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Mechanism and effectiveness of natamycin produced by *Streptomyces philanthi* RL-1-178 in controlling green mold disease caused by *Penicillium digitatum* on postharvest mandarin fruit (*Citrus reticulata* Blanco)

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ABSTRACT

Natamycin, also known as pimaricin, has been widely used as an antimycotic agent in the food industry. This study evaluates the potential of natamycin produced by *Streptomyces philanthi* RL-1-178 as a control agent against green mold disease caused by *Penicillium digitatum*. Its efficacy is compared with that of sodium benzoate, sodium propionate, Prochloraz®, and Propiconazole®, both *in vitro* and on mandarin fruit (*Citrus reticulata* Blanco). *In vitro* results demonstrated that natamycin was tested across a range of concentrations (16, 32, 64, 128, 256, and 512 μg/mL) and was effective at concentrations as low as 16 μg/mL, with complete inhibition of pathogen growth and spore germination observed at a minimum inhibitory concentration (MIC) of 256 μg/mL. This efficacy was comparable to that of sodium benzoate (2,000 μg/mL), Prochloraz® (500 μg/mL), and Propiconazole® (500 μg/mL). When applied at twice the MIC, natamycin effectively controls *P. digitatum*, with preventive applications proving more effective than curative ones. However, its curative efficacy was less than that of the tested fungicides, Prochloraz® and Propiconazole®, which achieved complete disease suppression even when applied after pathogen inoculation. Further research indicated that treating fungal spores with natamycin for 12 h before inoculating mandarin fruit prevented visible disease symptoms, demonstrating strong preventive capabilities. Significant disruptions in ergosterol biosynthesis and impairments in both enzymatic (superoxide dismutase, catalase) and non-enzymatic (oxidized and reduced glutathione, and their ratio) antioxidant defense mechanisms in *P. digitatum* cells post-treatment indicate the plasma membrane as a key target for its antifungal action. Additionally, natamycin compromised the integrity of the plasma membrane. Overall, our findings demonstrate the efficacy of natamycin in controlling green mold disease, suggesting its potential as a low-toxicity alternative to chemical fungicides for managing postharvest diseases. The study also highlights the use of natamycin produced by *S. philanthi* RL-1-178 as a biocontrol agent, offering a viable approach for postharvest disease management.

1. Introduction

Citrus reticulata Blanco, commonly known as mandarin orange, belongs to the Rutaceae family. The fruit, including the peel, pulp, and juice, is widely used for its nutritional and medicinal value [1,2]. The peel and essential oils from mandarin fruit are particularly noted for their bioactive properties. Bioactivities reported for these parts include antioxidant, antimicrobial, and anti-inflammatory effects [1,2]. Isolated compounds from mandarin, such as limonene, flavonoids, and carotenoids, have been identified as the primary contributors to these activities. Limonene, for example, exhibits notable antifungal and antibacterial properties, while the fruit's essential oils show strong antimicrobial effects [1,2]. These compounds play a vital role in enhancing the health benefits of the fruit and its extracts, making *C. reticulata* valuable for both medicinal and culinary applications.

Citrus fruits, widely distributed globally, are recognized as an essential commercial crop, valued for their rich content of vitamins, minerals, and dietary fiber that significantly contribute to human health [3–5]. However, they are vulnerable to various pathogenic fungi during postharvest storage, particularly *P. digitatum*, the causative agent of

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green mold disease, which results in substantial economic losses [6]. Indeed, green mold *disease*, triggered by *P. digitatum*, is especially damaging, accounting for up to 90 % of all postharvest losses in citrus fruits [3,6,7].

Currently, the management of postharvest diseases in citrus fruits largely relies on the extensive use of chemical fungicides during storage [8–10]. Fungicide treatments are essential for effectively controlling various fruit diseases, and this study focuses on two widely used fungicides in Thailand: Propiconazole® and Prochloraz®. Propiconazole®, a triazole fungicide, disrupts ergosterol biosynthesis in fungal cell membranes, compromising cell integrity, growth, and division, thus providing broad-spectrum control against pathogens such as *Colletotrichum* spp., *Sclerotinia* spp., *Cercospora* spp., *Alternaria* spp., and *P. digitatum* [11–14]. Prochloraz®, an imidazole fungicide, also targets ergosterol biosynthesis but through a slightly different mechanism [15], demonstrating similar efficacy against *P. digitatum* [16,17]. Both fungicides play critical roles in preventing and managing fruit diseases and have proven effective worldwide in controlling green mold disease [16–18].

However, excessive use of these chemicals has resulted in adverse effects, including environmental pollution, health risks, and food safety concerns, along with the emergence of drug-resistant pathogens [19–22]. Given increasing regulatory restrictions on chemical fungicides, there is an urgent need to explore alternative management strategies. Potential alternatives include biological control agents like beneficial bacteria and fungi, natural plant extracts, essential oils, and bio-based products. Additionally, methods such as controlled atmosphere storage, heat treatments, and UV radiation are being investigated for their efficacy in extending shelf life and reducing dependency on traditional chemical approaches [8].

Over the past few decades, the use of antagonistic microorganisms has been recognized as a successful strategy for controlling postharvest diseases. Various strains, including *Bacillus* spp. [6,23–25], *Pseudomonas* spp. [23,26,27], *Streptomyces* spp. [28,29], *Paenibacillus* spp. [30], *Aureobasidium* spp. [31], *Lactobacillus* spp. [32], and *Rhodotorula* spp. [33,34], have shown excellent efficacy in inhibiting *P. digitatum*. However, the widespread adoption of these biological agents in the market faces several challenges, including issues related to maintaining product integrity and shelf-life, which currently hinder their practical application [35].

In contrast, bio-based fungicides and substances generally regarded as safe, such as chitosan [27,36], cinnamic acid [37], acetic acid [28], gluconic acid [38], salicylic acid [39], sodium benzoate [8,40], and sodium propionate [41], have been employed to control postharvest decay. Among these, sodium benzoate and sodium propionate are commonly used antifungal agents in food preservation, valued for their ability to inhibit mold and yeast growth, thereby preventing spoilage and extending shelf life [42,43]. Sodium benzoate works by lowering the pH of the product environment, disrupting fungal cell metabolism and hindering their ability to grow and reproduce. Sodium propionate, often used in bakery products, targets the fungal cell membrane, interfering with essential cellular functions and effectively controlling mold growth [42,43]. Both compounds serve as crucial preservatives in the food industry, offering an alternative approach for fungal inhibition that may complement or serve as a comparison to agricultural fungicides like natamycin. Evaluating their relative effectiveness can provide insights into their optimal applications across various industries.

Natamycin, a naturally occurring antimycotic polyene, is primarily produced through the fermentation of *S. natalensis* [44]. Although *S. natalensis* is a well-known source of natamycin, *S. philanthi* RL-1-178 also synthesizes this compound and is particularly recognized for its potent antifungal and antiaflatoxigenic activity against *Aspergillus parasiticus* and *A. flavus* [45]. Consequently, *S. philanthi* RL-1-178 serves as a valuable strain for applications requiring targeted antifungal efficacy. Recognized as a food additive in over 40 countries and designated as a Generally Recognized as Safe (GRAS) product by the U.S. Food and Drug Administration (FDA) [46]. Natamycin is widely used to prevent yeast and mold contamination in daily food, owing to its very low toxicity to human and mammalian cells [47–51].

Recognized for its effectiveness in controlling postharvest decay of fruits, natamycin is particularly valued in agricultural research and applications. Its primary antifungal action involves the specific binding of ergosterol, crucial for maintaining fungal membrane integrity [47,48,50]. Beyond this, natamycin exhibits additional mechanisms that enhance its antifungal efficacy. For instance, it disrupts nutrient transport in fungi like *Saccharomyces cerevisiae* and *Aspergillus niger* [52], and it disturbs calcium homeostasis, which can lead to mitochondrial dysfunction in organisms such as *Leishmania* spp [53]. Natamycin can also disturb calcium homeostasis, leading to mitochondrial dysfunction against *Leishmaniasis* spp [53]. Moreover, natamycin has been reported to successfully control various fungal diseases of fresh fruit, including grey mold of mandarin fruit and blueberry fruit, crown rot of strawberry, green mold, and sour rot of citrus [48,50,54–56].

