (NA, Difco Laboratories) at 37°C, and *P. syringae* was cultured on Kings Medium B agar (KMB; King et al. 1954) at 24°C, both for 24 to 36 h before use.

Inoculation of citrus fruits. 'Eureka' lemons [*Citrus limon* (L.) Burm.] were obtained from orchards where no preharvest fungicides were used. Fruit were washed with water and/or 100 µg/ml sodium hypochlorite (NaOCl; diluted commercial household bleach) and placed into plastic trays inside commercial cardboard fruit boxes. The flavedo and albedo of each fruit were wounded (2 mm long, 0.5 mm wide, 2 mm

deep) using a stainless-steel wounding tool without penetrating the juice sacks. *P. digitatum* inoculum (1×10^6 conidia/ml) was prepared in sterile water amended with 0.01% Triton X-100 (Sigma-Aldrich), and 20-µl aliquots were pipetted into each wound. Fruit were incubated for 12 to 14 h at 20°C before treatment.

In vitro sanitation studies with *P. syringae* and *E. coli*. Sanitizer solutions were prepared in sterile deionized water 10 to 15 min before the start of the experiment. NaOCl concentrations were adjusted using free chlorine test strips (Serim Research, Elkhart, IN), and for peroxyacetic acid, peroxide test strips (Bartovation, New York, NY) were used. The pH of every solution was measured using a calibrated pH meter (SevenCompact pH/Ion meter S220; Mettler-Toledo International Inc., Columbus, OH). Aliquots of 990 µl sanitizer solutions were added to 1.8 ml microcentrifuge tubes. The tubes were either left at room temperature (24°C) or were heated to 48°C using a dry heating block (MyBlock, Benchmark Scientific, Inc., Edison, NJ). Bacterial suspensions were adjusted to 70% transmittance using a spectrophotometer (DU 730 Life Science UV/Vis Spectrophotometer; Beckman Coulter, Brea, CA), equivalent to approximately $1.5x10^8$ CFU/ml, and 10 µl was added to each sanitizer solution and to tubes with sterile distilled water that served as untreated controls. Tubes were inverted three times for mixing, and after incubation periods of 1 or 4 min, 10 µl reaction mixture was added to 990 µl of sterile deionized water to minimize any further chemical reaction. The diluted reaction mixtures were then plated onto 10-cm diameter NA plates (50 µl/plate) using a spiral plater (AutoPlate 4000; Advanced Biotech, Norwood, MA) set at the exponential deposition mode. Plates were enumerated for *E. coli* after 16 h at 37°C, and for *P.*

syringae after 24 h at 24°C using a standard spiral plater counting grid and were expressed as $\log_{10}$ number of CFU/ml solution. There were two or three replicates for each sanitizer-bacterium combination, and each experiment was done at least twice.

Efficacy of natamycin by itself or in the presence of sanitizing treatments in reducing green mold of inoculated lemon fruit in laboratory studies. Solutions of natamycin (1000 μg/ml) by itself or in mixtures with citric acid (1000 or 2000 μg/ml), lactic acid (1000 or 2000 μg/ml), NaOCl (100 μg/ml), or PAA (80 μg/ml) were prepared 16 h before treatment of fruit inoculated with *P. digitatum.* NaOCl and PAA concentrations were adjusted to the desired values using tests strips as described above. SDBS was added to selected treatments at a concentration of 55 μg/ml that was based on the ratio of citric acid and SDBS in a commercial product (i.e., Pro-San). Solutions were stored at 20°C in the dark to allow any chemical interactions to occur. Treatments were applied to fruit to run-off using a hand-operated atomizer (Model 15-RD; DeVilbiss Health Care, Somerset, PA). Control fruit were treated with water. Fruit were stored for seven days at 20ºC and then evaluated for green mold (i.e., soft, watery lesions around the inoculation site or conidia/mycelium-covered decay). Twelve fruit were used in each of 4 replications per treatment, and the experiment was done twice.

Efficacy of fungicide-sanitizer solutions in reducing green mold and sanitizing contaminated treatment solutions in experimental packingline studies. In the two studies conducted, treatment solutions (20 liters each) were prepared approximately 16 to 18 h before use and stored at 20°C in the dark. In the first study, fungicides (natamycin, fludioxonil, azoxystrobin) were used at registered rates by

