



## Photodynamic control of fungicide-resistant *Penicillium digitatum* by vitamin K3 water-soluble analogue

Xiran Li<sup>a,1</sup>, Lina Sheng<sup>a,1</sup>, Adrian Oscar Sbodio<sup>b</sup>, Zheng Zhang<sup>c</sup>, Gang Sun<sup>c</sup>, Bárbara Blanco-Ulate<sup>b,\*\*</sup>, Luxin Wang<sup>a,\*</sup>

<sup>a</sup> Department of Food Science and Technology, University of California Davis, Davis, CA, 95616, United States

<sup>b</sup> Department of Plant Sciences, University of California Davis, Davis, CA, 95616, United States

<sup>c</sup> Department of Biological and Agricultural Engineering, University of California Davis, Davis, CA, 95616, United States

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### ABSTRACT

*Penicillium digitatum*, the fungal pathogen that causes green mold, is responsible for substantial postharvest losses of citrus worldwide. Photodynamic technology is a promising alternative to fungicides to control postharvest fungal decay. In this study, we investigated the antifungal activity of menadione sodium bisulfite (MSB), which is a water-soluble form of vitamin K3, with and without light activation against a fungicide-resistant *P. digitatum* isolate 3189. Results showed that MSB alone effectively inhibited *P. digitatum* spore germination and mycelium growth at concentrations of 5 and 40 mg mL<sup>-1</sup>, respectively, but did not reduce the viability of the ungerminated spores. When light was applied in addition to MSB, the treatment of 30 mg mL<sup>-1</sup> of MSB and 30-min light irradiation resulted in a 5-log reduction of ungerminated spores regardless of the light type. In addition, light exposure decreased the required MSB concentrations for the control of spore germination and mycelium growth. Artificially-inoculated oranges with 3189 spores treated with 40 mg mL<sup>-1</sup> of MSB and exposed to 30-min of simulated sunlight had no disease symptoms of green mold up to 14 days later compared to controls. In summary, this study illustrates the promising antifungal effect of MSB both with and without light exposure. The *in vivo* study using oranges as model fruit demonstrates the application potential of MSB for the citrus produce industry.

### 1. Introduction

Food losses resulting from microbial spoilage are a significant global problem affecting growers, markets, and consumers. Fresh fruits and vegetables are particularly susceptible to spoilage as they are often grown and handled in an open and unprotected natural environment with relatively high levels of diverse microbial loads (Leff & Fierer, 2013). Based on the Food and Agriculture Organization (FAO) estimation, approximately 35–53% of fruits and vegetables are lost or wasted in different regions of the world (FAO, 2011). The magnitude of loss expands when considering the amount of energy input, including land resources, water, shipping costs, and other costs associated with pre- and post-harvest production, handling, and storage of food (Snyder & Worobo, 2015). Despite the contribution of microbial spoilage to waste and loss in the food supply, industry and regulatory agencies historically pay

less attention to spoilage microorganisms since food laws and regulations tend to focus more heavily on food safety and public health. Thus, fewer grants have been available to provide an incentive for basic research on food spoilage (Snyder & Worobo, 2018).

California is one of the leading citrus production states in the United States, contributing 52% of total US citrus production and bringing approximately \$7.2 billion to the state economy per year (Babcock, 2018; Ross, 2020). Fruit loss due to physiological deterioration and mold decay causes significant economic loss to citrus growers and packers in California (Buzby et al., 2015; Kanetis et al., 2008). *Penicillium digitatum* is an opportunistic fungal pathogen with a relatively short reproduction cycle. It can produce up to two billion spores on a single fruit within three to 5 day at 25 °C (Holmes & Eckert, 1999). Airborne spores reach the surface of healthy fruit and infect fruit through injuries caused by handling during harvest, transportation, and storage (Kanetis

\* Corresponding author.

\*\* Corresponding author.

E-mail addresses: [bblanco@ucdavis.edu](mailto:bblanco@ucdavis.edu) (B. Blanco-Ulate), [lxwang@ucdavis.edu](mailto:lxwang@ucdavis.edu) (L. Wang).