Although natamycin has been widely studied for its antifungal activity, including against *P. digitatum*, the potential of natamycin produced by *S. philanthi* RL-1-178 for controlling green mold in citrus has yet to be explored. Since different *Streptomyces* strains can produce natamycin with varying composition and potency, investigating its production by *S. philanthi* RL-1-178 is crucial to determine its unique efficacy and suitability for this application. This study aims to evaluate the effectiveness of natamycin derived from *S. philanthi* RL-1-178 as a biocontrol agent against *P. digitatum*-induced green mold disease in mandarin fruits, comparing it with sodium benzoate, sodium propionate, Prochloraz®, and Propiconazole®. Additionally, the study seeks to investigate the mechanisms behind natamycin's antifungal action, contributing to more sustainable postharvest disease management in citrus production. By addressing these objectives, the study highlights the importance of exploring *S. philanthi* RL-1-178 and its potential as an alternative biocontrol agent.

2. Material and methods

2.1. Pathogen

The fungal pathogen *P. digitatum*, responsible for typical green mold disease symptoms, was isolated from infected mandarin fruit (*C. reticulata* Blanco) obtained from the Lotus's Hatyai Supermarket, Songkhla, Thailand, using a single spore isolation method [28]. Only fruits exhibiting typical green mold disease symptoms were selected for pathogen isolation, while fruits without visible signs of infection were excluded. Cultures were subsequently grown on Difco™ potato dextrose agar (PDA) plates at 30 °C for seven days. Spores were harvested by suspending them in 10 mL of sterile 0.1 % Tween 80 solution, and the concentration was determined using a hemocytometer (a microscope slide with an etched grid used for counting cells). The spore count was performed by counting the spores within a defined area of the grid, providing a reliable and validated method for concentration determination. The spore concentration was then adjusted to achieve the required concentration with sterile distilled water.

2.2. Chemicals and reagents

In this study, natamycin was isolated and purified from *S. philanthi* RL-1-178 [45]. A stock solution of natamycin (8.48 mg/mL) was prepared in dimethyl sulfoxide (DMSO) and stored at 4 °C for use in the experiments.

Commercial preservatives, including sodium benzoate and sodium propionate (Aldrich, Milwaukee, WI, USA), were prepared in sterile distilled water at concentrations ranging from 500 to 2,000 μg/mL. These concentrations were selected based on their common use in postharvest treatments to control fungal growth, with sodium benzoate frequently applied at similar concentrations in food preservation and

storage to prevent spoilage. The chosen range represents concentrations typically effective in inhibiting fungal growth while being practical for application in postharvest handling.

Chemical fungicides, such as 45 % Prochloraz® (w/v) from AG-GRO (Thailand) Co., Ltd. and 25 % Propiconazole® (w/v) from SAIMA CHEMICAL CO., Ltd. (Japan), were also dissolved in sterile distilled water at concentrations ranging from 500 to 2000 μg/mL.

Sodium hypochlorite, metaphosphoric acid, n-heptane, dichloro dihydrofluorescein diacetate (DCFH-DA), dichlorofluorescein (DCF), N–N–N–N-tetramethylene diamine, quercetin, ortho-phthalaldehyde (OPA), NaOH, DMSO, Tween 80, potassium hydroxide (KOH), and EDTA were procured from and Hi-Media Laboratory, Mumbai, India.

2.3. Inhibitory effects of natamycin, commercial preservatives, and fungicides on the mycelial growth of P. digitatum

2.3.1. Effect of natamycin

The effects of natamycin at concentrations of 16, 32, 64, 128, 256, and 512 μg/mL against *P. digitatum* were investigated in both solid and liquid cultures. Solid and liquid cultures were chosen for testing to assess the antifungal activity of natamycin in two different growth environments, providing a comprehensive evaluation of its efficacy under varied conditions. For the solid culture, natamycin was incorporated into PDA to achieve the specified concentrations. A 5 μL droplet of spore suspension (10^5 spores/mL) was inoculated at the center of a 50 mm diameter PDA plate and incubated at 30 °C for 7 days [57]. DMSO was used as the control treatment. Colony size was measured after incubation, and the lowest concentration that completely inhibited *P. digitatum* growth after seven days was considered the MIC. The experiment included three replications and was repeated twice. To calculate the percentage of mycelial growth inhibition (%) by natamycin, the formula from Parizi et al. [58] was used: Percentage of inhibition = $[(R_1 - R_2)/R_1] \times 100$, where R_1 represents fungal growth in the control, and R_2 represents fungal growth in the natamycin treatment.

In the liquid culture experiments, the effects of natamycin on *P. digitatum* were tested in 5 mL glass test tubes, following the methodology adopted from Boukaew et al. [59], with a final volume of 2.0 mL. To initiate the experiment, 50 μL of each natamycin concentration (16, 32, 64, 128, 256, and 512 μg/mL) was mixed with 50 μL of fungal spore suspension (10^5 spores/mL) and 1.9 mL of potato dextrose broth (PDB). DMSO was used as a control in place of natamycin. The mixtures were incubated at 30 °C on a rotary shaker at 150 rpm to ensure even mixing and consistent fungal growth throughout the experiment. The lowest concentration that completely inhibited *P. digitatum* growth after three days of incubation was recorded as the MIC. After incubation, the mycelial mats were filtered through preweighed, dried filter paper, then dried at 60 °C for three days and weighed. The dry weight of the mycelia was used to calculate the percentage of mycelial growth inhibition (%) by natamycin, as previously described. The experiment was conducted with three replicates and repeated twice.

2.3.2. Effect of two commercial preservatives

The effects of sodium benzoate and sodium propionate on *P. digitatum* were evaluated. Specifically, a 5 μL droplet of spore suspension (10^5 spores/mL) was placed at the center of a 50 mm diameter Petri dish containing PDA medium, which was enhanced with various concentrations (ranging from 500 to 2000 μg/mL) of each preservative. Sterile distilled water was used as the control for the PDA medium. The incubation and the calculation of the percentage inhibition of mycelial growth followed the previously described method. The experiment was conducted with three replications and was repeated twice.

2.3.3. Effect of two chemical fungicides

The efficacy of Prochloraz® and Propiconazole® against *P. digitatum* was assessed. Specifically, a 5 μL droplet of spore suspension (10^5 spores/mL) was inoculated at the center of a 50 mm diameter Petri dish containing PDA medium, which was subsequently treated with various concentrations of each fungicide, ranging from 500 to 2000 μg/mL. Sterile distilled water serves as the control for the PDA medium. The incubation and the calculation of the percentage of mycelial growth inhibition were carried out following the method previously described. The experiment was conducted with three replications and was repeated twice.

2.4. Comparison of the inhibitory effects of natamycin with commercial preservatives and fungicides on P. digitatum spore germination

The efficacy of natamycin, together with sodium benzoate, sodium propionate, Prochloraz®, and Propiconazole®, in inhibiting the germination of *P. digitatum* spores was evaluated using a methodology outlined by Qin et al. [60]. In brief, spore suspensions of *P. digitatum* were distributed into the wells of a 24-well microtitration plate containing PDB to reach a final concentration of 10^5 spores/mL. These cultures were then treated with various concentrations of natamycin [1/4 MIC (64 μg/mL), 1/2 MIC (128 μg/mL), and MIC (256 μg/mL)], as well as 2,000 μg/mL of sodium benzoate and sodium propionate, and 500 μg/mL of Prochloraz® and Propiconazole®. DMSO was used as the control treatment. The plates were incubated on a rotary shaker at 150 rpm and 30 °C. Following a 20-h incubation period, the germination of approximately 150 spores was microscopically evaluated; spores were considered germinated if the germ tube length was equal to or greater than the diameter of the spore. Germination rates were calculated based on the percentage of germinated spores relative to the total spores assessed. The experiment was conducted with three replications and was repeated twice.