themselves or in mixture with a commercial citric acid-SDBS sanitizer (i.e., Pro-San). In the second study, natamycin was used in solutions of two concentrations of citric acid (825 and 2000 μg/ml) and SDBS. The amount of citric acid used was based on obtaining a pH of 3.0 or 3.5 before application, thus, different amounts were used for citric acid-SDBS by itself and when mixed with natamycin. Natamycin was also applied in combination with NaOCl that was added to obtain a measurement of 50 μg/ml free chlorine before storage of the solution, and the same amount was added just before use to test for both intermediate and short-term interactions. Additionally, sanitizers were also used by themselves. *E. coli* was added to treatment solutions to a final concentration of 1.6 $x10^6$ CFU/ml immediately before use. High-volume flooder treatments heated to 48 to 51°C with a tank-less water heater were applied on an experimental packlingline to lemon fruit 12 to 14 h after inoculation with *P. digitatum*. Treatment solutions were pumped from a 20-liter reservoir onto two stainless steel weirs positioned 20 cm above a moving roller bed. Solutions cascaded from the weirs onto the fruit with treatment durations of 12 to 15 s. Control fruit were left untreated, and an additional set of fruit was treated with heated water. Temperature and pH were recorded for each treatment solution before and after use. The free chlorine concentration in the natamycin-NaOCl treatment was measured after use. Treatment solution aliquots of 10-μl (three for each solution) were sampled 2 min (study 1) or 4 min (study 2) after the start of applications and mixed with 990 μl of sterile deionized water. Plating of solutions and enumeration of *E. coli* colonies was done as described above. Between treatments, the flooder equipment was cleaned with a 1% dish soap solution and then triple rinsed with water. Fruit were

evaluated for green mold after storage for 7 days at 20°C. Twelve fruit were used in each of 4 replications per treatment and each study was done twice.

Statistical analysis of data. Statistical analyses were performed using SAS (ver. 9.4; SAS Institute, Cary, NC) with a significance value of $\alpha = 0.05$. Percent disease incidence data from efficacy studies against *P. digitatum* were arcsine square-root transformed prior to analysis. Bacterial load (CFU/ml) was $\log_{10}$-transformed. One-way analyses were performed for in vitro experiments on sanitizer toxicity and treatments in fruit efficacy experiments using generalized linear mixed models with the GLIMMIX procedure. For these, treatment was treated as a fixed effect, and trial, replication (block), and the overall error term were treated as random effects. Fixed effects were tested for significance, and least squares treatment means were constructed using the lsmeans statement with the Tukey adjustment in PROC GLIMMIX. Contrast estimates for pairs of treatments were used to determine multiple comparison differences among treatment means.

RESULTS

In vitro sanitation studies with *P. syringae* and *E. coli*. Lactic acid, citric acid, PAA, and NaOCl at concentrations of 10 µg/ml were all highly toxic to *P. syringae* in 1-min exposures at ambient temperature (24°C). In comparison with the water control that contained 6.14 $\log_{10}$ CFU/ml, bacterial populations were significantly ($P<0.0001$) reduced by >4 $\log_{10}$ (Table 3.1). There was no significant difference among sanitizers, but NaOCl reduced populations to non-detectable levels.

In laboratory studies with *E. coli*, lactic acid at 2000 μg/ml was not effective (Table 3.1). PAA at the labeled rate of 80-μg/ml rate and citric acid at 2000 μg/ml slightly but significantly (P<0.0001) reduced bacterial populations from 6.21 $\log_{10}$ CFU in the control to 5.81 and 6.11 $\log_{10}$ CFU, respectively. The addition of SDBS (that itself had no bactericidal activity) to citric and lactic acids, however, completely inactivated *E. coli* (Table 3.1). NaOCl reduced populations of *E. coli* to non-detectable levels.

The sanitizing activities of NaOCl and PAA, as well as citric and lactic acids with or without the addition of SDBS were further evaluated in the presence of natamycin in 1-min exposures at 24°C (Table 3.2, study A) or at 48°C (Table 3.2, study B), and in 4-min-exposures at 48°C (Table 3.2, study C). Among mixtures used in 1-min exposures at 24°C, only natamycin-citric acid (1100 or 2200 μg/ml)-SDBS significantly (P <0.0001) reduced *E. coli* populations from the water control, but reductions were only 0.33 or 0.94 $\log_{10}$. The natamycin-NaOCl mixture showed no sanitizing activity in 1- or 4-min exposures at both temperatures (Table 3.2, studies A, B, C). When natamycin-citric acid-SDBS mixtures were heated to 48°C, however, bacterial reductions were more than 5 $\log_{10}$, and no bacteria were recovered using the 2200-μg/ml concentration of citric acid in 1-min exposures (Table 3.2, study B). In comparison, natamycin mixed with 2200 μg/ml citric acid without SDBS resulted in a 1-$\log_{10}$ reduction and mixed with PAA resulted in a 1.6 $\log_{10}$ reduction in CFU/ml. In 4-min heated exposures, reductions for the latter solutions were over 5.5-$\log_{10}$ (Table 3.2, study C).