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et al., 2008). While the direct elimination of spores is important because of their biological importance and persistence (Braga et al., 2015), containing expansion of the rotting is also helpful to extend the shelf life of the fruit. Once the germinated spores are established in fruit host tissues, lesions appear as soft rot on the peel, which later turn into white mycelium followed by an olive-colored area indicative of spore production (Costa et al., 2019). Over the years, various commercial fungicides have been used in citrus to minimize postharvest fungal decay (Harris, 2017; Kanetis et al., 2008). These include sodium o-phenylphenate (SOPP), thiabendazole (TBZ), sodium bicarbonate, pyrimethanil, fludioxonil, soda ash, and imazalil (IMZ). However, intensive use of fungicides both in the field and postharvest has led to the development of fungicide-resistant strains (Bus et al., 1991; Holmes & Eckert, 1999; Kinay et al., 2007; Palou et al., 2015; Zhu et al., 2006), including SOPP-resistant, TBZ-resistant, and IMZ-resistant *P. digitatum* (Holmes & Eckert, 1999; Kinay et al., 2007). The increasing trend for consumption of fresh fruit and vegetables, along with concerns about the use of synthetic chemicals and the increasing prevalence of fungicide-resistant fungi, have encouraged scientific research to develop novel technologies to control fungal decay.

Photodynamic inactivation (PDI), a non-thermal antimicrobial approach, has emerged as a promising alternative for the control of fungal infections of fresh produce because of its efficacy in reducing the microbial load and the low risk of developing antimicrobial resistance (Ghate et al., 2019). In the process of PDI, photosensitizers (PS) absorb energy from a specific wavelength of light and shift to a higher energy state, where it returns to the ground state by transferring energy to ground-state molecular oxygen or by electron transfer to neighboring substrates (usually a biomolecule) to form reactive oxygen species (ROS) (Klaussen et al., 2020). ROS can damage microbial cell walls, DNA, and biomolecules such as proteins and lipids, leading to cell death (Al-Asmari et al., 2017; Ghate et al., 2019; Lovell et al., 2010; Luksiene & Zukauskas, 2009). Molds, unlike bacteria, tend to grow on the surface of food or other solid substrates because of their higher demand for oxygen (Ghate et al., 2019). This provides an ideal condition for the application of photodynamic technology because 1) oxygen is the main element of photodynamic-related controls and 2) it mitigates the concerns of the limited penetration depth of certain types of light (Algorri et al., 2021; Rapacka-Zdonczyk et al., 2021). The use of PDI for the control of fungi species includes the inactivation of *Colletotrichum abscissum* conidia with methylene blue and solar irradiation (290–790 nm) (Gonzales et al., 2017), and *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. spores with curcumin and visible light (420 nm) (Al-Asmari et al., 2017). Most recently, Dibona-Villanueva and Fuentealba (2021) demonstrated the antifungal activity of a chitosan-riboflavin conjugate against *P. digitatum* under a broad spectrum of visible light. However, most of the PS currently in use are deeply colored, toxic, require multiple steps to synthesize, or have poor solubility, stability, and photostability in aqueous solutions (Kazantzis et al., 2020; Ormond & Freeman, 2013), all of which limit their applications in the fresh produce industry.

Menadione sodium bisulfite (MSB), also known as water soluble vitamin K3 is used clinically in China to treat hemorrhagic diseases caused by vitamin K deficiency and regarded as a safe source of vitamin K in animal nutrition in the U.S. and Europe (EFSA Panel on Additives Products or Substances used in Animal Feed, 2014; Pillai, Michaela, & Benz, 2021 2020; Zhang et al., 2018). The acute oral toxicity of MSB used in animal feed can be added up to levels as high as 1000 times of the dietary requirement without seeing any adverse effect (EFSA Panel on Additives Products or Substances used in Animal Feed, 2014). Zhang et al. (2021) recently reported that MSB showed good photoactivity and produced ROS efficiently when exposed to UV-A (315–400 nm) and UV-B (280–315 nm) irradiation. Because of its water solubility, low toxicity, and promising ROS production, in this study we evaluated MSB as a PS candidate to be applied onto the surface of fresh produce for the control of fungal infection. To do so, an IMZ- and TBZ-resistant *P. digitatum* isolate 3189 was selected as the model strain. The

photoinduced impact of MSB on the different life stages of *P. digitatum*, including spore germination, spore viability and mycelium growth, were evaluated in a series of *in vitro* studies. Based on the *in vitro* study results, the efficacy of photoactivated MSB against 3189 inoculated oranges was investigated *in vivo*.