2.5. Preventive and curative effects of natamycin against the development of P. digitatum in mandarin fruit

2.5.1. Plant material

Mandarin fruits (*C. reticulata* Blanco), with an average weight of approximately 28.04 g each, were sourced from the Lotus's Hatyai Supermarket, Songkhla, Thailand. Care was taken to select fruits of uniform size that exhibited no visible signs of disease or injury. Upon harvest, the fruits were either immediately used for testing or stored at 20 °C and 92–94 % relative humidity for up to 48 h [61]. Prior to the experiments, the fruits underwent a surface sterilization process, which involved immersing them in a sodium hypochlorite solution (3 %, v/v) for 3 min. Afterward, the fruit was thoroughly rinsed with sterile distilled water [62]. Finally, the fruits were gently dried using sterile techniques in a laminar airflow chamber for 30 min.

2.5.2. Preventive and curative effects of natamycin on green mold disease development in mandarin fruit

To assess the preventive effects of natamycin against *P. digitatum* on mandarin fruits, each experimental setup included five individual fruits. A sterilized needle [63] was used to introduce two wounds on each fruit, each 3 mm in diameter and about 3 mm deep. These wounds were immediately treated with 20 μL of different concentrations of natamycin [1/2 MIC (128 μg/mL), MIC (256 μg/mL), and 2 MIC (512 μg/mL)], as well as 2,000 μg/mL sodium benzoate and sodium propionate, and 500 μg/mL Prochloraz® and Propiconazole®. Subsequently, 30 min after the antifungal treatment, each wound was inoculated with 20 μL of a *P. digitatum* spore suspension at a concentration of 10^5 spores/mL. DMSO was used as the control treatment and applied to the wounds.

To assess the curative effects of natamycin against *P. digitatum* on mandarin fruits, we applied the same methodology as in previous tests but with one key variation. Initially, the fruits were inoculated with *P. digitatum* spores at a concentration of 10^5 spores/mL. Following the inoculation, antifungal treatments of 1/2 MIC, MIC, and 2 MIC concentrations of natamycin, as well as 2,000 μg/mL sodium benzoate and sodium propionate, and 500 μg/mL Prochloraz® and Propiconazole®,

were applied 30 min later. DMSO was used as the control treatment and applied to the wounds.

To achieve high humidity levels of 90–95 % [64,65], the treated mandarin fruits were stored in plastic containers lined with polyethylene and kept in an incubator maintained at 30 °C. After a 12-day incubation period [25], the disease severity (DS) was assessed by measuring the average diameter of lesions for each treatment [24]. Each treatment was replicated three times, with 5 fruits used per replicate, for a total of 15 fruits per treatment [24].

2.6. Impact of varied time durations of natamycin treatment on green mold disease development in mandarin fruit

In each experimental setup, five mandarin fruits were used, each with two wounds as described previously. A solution containing 100 μL of natamycin at MIC (256 μg/mL) and 100 μL of a spore suspension from *P. digitatum*, adjusted to a concentration of 10^5 spores/mL, was carefully prepared. This blend was then incubated at 30 °C with a rotation of 150 rpm over intervals of 0, 4, 8, and 12 h. After each incubation period, 20 μL of the mixture was applied to each wound on the mandarin fruits. DMSO water served as the control. To ensure high humidity levels of 90–95 %, the treated fruits were placed in plastic containers lined with polyethylene and kept in a 30 °C incubation environment. For twelve days post-inoculation [25], disease severity was evaluated using the methods specified in the previous section. Each treatment was replicated three times, with 5 fruits used per replicate, for a total of 15 fruits per treatment [24].

2.7. Mechanism of action of natamycin against P. digitatum

We investigated the mechanisms of action of natamycin at concentrations of 1/2 MIC and MIC in combating *P. digitatum*. To do this, *P. digitatum* spores were cultured in 100 mL of PDB at a density of 10^5 spores/mL and incubated at 30 °C on a rotary shaker set to 150 rpm for three days. After incubation, the mycelia were harvested and rinsed with sterile distilled water. Then, 1 g (wet weight) of the clean mycelia was resuspended in 5 mL of PDB containing natamycin at concentrations of 1/2 MIC (128 μg/mL) and MIC (256 μg/mL). The control setup included DMSO as a solvent control. These suspensions were incubated under the same conditions for an additional two days. Afterward, the mycelia's wet weights were examined to assess ergosterol content and to evaluate both enzymatic and non-enzymatic defense responses, which are elaborated upon in subsequent sections.

2.7.1. Evaluation of ergosterol content

Treated and untreated fungal biomass (as obtained in Section 2.7) was used to measure ergosterol production. To extract ergosterol, 5 mL of ethanolic KOH solution was added to the mycelial mat (2 g wet weight), mixed by swirling for 2 min, and then heated for 1 h at 80 °C. Following this, n-heptane and distilled water (2:5 v/v) were added to each flask, and the mixture was swirled for 2 min before being allowed to stand at 30 °C to extract membrane sterols [66,67]. The sterol content was measured by scanning the upper n-heptane layer between 230 and 300 nm using a UV–Visible spectrophotometer [66]. The experiment was performed in triplicate and repeated twice.

2.7.2. Assessment of enzymatic and non-enzymatic defense systems

2.7.2.1. Preparation of fungal biomass and enzyme extracts. Treated and untreated fungal biomass (1.5 g wet weight, as obtained in Section 2.7) was homogenized in 3 mL of phosphate-buffered saline (100 mM, pH 7.4) and centrifuged at 8,000 rpm for 10 min at 4 °C [66]. The control setup included DMSO as a solvent control. The resulting supernatant was collected for enzymatic and non-enzymatic analyses.

2.7.2.2. Determination of cellular ROS, CAT, SOD, and glutathione (reduced and oxidized). The analysis of reactive oxygen species (ROS), catalase (CAT), superoxide dismutase (SOD), and glutathione (both reduced and oxidized) was performed to assess the oxidative stress response in *P. digitatum* following treatment with natamycin. These parameters are essential indicators of oxidative damage and the antioxidant defense system in the fungus. By measuring these factors, we aimed to understand the mechanism of action of natamycin, focusing on how it induces oxidative stress and triggers defense responses in the pathogen. This approach helps elucidate the biochemical pathways involved in natamycin's antifungal activity, thereby providing a deeper understanding of its effects on *P. digitatum*.

To estimate intracellular ROS levels, the methodology of Keston and Brandt [68] was employed using the DCFH-DA fluorescent dye assay. Fluorescence intensity was measured with excitation and emission wavelengths of 485 and 530 nm, respectively, and the concentration of DCF was determined using a standard curve. CAT activity was assessed following the protocol of Beers and Sizer [69], with activity expressed as units per minute per milligram of protein, based on a molar extinction coefficient of 43.6 M^{-1} cm^{-1} [66]. SOD activity was evaluated using the quercetin auto-oxidation method of Kostyuk and Potapovich [70], where the reaction mixture contained phosphate buffer (0.016 M, pH 10), EDTA (0.08 mM), N,N,N′,N′-tetramethylene diamine, and 100 μL of quercetin solution (1.5 mg/10 mL DMSO). Inhibition of quercetin auto-oxidation was measured at 406 nm at 0 and 20 min, with enzyme activity expressed as inhibition per milligram of protein [66].

For glutathione estimation, seven-day-old *P. digitatum* biomass was homogenized in phosphate buffer (0.1 M, pH 8), EDTA (0.005 M), and metaphosphoric acid (25 %), then centrifuged at 10,000 g for 10 min at 4 °C. The fluorescence of the supernatant was measured for glutathione at 420 nm with excitation at 350 nm [71]. To assess oxidized glutathione, the reaction mixture included 500 μL of the supernatant and 200 μL of 0.04 M NEM, incubated at room temperature for 20 min. The addition of OPA and NaOH solution followed by a 30-min incubation allowed for fluorescence measurement at 420 nm. Total protein content was determined using the methodology of Lowry et al. [72]. All experiments were performed in triplicate and repeated twice.