Efficacy of natamycin in the presence of sanitizing treatments in reducing green mold of inoculated lemon fruit in laboratory studies. Untreated, inoculated lemon fruit developed 77.7% incidence of green mold, and spray applications with natamycin at 1000 μg/ml significantly (P<0.0001) reduced the incidence to 17.7% (Fig. 3.1). When natamycin solutions were incubated for 16 h before use with citric or lactic acid (both at 1000 μg/ml) with or without SDBS or with NaOCl, the efficacy was statistically similar to natamycin by itself, and incidences ranged from 9.4% for natamycin-lactic acid to 19.8% for natamycin-lactic acid-SDBS. Application of natamycin mixtures with 2000 μg/ml lactic acid-SDBS were effective in reducing green mold from 98% incidence in the control to 12.5%, however, phytotoxicity was observed as browning and pitting of the fruit rind (*data not shown*). Phytotoxicity was also observed in lactic acid-SDBS mixtures with azoxystrobin and fludioxonil (*data not shown*). A similar level of decay control was obtained for natamycin mixed with citric acid at concentrations as high as 6,600 μg/ml, and no phytotoxicity was present (*data not shown*). The addition of PAA to natamycin resulted in a numerically reduced efficacy (i.e., 37.5% incidence) as compared to natamycin by itself, and this treatment was significantly (P<0.0001) less effective than the natamycin-organic acid mixtures with the exception of natamycin-lactic acid-SDBS. In the presence of citric or lactic acids, the pH of the natamycin solution dropped from 5.6 to between 2.7 and 2.8, the pH in the presence of PAA was 3.7, whereas when using NaOCl it increased to 7.3.

Efficacy of recirculating fungicide-sanitizer solutions in reducing green mold of inoculated lemon fruit and and bacterial load in experimental packingline studies. Heated (average 49°C) treatments were compared as flooder treatments in experimental packingline studies. In the first study, natamycin, fludioxonil, and azoxystrobin used by themselves were all highly effective in reducing green mold and significantly ($P< 0.0001$) reduced the incidence to between 1.0 and 7.2% as compared with 84.7% for the untreated control (Fig. 3.2.A). All three fungicides were similarly highly effective when solutions were mixed with 1000 μg/ml citric acid and 55 μg/ml SDBS 16 to 18 h before use as compared by themselves and were not significantly different from each other (P=1.0000) . Citric acid-SDBS by itself reduced decay incidence to 49.0%

Citric acid-SDBS at a pH of 3.8 to 3.9 displayed a high sanitizing activity against *E. coli* with a significant ($P< 0.0001$) reduction in bacterial load from 6.2 $\log_{10}$ CFU/ml in the non-heated water control to 1.9 $\log_{10}$ CFU/ml, i.e., a 4.3-$\log_{10}$ reduction (Fig. 3.2.B). Similar reductions in bacterial load were obtained in fludioxonil- and azoxystrobin-citric acid-SDBS mixtures (i.e., 4.1 and 3.9 $\log_{10}$, respectively) at a pH of 3.6 to 3.7. Bacterial populations in the natamycin-sanitizer mixture (pH 3.7 to 3.8), however, were similar in size to those in the untreated water control.

In the second packingline study, treatment of fruit with citric acid-SDBS (initial pH 3.5 or 3.0) heated to 49°C did not significantly (P=1.0000) decrease the incidence of green mold from the untreated control, but heated water by itself significantly (P=0.0313) reduced the decay from 97% in the control to 74% (Fig. 3.3.A). Natamycin by itself or

mixed with NaOCl or with two rates of citric acid (825 and 2000 μg/ml)-SDBS were all similarly effective in reducing green mold (Fig. 3.3.A). The incidence was significantly ($P< 0.0001$) reduced from 97% in the control to between 28.1% (i.e., natamycin-2000 μg/ml citric acid-SDBS) and 42.7% (i.e., natamycin-NaOCl).

Incubation of *E. coli* in water heated to 49°C for 4 min had no effect on bacterial viability (Fig. 3.3.B). The high and low concentrations of citric acid mixed with SDBS were highly toxic to *E. coli* after 4 min of exposure to the bacteria, significantly ($P< 0.0001$) reducing populations from 6.2 $\log_{10}$ CFU/ml in the control to 0 or 0.76 $\log_{10}$ CFU/ml, respectively (Fig. 3.3.B). This corresponds to >5 $\log_{10}$ reductions. At both concentrations of citric acid, at pH 3.5 or 3.0, the addition of natamycin significantly ($P<0.0001$) decreased the sanitizing activity of citric acid-SDBS, and bacterial counts for natamycin mixed with the low concentration of citric acid were statistically similar to the water control. Natamycin mixed with 2000 μg/ml citric acid and 55 μg/ml SDBS, however, resulted in 2.19 $\log_{10}$ viable bacteria remaining in the solution, corresponding to a 4-$\log_{10}$ reduction as compared with the control. Furthermore, the natamycin mixture with NaOCl was also not effective in reducing bacterial viability (Fig. 3.3.B). The free chlorine concentration in the natamycin-NaOCl mixture was measured between 40 to 50 μg/ml following treatment for the two experimental repeats.

In both experimental packingline studies, the pH of treatment solutions slightly increased after use. In the first study, the pH increased by 0.1 for all treatments; whereas in the second study, pH increases were between 0.3 and 0.5 for the citric acid treatments,

1.1 for natamycin-NaOCl, and 0.5 to 0.6 for the water and natamycin treatments, respectively.