2.7.3. Measurement of electrical conductivity

Cellular leakage was evaluated using a modified protocol adapted from Lewis and Papavizas [73]. Spores of *P. digitatum* were initially cultured in 100 mL of PDB at a concentration of 10^5 spores/mL and incubated at 30 °C on a rotary shaker (150 rpm) for three days. After incubation, the mycelia were collected, washed with sterile distilled water, and 2.5 g (wet weight) of the mycelia were resuspended in 25 mL of sterile distilled water. The suspension was supplemented with natamycin at concentrations of 1/2 MIC (128 μg/mL) and MIC (256 μg/mL), and incubated at 30 °C for 0, 2, 4, 6, and 8-h intervals [74]. The control setup included DMSO as a solvent control. The mycelia were then filtered through a double layer of sterile cheesecloth, and the resultant filtrates were analyzed for electrolyte leakage using a conductivity meter (EC 700, APENA), following the methodology refined by Lee et al. [75]. Conductivity measurements were performed in triplicate and repeated twice.

2.8. Statistical analysis

The data from the *in vitro* and *in vivo* experiments were subjected to one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) version 26 (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY, USA: IBM Corp). Assumptions for normality were visually assessed using histograms and Q-Q plots, which were generated through SPSS graphical tools. Homogeneity of variances was tested using Levene's test, which was performed automatically as part of the ANOVA procedure. The significance of differences among the various treatments and controls was determined using the Tukey HSD

test, with differences considered significant at $p < 0.05$.

3. Results

3.1. Inhibitory effects of natamycin, commercial preservatives, and fungicides on the mycelial growth of P. digitatum

The MIC of natamycin against *P. digitatum* was evaluated in both solid and liquid cultures, as detailed in Table 1. In solid culture, increasing concentrations of natamycin (16–512 μg/mL) significantly inhibited the growth of *P. digitatum* ($p < 0.05$), with effective inhibition starting at 16 μg/mL and complete inhibition (100 %) observed at 256 μg/mL. Similar results were obtained in liquid culture, where diverse concentrations of natamycin markedly reduced mycelial growth compared to controls. Notably, concentrations of 16–128 μg/mL resulted in 44.59–83.77 % inhibition in liquid culture, exceeding the 29.35–75.00 % inhibition observed in solid culture. Complete inhibition (100 %) of pathogen growth was also achieved at 256 μg/mL, confirming the MIC of natamycin against *P. digitatum* to be 256 μg/mL. Concentrations exceeding 256 μg/mL effectively suppressed further fungal proliferation.

Table 2 illustrates the significant inhibitory effects of sodium benzoate and sodium propionate on the mycelial growth of *P. digitatum* ($p < 0.05$). Across the range of concentrations tested (500–2,000 μg/mL), sodium benzoate demonstrated substantial inhibition of *P. digitatum*, ranging from 17.39 % to complete (100 %) inhibition, while sodium propionate exhibited comparatively lower inhibition, ranging from 6.52 % to 16.30 %. Notably, at the highest concentration tested (2,000 μg/mL), sodium benzoate achieved complete inhibition (100 %) of *P. digitatum*, whereas sodium propionate only reached 16.30 % inhibition. These findings strongly indicate the potent antifungal efficacy of sodium benzoate against *P. digitatum*.

The antifungal efficacy of both Prochloraz® and Propiconazole® against *P. digitatum* was investigated, and the results are presented in Table 3. No significant inhibition of *P. digitatum* was observed across increasing concentrations (500–2,000 μg/mL) of both fungicides ($p > 0.05$). Particularly noteworthy was the complete inhibition (100 %) of *P. digitatum* achieved by both fungicides at the lowest concentration tested (500 μg/mL).

3.2. Comparison of the inhibitory effect of natamycin with commercial preservatives and fungicides on the germination of P. digitatum spores

The antifungal efficacy of natamycin at concentrations of 1/4 MIC (64 μg/mL), 1/2 MIC (128 μg/mL), and MIC (256 μg/mL) was evaluated against *P. digitatum* spore germination and compared to that of sodium benzoate, sodium propionate, Prochloraz®, and Propiconazole® (Table 4). The control treatment showed a spore germination rate of 87.56 % for *P. digitatum*. However, following the application of antifungal agents, a significant reduction in spore germination was observed ($p < 0.05$). Natamycin at 1/2 MIC demonstrated substantial inhibition of spore germination (12 %), significantly outperforming both 1/4 MIC (31.56 %) and sodium propionate (67.78 %) ($p < 0.05$). Notably, at MIC, natamycin completely inhibited spore germination (100 %), showing efficacy comparable to sodium benzoate, Prochloraz®, and Propiconazole®. These results suggest that natamycin could serve as an effective alternative to traditional chemical preservatives and fungicides in controlling *P. digitatum*, with results equal to or superior in terms of spore inhibition. Given that 1/4 MIC showed considerably less activity against *P. digitatum*, it was not selected for further study.

3.3. Preventive and curative effects of natamycin against development of P. digitatum in mandarin fruit

The impact of natamycin at concentrations of 1/2 MIC (128 μg/mL), MIC (256 μg/mL), and 2 MIC (512 μg/mL) on the development of *P. digitatum* on mandarin fruit was compared with that of sodium benzoate, sodium propionate, Prochloraz®, and Propiconazole®. The results of both preventive (Table 5A) and curative (Table 5B) activity tests are presented in Table 5. In our study, natamycin demonstrated significant biocontrol activity against *P. digitatum* on mandarin fruit in both preventive and curative applications. In terms of preventive activity (Table 5A), we observed a decrease in lesion diameter with increasing natamycin concentration. Notably, at a 2 MIC concentration, the lesion area was recorded at 0.69 cm^2, achieving a control efficacy of 85.58 %, comparable to that of sodium benzoate but lower than Prochloraz® and Propiconazole® (both 100 %), highlighting its effectiveness in preventing the development of *P. digitatum*. However, in curative activity tests (Table 5B), natamycin exhibited slightly lower control efficacy (24.41 %–77.53 %) compared to its preventive effect (27.18 %–85.58 %). At a 2 MIC concentration, the lesion area was limited to 1.08 cm^2, achieving a control efficacy of 77.53 %. This efficacy was not significantly different ($p > 0.05$) from that of sodium benzoate (79.47 %) but was lower than that of both chemical fungicides (100 %). Overall, while fungicidal treatments displayed higher control efficacy in both preventive and curative tests, our findings indicated that natamycin was more effective as a preventive treatment than as a curative one against *P. digitatum* on mandarin fruit. When applied at twice the MIC, natamycin effectively controlled green mold disease, though it was slightly less potent than fungicides in both preventive and curative applications.

3.4. Impact of varied time durations of natamycin treatment on green mold disease development in mandarin fruit

This study investigated the impact of different natamycin treatment durations (0–12 h) on *P. digitatum* spores before inoculating mandarin fruit, specifically observing lesion area (Fig. 1A), control efficacy (Fig. 1B), and disease symptom development (Fig. 1C), as depicted in Fig. 1. As shown in Fig. 1, increasing treatment duration significantly ($p < 0.05$) inhibited green mold disease development. In the untreated control (Fig. 1A–a), the lesion area was recorded at 4.76 cm^2. With natamycin treatment durations from 0 to 12 h, lesion area significantly

Table 1
Mycelial growth of *P. digitatum* in PDA and PDB media after 7 and 3 days of incubation at 30 °C, respectively, showing the effects of natamycin at concentrations ranging from 16 to 512 μg/mL.

Parameters	Natamycin concentration (μg/mL) in PAD							Parameters	Natamycin concentration (μg/mL) in PDB						
	Control	16	32	64	128	256	512		Control	16	32	64	128	256	512
Colony diameter (mm)	15.33^{a} ± 0.82	10.83^{b} ± 0.98	10.17^{c} ± 0.75	6.17^{d} ± 1.60	3.83^{e} ± 0.75	0.0^{f} ± 0.0	0.0^{f} ± 0.0	Mycelial dry weight (mg/2 mL PDB)	4.62^{a} ± 0.02	2.56^{b} ± 0.08	1.52^{c} ± 0.05	0.98^{d} ± 1.60	0.75^{e} ± 0.05	0.0^{f} ± 0.0	0.0^{f} ± 0.0
Inhibition of mycelial growth (%)	–	29.35	44.57	59.78	75.00	100	100	Inhibition of mycelial growth (%)	–	44.59	67.10	78.79	83.77	100	100

Note: The presented data represent the mean of three replicates ± standard deviation (SD). Values within the same row that share the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

Table 2
Mycelial growth of *P. digitatum* on PDA medium after 7 days at 30 °C, showing the effects of sodium benzoate and sodium propionate concentrations ranging from 500 to 2,000 μg/mL.