DISCUSSION

In this study, I demonstrate that aqueous solutions of natamycin as well as of azoxystrobin or fludioxonil can be successfully sanitized with citric acid-SDBS if the pH is reduced to 3.0 to 3.3 (measured before and after use, respectively). In treatment mixtures prepared 16 to 18 h before use, natamycin remained effective in reducing green mold of lemon fruit, and *E. coli* populations were reduced by 4 $\log_{10}$ CFU/ml as compared with a water control. I used *P. syringae* and a non-pathogenic strain of *E. coli* as surrogates for human pathogens in the sanitation studies and expect similar results for *P. aeruginosa* and pathogenic strains of *E. coli*. The sanitizing agents tested were all much more active against *P. syringae* than against *E. coli.* Furthermore, comparative studies with citric acid done by others indicated that *E. coli* O157:H7 was more acid-resistant than *L. monocytogenes* and *Salmonella* sp. (Putnam et al. 2018). Therefore, I used *E. coli* as the more resilient species in all my fruit studies. Laboratory studies indicated that the addition of SDBS was critical for high sanitation efficacy. Surfactants are known to improve the efficacy of sanitizers by fluidizing and weakening bacterial cell membranes, thereby increasing the effects of organic acid exposure (Jackson-Davis et al. 2018; Predmore and Li 2011). Additionally, laboratory studies also demonstrated that heated treatments were significantly more effective than those at ambient temperature of 24°C. Therefore, packingline flooder treatments were used at an average temperature of

49°C. Most citrus packinghouses are adapted to do these heated treatments that are currently often used for other postharvest fungicides (D'Aquino et al. 2006; Smilanick et al. 1997).

When other commonly used sanitizers were mixed with natamycin, efficacy in decay control was decreased (i.e., PAA), or the sanitizing power was inactivated (i.e., NaOCl). PAA and NaOCl are both oxidizing agents that target lipids, carbohydrates, and peptide linkages. This results in denatured proteins and inactivated enzymes that affect essential cell components including membranes (Wallis-Lage 2010). Natamycin is reported to be unstable when exposed to oxidizers (Dekker and Ark 1959), and this can explain the reduced efficacy against green mold in the presence of PAA. NaOCl is a stronger oxidizer than PAA and may allow the formation of an oxidized metabolite of natamycin that still has antifungal activity. Detailed residue analyses may help to understand why the activity of natamycin is maintained in the presence of NaOCl. In contrast, the sanitizing power of NaOCl in mixture with natamycin was completely lost. Natamycin is a large, complex organic molecule that itself or through the presence of certain components of the formulation may interfere with the oxidizing activity of NaOCl as is known for various organic materials (Suslow 1997). In the experimental packingline studies, the average pH of the natamycin-NaOCl treatment solution increased from 7.3 to 8.4 after treatment. A low ratio of hypochlorous acid (HOCl):hypochlorite (OCl^-) present at these high pH values could have contributed to the lack of sanitizing activity in this particular study. In the in vitro studies, however, a lack of sanitizing activity was also evident in natamycin-NaOCl mixtures at pH values ranging from 6.7 to 7.0 that are

considered ideal for NaOCl sanitation. These incompatibilities and known effects of oxidizers as human irritants necessitated the evaluation of alternative sanitation methods.

The inhibitory action of organic acids is based on lowering the pH of solutions to levels that affect ionic gradients or membrane permeability of the bacterial cell and may also inhibit or shut down electron transport systems within the cells (Fatica and Schneider 2009). Many foodborne pathogens are inactivated at a highly acidic pH and cannot grow under these conditions. In studies, in addition to citric acid-SDBS, lactic acid-SDBS also showed good sanitizing activity. Because phytotoxicity was observed on the fruit rind as browning and pitting when lemon fruit were treated with 2000 µg/ml lactic acid by itself or in mixtures with natamycin, fludioxonil, or azoxystrobin, this sanitizing agent was no longer pursued for evaluation.

Organic acid compounds are most active at a pH below pH 3.0 (Fatica and Schneider 2009). Maintaining a pH of 3 by adjusting the amount of citric acid used in the treatment solution was critical in my studies. Natamycin is considered stable at pH 3 to 9 (Stark 2000). Hydrolyzation and degradation of the molecule has been reported at lower pH, and the mycosamine moiety is split off (Stark 2000). For example, after incubation at pH 2 and 37°C for 72 h, the molecule was degraded, whereas at pH 4.5, it was relatively stable with a 15% reduction of the parent molecule (Raab 1972). Thus, although hydrolysis of natamycin under low-pH conditions is a concern, there was no negative impact under my experimental timeframes.

Because citric acid is readily available, inexpensive, and is already used in packinghouses to adjust the pH of NaOCl solutions, citric acid sanitation can be easily

integrated into fruit processing. Its activity is not affected by hard water or organic residues, there are no toxic degradation byproducts and no irritating odors, and no rinsing of treated commodities is required (Fatica and Schneider 2009). Furthermore, citric acid is considered Generally Recognized as Safe (GRAS) by the US-FDA, and as for natamycin, no residue tolerance is needed in the United States. Still, there are potential drawbacks with recycling natamycin-citric acid flooders with the need for monitoring pH and replenishing the organic acid to maintain a proper pH (i.e., 3 to 3.3). In citrus packinghouses, fruit are commonly treated with alkaline sodium carbonate or bicarbonate salts that temporarily increase the pH in surface wounds even after rinsing the fruit with potable water. At pH 8 and above, growth of citrus decay pathogens is inhibited. Fungicide drenches are typically set up after alkaline dips and thus, a highly acidic recycling drench will be difficult to maintain because of carryover of the salt solution by the fruit in the sequential processing. With proper monitoring of pH, sanitizing natamycin flooder solutions with citric acid will therefore be an easily integrated option for packinghouses that do not use alkaline salt treatments.

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Table 3.1. In vitro toxicity of organic acids and oxidizing santizers to *Escherichia coli* and *Pseudomonas syringae* in 1-min direct aqueous exposures at 24°C.

Bacteria	Treatment[a]	(µg/ml)	pH	$\log_{10}$ CFU/ml[b]	LSCE[c]
P. syringae	Water	---	5.87	6.14	a
	Lactic acid	10	4.06	1.75	b
	Citric acid	10	4.17	0.63	b
	Peroxyacetic acid	10	4.07	0.74	b
	NaOCl	10	6.86	0.00	b
E. coli	Water	---	5.87	6.21	a
	SDBS	100	6.85	6.22	a
	Lactic acid	2000	2.52	6.19	a
	Lactic acid + SDBS	2000 + 100	2.53	0.00	d
	Citric acid	2000	2.49	6.11	b
	Citric acid + SDBS	2000 + 100	2.50	0.00	d
	Peroxyacetic acid	80	3.51	5.81	c
	NaOCl	100	7.00	0.00	d

[a] Initial bacterial concentrations were 1.4 x 10^6 CFU/ml (6.1 $\log_{10}$) for *P. syringae* and 1.5 x 10^6 CFU/ml (6.2 $\log_{10}$) for *E. coli*. Sodium hypochlorite (NaOCl) was measured as free chlorine. Samples were taken after 1 min of exposure, diluted 1:100 with deionized water, and plated onto nutrient agar.

[b] Bacterial colonies were enumerated after 24 h at 24°C for *P. syringae* and 16 h at 37°C for *E. coli*. Data from at least two experiments were averaged.

[c] Values followed by the same letter are not significantly different based on lsmeans and contrast estimates (LSCE) of the GLIMMIX procedure in SAS v9.4 ($P < 0.05$).

Table 3.2. In vitro toxicity of organic acids and oxidizing santizers in mixtures with natamycin to *Escherichia coli* in 1- or 4-min direct aqueous exposures at 24°C or 48°C.

Study	Treatment[a]	Concentration (µg/ml)	Exposure time (min)	pH	Temperature (°C)	log_{10} CFU/ml[b]	LSCE[c]
A	Water	---	1	5.74	24	6.50	a
	Natamycin (SC)	1000	1	5.49	24	6.52	a
	Natamycin + NaOCl	1000 + 100	1	6.71	24	6.52	a
	Natamycin + peroxyacetic acid	1000 + 80	1	3.69	24	6.37	ab
	Natamycin + citric acid	1000 + 1100	1	2.79	24	6.44	ab
	Natamycin + citric acid + SDBS	1000 + 1100 + 55	1	2.82	24	6.17	b
	Natamycin + citric acid	1000 + 2200	1	2.62	24	6.25	ab
	Natamycin + citric acid + SDBS	1000 + 2200 + 110	1	2.64	24	5.56	c
B	Water	---	1	5.63	48	6.39	a
	Natamycin (SC)	1000	1	5.47	48	6.48	a
	Natamycin + NaOCl	1000 + 100	1	7.02	48	6.45	a
	Natamycin + peroxyacetic acid	1000 + 80	1	3.71	48	4.77	b
	Natamycin + citric acid	1000 + 1100	1	2.81	48	5.70	ab
	Natamycin + citric acid + SDBS	1000 + 1100 + 55	1	2.82	48	0.83	c
	Natamycin + citric acid	1000 + 2200	1	2.64	48	5.36	b
	Natamycin + citric acid + SDBS	1000 + 2200 + 110	1	2.65	48	0.00	c
C	Water	---	4	5.63	48	6.39	a
	Natamycin (SC)	1000	4	5.47	48	6.46	a
	Natamycin + NaOCl	1000 + 100	4	7.02	48	6.27	a
	Natamycin + peroxyacetic acid	1000 + 80	4	3.71	48	0.00	c
	Natamycin + citric acid	1000 + 1100	4	2.81	48	3.94	b
	Natamycin + citric acid + SDBS	1000 + 1100 + 55	4	2.82	48	0.00	c
	Natamycin + citric acid	1000 + 2200	4	2.64	48	0.83	c
	Natamycin + citric acid + SDBS	1000 + 2200 + 110	4	2.65	48	0.00	c

[a] The initial bacterial concentrations was 1.5 x 10^6 CFU/ml (6.2 log_{10}). Sodium hypochlorite (NaOCl) was measured as free chlorine. Samples were taken after 1 or 4 min of exposure, diluted 1:100 with deionized water, and plated onto nutrient agar.