Parameters	Sodium benzoate concentration (μg/mL)					Sodium propionate concentration (μg/mL)				
	Control	500	1,000	1,500	2,000	Control	500	1,000	1,500	2,000
Colony diameter (mm)	$15.33^{a} \pm 0.82$	$12.67^{b} \pm 1.86$	$8.83^{c} \pm 1.33$	$4.17^{d} \pm 0.98$	$0.0^{e} \pm 0.0$	$15.33^{a} \pm 0.82$	$13.83^{ab} \pm 0.98$	$14.33^{ab} \pm 1.03$	$13.00^{b} \pm 1.10$	$12.83^{b} \pm 0.41$
Inhibition of mycelial growth (%)	–	17.39	42.39	72.83	100.00	–	6.52	9.78	15.22	16.30

Note: The presented data represent the mean of three replicates ± standard deviation (SD). Values within the same row that share the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

Table 3
Mycelial growth of *P. digitatum* on PDA medium after 7 days at 30 °C, showing the effects of Prochloraz® and Propiconazole® concentrations ranging from 500 to 2,000 μg/mL.

Parameters	Prochloraz® concentration (μg/mL)					Propiconazole® concentration (μg/mL)				
	Control	500	1,000	1,500	2,000	Control	500	1,000	1,500	2,000
Colony diameter (mm)	$15.33^{a} \pm 0.82$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$	$15.33^{a} \pm 0.82$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$	$0.0^{b} \pm 0.0$
Inhibition of mycelial growth (%)	–	100	100	100	100	–	100	100	100	100

Note: The presented data represent the mean of three replicates ± standard deviation (SD). Values within the same row that share the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

Table 4
Effect of natamycin (1/4 MIC, 1/2 MIC, and MIC), commercial preservatives (sodium benzoate and sodium propionate at 2,000 μg/mL), and chemical fungicides (Prochloraz® and Propiconazole® at 500 μg/mL) on spore germination of *P. digitatum* after 24 h incubation at 30 °C.

Treatment	Concentration (μg/mL)	Spore germination (%)
Control	–	$87.56^{a} \pm 5.86$
Natamycin	64 (1/4 MIC)	$31.56^{c} \pm 3.03$
Natamycin	128 (1/2 MIC)	$12.00^{d} \pm 2.4$
Natamycin	256 (MIC)	$0.0^{e} \pm 0.0$
Sodium benzoate	2,000	$0.0^{e} \pm 0.0$
Sodium propionate	2,000	$67.78^{b} \pm 6.10$
Prochloraz®	500	$0.0^{e} \pm 0.0$
Propiconazole®	500	$0.0^{e} \pm 0.0$

Note: The presented data represent the mean of three replicates ± standard deviation (SD). Values within the same column that share the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

($p < 0.05$) decreased from 4.76 cm^2 to 0 cm^2 (Fig. 1A–a-e). After 8 h of treatment, *P. digitatum* lesion area was reduced to 0.49 cm^2, achieving a control efficacy of 89.64 % (Fig. 1B–d). Extending the treatment to 12 h resulted in complete inhibition of *P. digitatum* spores, with no visible disease symptoms on inoculated mandarin fruit (Fig. 1C–e). Fig. 1C shows that while the control (Fig. 1a) had the highest lesion area, increasing treatment time progressively reduced symptoms, with the smallest lesion observed at 12 h (Fig. 1e). This progression highlights the strong time-dependent relationship among Fig. 1A, B, and 1C.

3.5. Mechanism action of natamycin against P. digitatum

3.5.1. Effect of natamycin on ergosterol content of P. digitatum

The study investigated the impact of natamycin on the biosynthesis of membrane ergosterol in *P. digitatum* cells (Fig. 2). Natamycin significantly inhibited ($p < 0.05$) ergosterol biosynthesis in a dose-dependent manner. For fungal pathogens treated with 1/2 MIC (128 μg/mL) of natamycin, the reduction in ergosterol content was 39.34 %. When the concentration was increased to the MIC level (256 μg/mL), the reduction percentage of ergosterol further increased to 68.58 %.

Table 5
Comparative efficacy of preventive **(A)** and curative **(B)** treatments using natamycin (1/2 MIC, MIC, and 2 MIC) and commercial fungicides in controlling green mold disease in mandarin fruits after 12 days of storage at 30 °C under humid conditions.

(A)

Treatment	Lesion diameter (cm^2)	Control efficacy (%)
Control	$4.81^{a} \pm 0.54$	–
Natamycin at 128 μg/mL (1/2 MIC)	$3.50^{b} \pm 0.12$	27.18
Natamycin at 256 μg/mL (MIC)	$2.15^{c} \pm 0.07$	55.20
Natamycin at 512 μg/mL (2 MIC)	$0.69^{d} \pm 0.09$	85.58
Sodium benzoate at 2,000 μg/mL	$0.80^{d} \pm 0.05$	83.36
Sodium propionate at 2,000 μg/mL	$3.79^{b} \pm 0.27$	21.08
Prochloraz® at 500 μg/mL	$0^{d} \pm 0.0$	100
Propiconazole® at 500 μg/mL	$0^{d} \pm 0.0$	100

(B)

Treatment	Lesion diameter (cm^2)	Control efficacy (%)
Control	$4.81^{a} \pm 0.54$	–
Natamycin at 128 μg/mL (1/2 MIC)	$3.63^{b} \pm 0.32$	24.41
Natamycin at 256 μg/mL (MIC)	$2.35^{c} \pm 0.17$	51.04
Natamycin at 512 μg/mL (2 MIC)	$1.08^{d} \pm 0.09$	77.53
Sodium benzoate at 2,000 μg/mL	$0.99^{d} \pm 0.07$	79.47
Sodium propionate at 2,000 μg/mL	$3.99^{ab} \pm 0.79$	16.92
Prochloraz® at 500 μg/mL	$0^{e} \pm 0.0$	100.00
Propiconazole® at 500 μg/mL	$0^{e} \pm 0.0$	100.00

Note: The presented data represent the mean of three replicates ± standard deviation (SD) ($n = 15$). Values within the same column that share the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

3.5.2. Effect of natamycin on the enzymatic and nonenzymatic defense systems of P. digitatum in vitro

The influence of natamycin at concentrations of 1/2 MIC (128 μg/mL) and MIC (256 μg/mL) on the enzymatic and nonenzymatic defense systems of *P. digitatum* was investigated *in vitro* (Fig. 3). Treatment with natamycin resulted in increased levels of ROS at both 1/2 MIC and MIC, with ROS concentrations reaching 4.03 and 5.72 μM/mg protein, respectively, compared to 1.28 μM/mg protein in the control (Fig. 3A). Furthermore, there was a significant increase ($p < 0.05$) in the activity of