[b] Bacterial colonies were enumerated after 16 h at 37°C. Data from at least two experiments were averaged.

[c] Values followed by the same letter are not significantly different as denoted by the lsmeans statement and contrast estimates (LSCE) of the GLIMMIX procedure in SAS v9.4 ($P < 0.05$). Each study was analyzed separately.

Treatment	Concentration (µg/ml)	pH
Water	---	5.8
Natamycin	1000	5.6
Natamycin + CA	1000 + 1000	2.7
Natamycin + CA + SDBS	1000 + 1000 + 55	2.8
Natamycin + LA	1000 + 1000	2.8
Natamycin + LA + SDBS	1000 + 1000 + 55	2.8
Natamycin + NaOCl	1000 + 100	7.3
Natamycin + PAA	1000 + 80	3.7

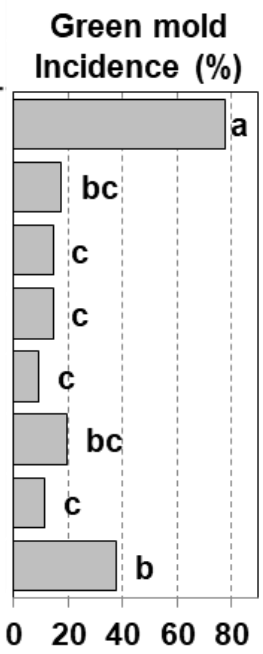

Fig. 3.1. Laboratory studies on the efficacy of natamycin in combination with citric acid (CA) or lactic acid (LA) and the surfactant sodium dodecylbenzenesulfonate (SDBS) as compared to using sodium hypochlorite (NaOCl) measured as free chlorine or peroxyacetic acid (PAA) for managing green mold of lemon fruit caused by *Penicillium digitatum*. Treatment solutions were stored in the dark at 20°C for 16 h before use. Fruit were wound-inoculated (20 µl of 1 x 10^6 conidia/ml), treated after 12 to 13 h using a hand sprayer, and incubated for 7 days at 20ºC. pH values are the average immediately before and after treatment. Horizontal bars followed by the same letter are not significantly different ($P > 0.05$) as determined by using 'lsmeans' with the Tukey adjustment in the GLIMMIX procedure in SAS v9.4.

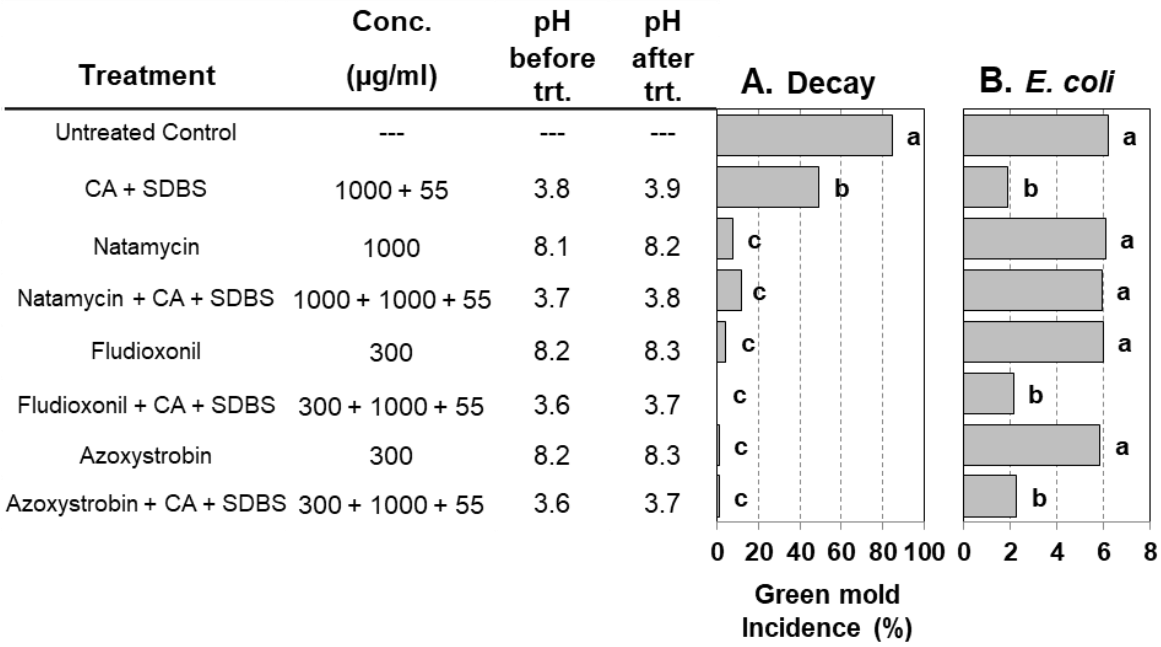

Fig. 3.2. Experimental packingline study on the compatibility of natamycin, fludioxonil, and azoxystrobin in combination with a commercial formulation of citric acid (CA) and dodecylbenzenesulfonate (SDBS) for **A,** managing green mold of lemon fruit caused by *Penicillium digitatum* and **B,** as a sanitizer against *E. coli*. Treatment solutions were stored in the dark at 20°C for 16 to 18 h and were then amended with *E. coli* to a final of 1.57×10^6 CFU/ml (6.2 $\log_{10}$) before use. Fruit were wound-inoculated (20 µl of 1×10^7 conidia/ml), incubated for 12 to 14 h at 20°C, treated with a heated (average 49°C) re-cycling flooder on an experimental packingline, and incubated for 7 days at 20°C. Solutions were sampled after 2 min treatment time for bacterial enumeration and compared to the initial concentration (i.e., the untreated control). Horizontal bars followed by the same letter are not significantly different ($P > 0.05$) as determined by using 'lsmeans' with the Tukey adjustment in the GLIMMIX procedure in SAS v9.4.

Treatment	Conc. (µg/ml)	pH before trt.	pH after trt.
Untreated Control	---	---	---
Water	---	8.2	8.7
Natamycin	1000	8.0	8.6
Natamycin + NaOCl	1000 + (50+50)	7.3	8.4
CA + SDBS	825 + 55	3.5	4.0
Natamycin + CA + SDBS	1000 + 825 + 55	3.5	3.9
CA + SDBS	1650 + 55	3.0	3.4
Natamycin + CA + SDBS	1000 + 2000 + 55	3.0	3.3

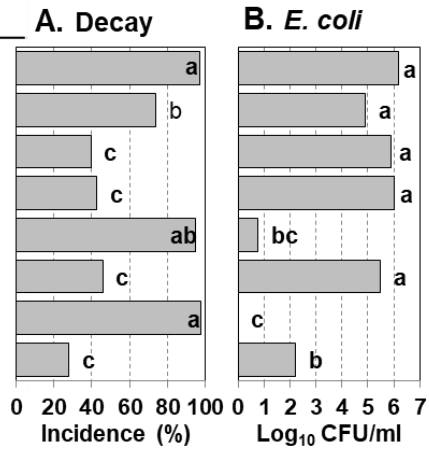

Fig. 3.3. Experimental packingline study on the compatibility of natamycin in combination with citric acid (CA) - dodecylbenzenesulfonate (SDBS) or sodium hypochlorite (NaOCl) for **A,** managing green mold of lemon caused by *Penicillium digitatum* and **B,** as a sanitizer against *E. coli* in 4-min exposures. Treatment solutions were stored in the dark at 20°C for 16 to 18 h. NaOCl was added to natamycin before storage and before use at 50 µg/ml free chlorine each time. Solutions were amended with *E. coli* to a final of 1.57×10^6 CFU/ml (6.2 $\log_{10}$) immediately before use. Fruit were wound-inoculated (20 µl of 1×10^7 conidia/ml), incubated for 12 to 14 h at 20°C, treated with a heated (average 49°C) re-cycling flooder on an experimental packingline, and incubated for 7 days at 20°C. Solutions were sampled after 4 min treatment time for bacterial enumeration and compared to the initial concentration (i.e., the untreated control). Horizontal bars followed by the same letter are not significantly different ($P > 0.05$) as determined by using 'lsmeans' with the Tukey adjustment in the GLIMMIX procedure in SAS v9.4.

GENERAL CONCLUSION

For a new fungicide to be commercially successful, it must be efficacious but also compatible with current disease management practices. In its use as a food preservative for many decades in the food industry, no viable resistance to natamycin has ever observed. As a recently registered biofungicide for postharvest use on citrus and stone fruits in the United States, the efficacy of natamycin against fruit decays and its best use practices need to be fully elucidated. On citrus, the two most economically important postharvest pathogens are *Penicillium digitatum* and *Geotrichum citri-aurantii*, which can cause significant crop loss if not managed with agrochemicals. Studies were conducted to determine whether natamycin could be an effective addition to a citrus packinghouse's disease management program. This dissertation demonstrates the first studies to compare in vitro toxicity of natamycin to sub-populations of some of the most important postharvest pathogens, investigate the efficacy of natamycin against postharvest decays of citrus fruits and its compatibility with various application methods currently used in citrus packinghouses, and determine the suitability of organic acids as alternative sanitizers in mixtures with surfactants for use in recirculating fungicide application systems.