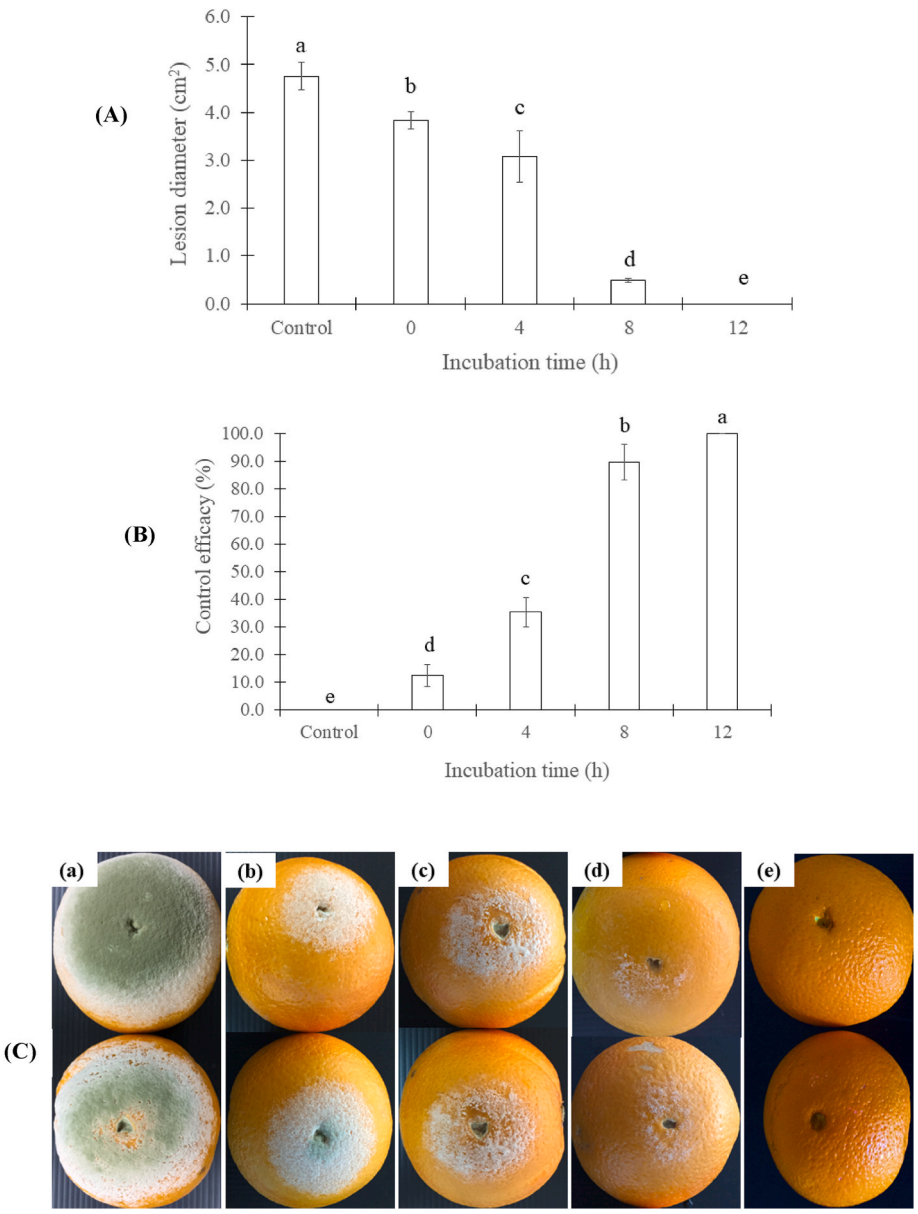

Fig. 1. Effect of different natamycin treatment durations on green mold disease development in mandarin fruit after a 12-day storage period at 30 °C under humid conditions. Natamycin (256 μg/mL) was mixed with *P. digitatum* spores, and after varied incubation periods (0, 4, 8, and 12 h), the mixture was inoculated into mandarin fruit. **(A)** Lesion diameters, **(B)** Control efficacy, and **(C)** Disease symptoms are shown. Inoculation treatments are as follows:
a: *P. digitatum* + DMSO (control).
b: Natamycin + *P. digitatum* mixed for 0 h before inoculation.
c: Natamycin + *P. digitatum* mixed for 4 h before inoculation.
d: Natamycin + *P. digitatum* mixed for 8 h before inoculation.
e: Natamycin + *P. digitatum* mixed for 12 h before inoculation.
Data represent the mean of three replicates ± standard deviation (SD) (n = 15). Values with the same letter are not significantly different, as determined by ANOVA with Tukey's HSD test ($p > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cellular antioxidant enzymes such as SOD and CAT. In the control, SOD and CAT levels were 0.30 and 66.09 units/min/mg protein, respectively, whereas at 1/2 MIC and MIC, these levels increased to 1.0, 83.92, and 2.74, 113.41 units/min/mg protein, respectively (Fig. 3B and C). Additionally, there were significant ($p < 0.05$) alterations in the levels of GSH, GSSG, and the GSH/GSSG ratio in *P. digitatum* cells after treatment with natamycin. Treated cells showed marked increases in GSH levels, from 0.21 μM/mg protein in the control to 0.42 and 0.88 μM/mg protein at 1/2 MIC and MIC, respectively (Fig. 3D). Similarly, the GSH/GSSG ratio increased (Fig. 3F). Conversely, the cellular GSSG levels decreased in response to the 1/2 MIC (0.13 μM/mg protein) and MIC (0.10 μM/mg protein) treatments compared to the control (0.25 μM/mg protein) (Fig. 3E).

3.5.3. *Effect of natamycin on cell membrane permeability of P. digitatum*

The effect of natamycin at concentrations of 1/2 MIC (128 μg/mL) and MIC (256 μg/mL) on the cell membrane permeability of *P. digitatum* was investigated, as presented in Fig. 4. Data from Fig. 4 demonstrate that the control group showed no significant variations in electrical conductivity during the 8-h measurement period. However, there was a significant increase in conductivity in groups treated with natamycin at both 1/2 MIC and MIC concentrations. Additionally, the electrical

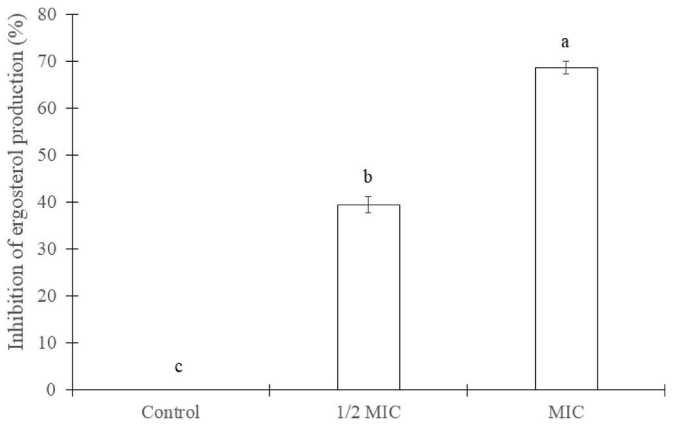

Fig. 2. Effect of natamycin concentrations (1/2 MIC, 128 μg/mL; MIC, 256 μg/mL) on the inhibition of ergosterol production in *P. digitatum* cells. The cells were cultured in PDB at 30 °C for 2 days. The data represent the mean of three replicates (±SD). Values with the same letter are not significantly different, as determined by ANOVA with Tukey's HSD test ($p > 0.05$).

conductivity continued to increase with prolonged exposure time to natamycin.

4. Discussion

Natamycin is widely used as an antifungal agent [76–78] and has been successfully applied to control postharvest diseases [55,79–81]. While recent studies have confirmed the effectiveness of natamycin in managing postharvest green mold disease in citrus caused by *P. digitatum* [48,54], our research explores the potential of natamycin derived from *S. philanthi* RL-1-178 [45] as a biofungicide for mandarin fruit. Our results show that natamycin effectively halts the progression of green mold disease on mandarin fruit by targeting key components of the fungal cell membrane, such as ergosterol, and impairing both enzymatic and non-enzymatic defense mechanisms. Additionally, it disrupts the integrity of the plasma membrane.

The sensitivity of fruit fungal pathogens to natamycin has been increasingly reported in recent years. Our *in vitro* experiments demonstrated the inhibitory effect of natamycin on *P. digitatum* in a dose-dependent manner. At a concentration of 256 μg/mL (MIC), natamycin completely inhibited (100 %) mycelial growth and spore germination. This level of efficacy was comparable to that of chemical fungicides such as Prochloraz® and Propiconazole® at 500 μg/mL. Natamycin also showed greater inhibition than common preservatives like sodium benzoate (2,000 μg/mL). In contrast, sodium propionate (2,000 μg/mL) was less effective, likely due to its more limited mode of action, which primarily disrupts fungal cell membranes and metabolic processes. It may require higher concentrations or longer exposure to achieve efficacy similar to that of fungicides like Prochloraz® and Propiconazole®, which target specific stages of fungal growth. Additionally, sodium propionate's antifungal spectrum may not be as broad or effective against *P. digitatum* as natamycin or synthetic fungicides. Other treatments may also have synergistic effects that contribute to their enhanced antifungal activity.

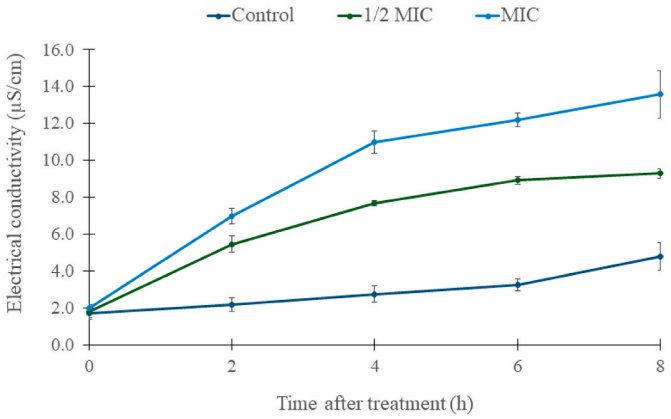

Fig. 4. Effect of natamycin concentrations (1/2 MIC, 128 μg/mL; MIC, 256 μg/mL) on cellular leakage in *P. digitatum*. Three-day-old mycelia were treated with natamycin for 0, 2, 4, 6, and 8 h in distilled water. The mycelia were filtered, and the filtrate solutions were used to measure electrical conductivity. Data are presented as the mean of three replicates (±SD).

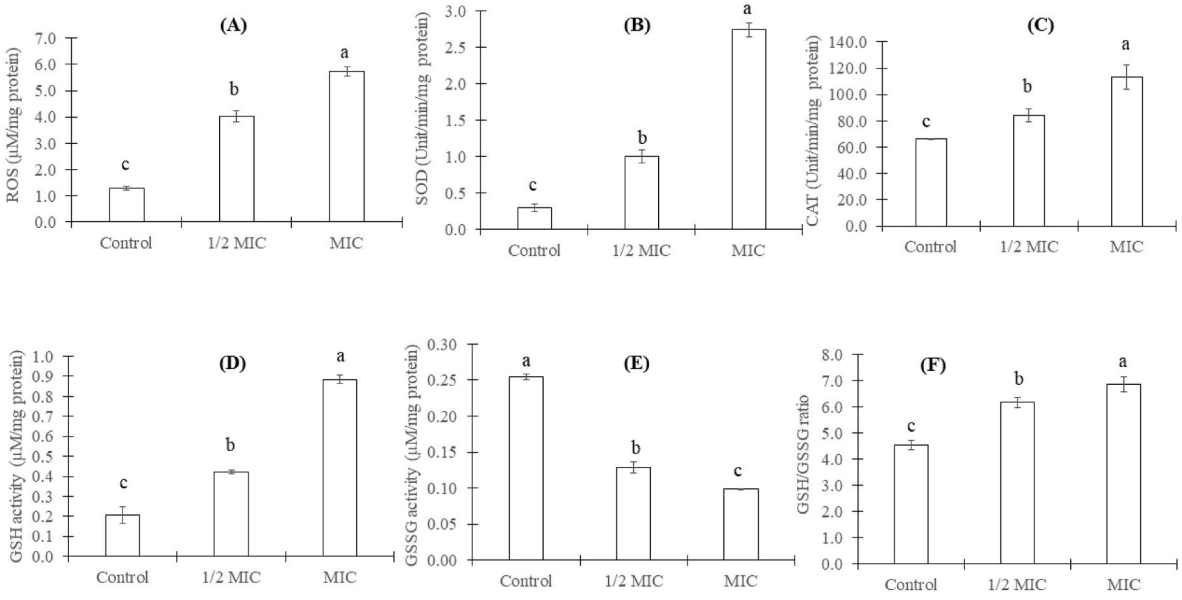

Fig. 3. Effect of natamycin concentrations (1/2 MIC, 128 μg/mL; MIC, 256 μg/mL) on the levels of reactive oxygen species (ROS) **(A)**, superoxide dismutase (SOD) **(B)**, catalase (CAT) **(C)**, reduced glutathione (GSH) activity **(D)**, oxidized glutathione (GSSG) activity **(E)**, and the GSH/GSSG ratio **(F)** in *P. digitatum* cells cultured in PDB at 30 °C for 2 days. The data are presented as the mean of three replicates (±SD). Values with the same letter are not significantly different, as determined by ANOVA with Tukey's HSD test ($p > 0.05$).

Our findings at 256 mg/L represent a higher concentration than those reported in previous studies. For instance, Du et al. [48] demonstrated complete inhibition of mycelial growth and spore germination of *P. digitatum* at a concentration of 3 mg/L, while He et al. [82] reported that concentrations of 3 mg/L and 2.5 mg/L achieved complete inhibition against *P. expansum*. This suggests that the effective concentration of natamycin required for complete inhibition may vary depending on the strain and experimental conditions. If no studies report a concentration as high as ours, this may indicate variability in pathogen sensitivity or a potential need for adjustment based on application context.

Beyond its effectiveness against *P. digitatum*, natamycin has demonstrated broad-spectrum antifungal activity. For instance, Cong et al. [83] reported that 20 mg/L of natamycin effectively suppressed *A. alternata* and *Fusarium semitectum* growth, while Ojaghian et al. [84] showed complete inhibition of *S. sclerotiorum* at 2.5 mg/L. Similarly, Cao et al. [79] found that 1.25 and 2.5 mg/L inhibited *C. fructicola* growth and spore germination, and Pan et al. [81] observed effective inhibition of both *Botryosphaeria dothidea* mycelial growth and spore germination at 2 mg/L. Saito et al. [56] identified MICs for *Botrytis cinerea* ranging from 5.0 to 10.0 mg/L for mycelial growth, and 0.7–1.0 mg/L for spore germination. These studies highlight the versatility of natamycin in controlling various fungal pathogens, supporting its potential as an alternative for managing postharvest diseases and maintaining food quality standards.

In our study, initial *in vitro* experiments laid the groundwork for using natamycin as a biocontrol agent against *P. digitatum*. Subsequent *in vivo* trials on mandarin fruit confirmed its efficacy over a 12-day period at 30 °C, with natamycin showing strong biocontrol activity. At a concentration of 2 MIC (512 μg/mL), natamycin achieved significant biocontrol effects (85.58 % for preventive and 77.53 % for curative applications), outperforming sodium propionate (21.08 % and 16.92 %, respectively) and showing similar efficacy to sodium benzoate (83.36 % and 79.47 %, respectively). However, it was less effective than chemical fungicides, which achieved complete control (100 %) of green mold disease. Despite being less effective than chemical fungicides, natamycin offers several advantages for postharvest disease management. As a natural product, it has a favorable environmental profile compared to synthetic fungicides, which may have long-term ecological impacts and contribute to resistance. Moreover, natamycin's lower toxicity makes it a safer alternative for use in food storage and production.

From a practical standpoint, natamycin could play a significant role in integrated pest management (IPM) systems, particularly in preventive applications where its efficacy (85.58 %) is higher. This makes it an excellent candidate for early-stage treatments aimed at reducing the initial incidence of postharvest diseases. However, its lower efficacy in curative applications (77.53 %) suggests that it may be less effective for treating advanced infections, where more potent fungicides might be necessary. Natamycin's relatively low toxicity and favorable environmental profile offer it as an attractive alternative to synthetic fungicides, especially for organic farming systems. Additionally, its cost-effectiveness, combined with its stability under various storage conditions, makes it a practical option for smaller-scale operations. For large-scale use, natamycin could be particularly beneficial when incorporated into a broader disease management strategy, potentially reducing the reliance on synthetic chemicals and offering a more sustainable solution. However, further research into optimal application methods, dosage adjustments for different fungal species, and cost-effectiveness across various scales of operation will be important to fully realize its potential in large-scale agricultural settings.

Although natamycin demonstrated substantial antifungal activity *in vitro*, the concentration required for effective *in vivo* control (2 MIC, 512 μg/mL) was higher than the MIC observed *in vitro*. This discrepancy can be attributed to the complex environmental conditions at fruit wound sites, such as pH, nutrient availability, and physical barriers, which may necessitate higher doses. These findings are consistent with those of Du et al. [48] and He et al. [82], who reported that higher concentrations are generally required for effective *in vivo* control compared to *in vitro* settings.