Baseline sensitivities of mycelial growth for seven postharvest decay pathogens to natamycin were determined. *M. fructicola* was found to be the most sensitive to natamycin (0.17 μg/ml mean EC_{50} value), *P. digitatum* was intermediate (1.54 μg/ml mean EC_{50} value), and *G. citri-aurantii* was the least sensitive (3.20 μg/ml mean EC_{50} value). The distributions of EC_{50} values for all species were unimodal, and no resistant

outliers were detected, indicating a very low resistance potential. This was further confirmed with mass platings of *P. digitatum* and *G. citri-aurantii* conidia onto natamycin amended agar showing that no colonies developed at concentrations higher than the EC_{95} values. Microscopic observations indicated that natamycin arrested fungal spores at the pre-germination swelling stage as compared with propiconazole that inhibited germ tube elongation. The broad-spectrum activity against various postharvest fungal pathogens, in particular against *G. citri-aurantii* where few treatment options are available, and a reduced risk of resistance development in targeted pathogens support its integration into packinghouse disease management programs.

Overall in laboratory experiments, spray applications with 1000 µg/ml natamycin were moderately to highly effective against green mold decay, but sporulation of the pathogen was not inhibited. Treatments with natamycin were determined to be significantly more effective against green mold on grapefruit and lemons than on oranges and mandarins. Natamycin was compatible with a storage fruit coating in mixtures and in sequential application of the fungicide and fruit coating, but was significantly less effective when applied in mixture with a packing fruit coating. Sour rot of lemons and mandarins was significantly reduced from the untreated control by natamycin itself (1000 µg/ml), but propiconazole (540 µg/ml) and propiconazole + natamycin (540 + 500 µg/ml) mixtures were generally more effective. In experimental and commercial packingline studies, natamycin applied in a storage fruit coating or as an aqueous flooder treatment in mixture with fludioxonil or propiconazole was highly effective, typically resulting in a >85% reduction of green mold and sour rot on lemon. Overall, natamycin was found to

be effective by itself against green mold and sour rot, but was generally more effective in mixtures with other fungicides. Unlike other postharvest fungicides, natamycin has the ability to reduce total populations of targeted pathogens with a very low risk of resistance development. In mixtures, it may greatly reduce the risk for selecting resistance to single-site mode of action fungicides that are more prone to resistance development. Thus, the most important role of natamycin is its use in mixtures with other fungicides to provide an anti-resistance strategy in packinghouse disease management.

In a packinghouse, a pressing issue is the proper sanitation of recirculating fungicide systems to meet food safety audits as required by the Food Safety Modernization Act. Since natamycin is incompatible with oxidative sanitizers, organic acids (with a surfactant added) were determined to be a viable alternative. In in vitro experiments, *P. syringae* was more susceptible to acidic conditions near pH 4.0 than *E. coli*. *E. coli* populations were still effectively reduced by more than 5 $\log_{10}$ CFU/ml in heated natamycin solutions containing citric acid and SDBS. In fruit efficacy experiments, natamycin was effective and apparently stable at pH values as low as 3.0 for up to 16 to 18 h and was compatible with citric acid-SDBS mixtures in treatments against green mold using a recirculating flooder application. Samplings from heated natamycin-citric acid-SDBS mixtures demonstrated that a pH of 3.0 of the treatment solution was more effective (i.e., a 4.0-$\log_{10}$ reduction) in reducing the *E. coli* load than a pH of 3.5 (i.e., a 0.7-$\log_{10}$ reduction). These results support the use of citric acid-SDBS an alternative sanitizer for heated natamycin solutions. An organic acid-based sanitizer, however, will not be viable if a packinghouse utilizes an alkaline sodium carbonate or

sodium bicarbonate fruit pre-treatment due to the likely carryover of alkalinity that will increase the pH of a recirculating fungicide solution.

In summary, natamycin was determined to be less toxic against fungal decay fungi in vitro and less effective against green mold and sour rot decays than some conventional synthetic fungicides in fruit efficacy experiments, but was found to be an effective mixture partner with other fungicides such as azoxystrobin, fludioxonil, or propiconazole for managing green mold and with propiconazole for managing sour rot. I have determined that natamycin is best applied in a packinghouse as a non-recovery spray in combination with other fungicides, in mixture with a storage coating or before subsequent application with a storage or pack coating. For packinghouses utilizing a recirculating fungicide system without a sodium carbonate or sodium bicarbonate fruit pre-treatment step, an organic acid-based sanitizer such as citric acid with SDBS may be used with natamycin in a heated system as an alternative to oxidative sanitizers that are incompatible.

The findings published in this dissertation support the registration of natamycin as a postharvest fungicide treatment for citrus. In contrast to the biofungicides polyoxin-D and potassium phosphite (a phosphonate fungicide) that are active against a limited number of decays, the biofungicide natamycin is effective as a residual treatment against a broad number of plant pathogens. Still, registered synthetic fungicides are mostly considered highly effective if no resistance in the pathogen population is present. Although currently not certified for organic use in the United States, natamycin could be approved in the future and could revolutionize postharvest handling of organic fruits

where currently only limited options for the control of postharvest decay fungi are available.