The data presented in our study further supports the potential of natamycin for successfully controlling pathogens in postharvest fruits and mushrooms. For instance, He et al. [82] demonstrated that 100 mg/L of natamycin could completely inhibit the occurrence of grey mold disease in grapefruits. In contrast, a higher concentration of 200 mg/L was necessary to control blue mold disease in jujube fruits [82]. Similarly, Du et al. [48] found that 300 mg/L of natamycin significantly reduced decay incidences and lesion diameters caused by *P. digitatum* in citrus. Natamycin has also been effectively employed as an alternative biofungicide for managing a range of pre-harvest diseases. These include crown rot in strawberries caused by *C. acutatum*, as reported by Haack et al. [55], anthracnose disease in apples due to *C. fructicola* [79], and dry bubble disease in mushrooms resulting from *Verticillium fungicola* [80]. Additionally, Saito et al. [50] demonstrated that natamycin significantly reduced overall fruit decay caused by natural infections without adversely affecting fruit quality. Moreover, Pan et al. [81] reported that the inhibitory effect of natamycin on *B. dothidea* inoculated in kiwifruit was dose-dependent, with 500 mg/L significantly reducing the incidence of soft rot. Overall, natamycin has proven effective as a postharvest treatment across a range of produce, including shiitake mushrooms, stone fruits, and citrus, demonstrating its broad-spectrum efficacy [80,83,85].

In our study, we investigated the impact of varying durations of natamycin treatment on *P. digitatum* spores prior to inoculation onto mandarin fruit. Our objective was to better understand the mechanisms by which natamycin affects the viability and behavior of *P. digitatum* spores, as well as its potential as a control measure for postharvest diseases. Our findings showed that treating *P. digitatum* spores with natamycin for 12 h resulted in complete inhibition of spore germination, with no visible disease symptoms upon inoculation onto mandarin fruit. These results suggest that natamycin is a promising agent for preventing *P. digitatum* infection during storage and handling. However, while natamycin demonstrates strong antifungal activity, it is important to consider the potential for resistance development over time, particularly when used as a biocontrol agent in IPM systems. The long-term efficacy of natamycin in preventing resistance should be closely monitored, as repeated or prolonged exposure to any antifungal agent, including biocontrol agents, could lead to the selection of resistant strains. Further studies are needed to evaluate the risk of resistance development, the molecular mechanisms behind it, and how resistance could affect its long-term utility in agricultural settings. Additionally, exploring the effectiveness of natamycin in combination with other biocontrol agents or chemical fungicides may help mitigate the risk of resistance while enhancing its overall efficacy.

The remarkable efficacy of natamycin in inhibiting the growth of *P. digitatum*, observed both *in vitro* and in mandarin fruit, highlights its potential as an effective biocontrol agent. Given its impressive performance, it was crucial to explore the mechanisms underlying its antifungal action against *P. digitatum*. Our study aims to elucidate the specific mechanisms through which natamycin exerts its inhibitory effects on this fungal pathogen. Ergosterol, a sterol found in the cell membranes of fungi, plays a crucial role in maintaining cell membrane integrity [86]. Natamycin is widely known for its ability to compromise the integrity of mold cell membranes by irreversibly binding to membrane sterols, especially ergosterol, which is prevalent in the cell membranes of these fungi. This binding significantly increases membrane permeability, leading to the leakage of essential cellular constituents, including actions. Such leakage precipitates a substantial drop in intracellular pH, ultimately resulting in cell lysis [49]. Our findings corroborate these established effects, demonstrating that natamycin significantly impairs ergosterol biosynthesis in *P. digitatum*. This impairment highlights the plasma membrane as a critical antifungal target of natamycin, underlining its potent activity and specificity in inhibiting fungal growth by targeting essential components of fungal cell

structure and metabolism.

The ROS in triggering apoptosis by disrupting cellular oxygen metabolism is extensively documented [87]. Based on this premise, our research hypothesized that elevated ROS levels induced by natamycin might lead to apoptosis in *P. digitatum* cells. The findings from our study support this hypothesis, showing that *P. digitatum* cells treated with natamycin at 1/2 MIC (128 μg/mL) and MIC (256 μg/mL) concentrations exhibited significantly higher ROS levels than the control group. This increase in ROS can lead to a series of detrimental cellular effects. Yoo et al. [88] outlined that elevated ROS levels can cause multiple cellular disruptions, including nucleic acid fragmentation, swelling of cellular tissue, condensation of chromatin fibrils, depletion of ATP pools, inhibition of ATPase activity within the mitochondrial inner membrane, and degradation of the phosphatidylserine lipid layer, ultimately leading to cell death or apoptosis. Building on this, our research also detected a notable increase in the activity of critical cellular antioxidant enzymes, such as SOD and CAT, in cells treated with natamycin. The elevated activity of antioxidant enzymes, stimulated by treatment with natamycin, seems to alleviate the oxidative stress triggered by increased ROS levels. Additionally, this treatment led to significant alterations in the concentrations of GSH, GSSG, and their ratio. The interplay between GSH and GSSG, governed by the thiol groups of cysteine residues and their allocation within cellular organelles, is vital for regulating the cell's redox balance and its overall capability to counter oxidative stress [89]. These findings suggest that the antifungal effectiveness of natamycin is tied to complex interactions within cellular oxidative dynamics, highlighting its potency as a powerful agent for controlling fungal infections through the induction of oxidative stress and apoptosis in pathogenic fungi.

The cytoplasmic membrane of fungi acts as a crucial mechanical and osmotic barrier, facilitating the selective transport of substances into and out of cells, and is essential for processes such as surface adsorption, synthesis, and secretion. Previous research has demonstrated that antifungal agents primarily inhibit microbial growth by disrupting cell membrane permeability and altering the synthesis of membrane components [90]. This disruption can cause the leakage of internal components and electrolytes, leading to changes in electrical conductivity. Our findings indicate that the observed variations in conductivity with natamycin reflect alterations in the permeability of the *P. digitatum* cell membrane. The observed effects of natamycin on the cell membrane permeability align with its documented impact on the cell membrane integrity of *B. cinerea* and *P. expansum* [82], as well as *C. fructicola* [79]. These findings corroborate the hypothesis that natamycin impedes *P. digitatum* by damaging the cell membrane, resulting in the leakage of crucial intracellular components and electrolytes like K^+, Ca^{2+}, and Na^+ [91]. This leakage could profoundly impact transmembrane material transport, energy metabolism, and the balance of membrane potential [92], suggesting substantial consequences for the cellular functions and survival of the fungus. Thus, the disruption of membrane integrity is likely a key factor in the antifungal efficacy of natamycin.

5. Conclusion

This study highlights natamycin's potential as a safer, less toxic alternative to conventional fungicides for managing postharvest green mold disease in mandarin fruits. Its broad-spectrum antifungal activity, particularly against *P. digitatum*, and its mechanism of disrupting membrane integrity while modulating antioxidant defenses distinguish it from traditional chemical treatments. However, the *in vivo* effectiveness of natamycin was lower than *in vitro*, indicating that higher concentrations may be necessary for effective control in fruit wounds. The nutrient-rich nature of fruit wounds complicates natamycin's efficacy, requiring careful optimization of concentrations. To address these challenges, future research should focus on combining natamycin with other biocontrol agents to enhance its effectiveness. Variability in the pathogen's response under *in vivo* conditions also presents challenges, underscoring the need to better understand the environmental factors involved. Such insights will help refine application strategies and maximize natamycin's potential in postharvest disease management. Further studies are essential to explore synergistic combinations of natamycin with other natural preservatives or biocontrol agents, and to clarify its action on fruit tissues. Additionally, research on optimizing application methods, stability under commercial storage conditions, and cost-effectiveness is crucial for scaling up its use in large-scale postharvest disease management. These efforts will ensure natamycin's practical applicability in the agricultural industry, particularly for managing postharvest diseases in a variety of fruit commodities.

CRediT authorship contribution statement

Sawai Boukaew: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Krittin Chumkaew:** Writing – review & editing, Formal analysis, Data curation. **Sirasit Srinuanpan:** Writing – review & editing.

Availability of data and materials

Not applicable.

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Declaration of competing interest

The authors declare no competing interests regarding the publication of this study.

Data availability

The authors do not have permission to share data.

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