## 2. Materials and methods

### 2.1. Fungal culture and the preparation of spore suspension

The fungicide-resistant *P. digitatum* 3189 isolate was provided by Prof. James E. Adaskaveg (University of California, Riverside). It was isolated from *Citrus limon* fruit sampled in a packinghouse in Bakersfield, California, in May 2003. The 3189 isolate displays resistance against IMZ (EC<sub>50</sub> IMZ > 1.5 ppm) and TBZ (EC<sub>50</sub> TBZ > 10 ppm) but is sensitive to fludioxonil and azoxystrobin. The axenic fungal culture was grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, US) for 10 days under ambient temperature (22 ± 1 °C). Spores were then harvested by washing the agar surface with sterile Milli-Q water containing 0.1% Tween 20 and then passing the suspension through multiple layers of sterile cotton cloth to remove hyphal fragments. The resulting spore suspension was counted in a hemocytometer (INCYTO, Cheonan-si, Chungcheongnam-do, South Korea) and adjusted to 1 × 10<sup>6</sup> spores mL<sup>-1</sup> with sterile Milli-Q water.

### 2.2. Light sources and dose calculation

The UV-A light was provided by four UV-A lamps (320–400 nm, 18W, Actinic BL, Philips, Holland). The exposure distance of UV-A light was 8 cm, and the average UV-A light intensity received by the 96 well plate, the Petri dish and the fruit was 6.8 ± 0.2 mW cm<sup>-2</sup>. The light dose was calculated as:

$$\text{light dose (J cm}^{-2}\text{)} = \text{Irradiation time (s)} \times \text{irradiance (mW cm}^{-2}\text{)}$$

Based on the calculation, a 15- or 30-min UV-A light exposure yielded a total light dosage of 61 kJ m<sup>-2</sup> and 120 kJ m<sup>-2</sup>, respectively. The simulated sunlight was generated by SUNTEST CPS+ (Atlas Material Testing Technology, Mount Prospect, IL, US) simulating the UV-A and UV-B components of the solar light (300–400 nm). The illumination distance was 20 cm. The total cumulative dose was measured and generated automatically by the machine, and the doses applied for 15- or 30-min simulated sunlight exposures were 58 kJ m<sup>-2</sup> and 117 kJ m<sup>-2</sup>, respectively.

### 2.3. Spiral gradient dilution (SGD) method to determine fungal growth inhibition

The spiral gradient dilution method was used to estimate the effective concentrations of MSB required to cause inhibition of the target fungus growth by 50% (EC<sub>50</sub>) and 99% (EC<sub>99</sub>). Spiral gradient plates were prepared as described by Förster et al. (2004). Briefly, 50 ml of PDA was poured into each petri dish (15 cm in diameter) to create a layer of agar with 3.3 mm thickness. MSB was purchased from MilliporeSigma (Burlington, NJ, US). MSB solution was prepared in sterile Milli-Q water at 100 mg mL<sup>-1</sup>. A total of 50 µL of MSB solution was then spirally plated onto each PDA plate. The MSB solution applied from the center of each plate was 100 mg mL<sup>-1</sup>, with the MSB concentration decreasing as it got spirally plated toward the edge of the plate. For the control plates, sterile Milli-Q water was applied using the same method. Plates were then incubated at 25 °C for 4 h to allow the MSB solution/Milli-Q water to be fully absorbed into the agar and form a radial gradient over the plate. A sterile cork borer, approximately 28 mm in diameter, was used to remove the center of the plate, preventing fungus growth across the center of the plate into the opposite inoculation area. Ten mL of spore suspension (1 × 10<sup>6</sup> spores mL<sup>-1</sup>) were

dispensed at the peripheral of the agar surface and evenly spread to the center of the plate along the radius with a disposable plastic pellet pestle (Thermo Fisher Scientific Inc. Waltham, MA, U.S.). The spore suspension was applied on a plate in sextuplicate, and two plates were prepared for each treatment. Inoculated plates were subjected to 15- or 30-min of simulated sunlight (SS15 and SS30) or UV-A (UV-A15 and UV-A30) before being incubated in the dark at ambient temperature for four days. The dark controls were directly incubated in the dark. The EC<sub>50</sub> and EC<sub>90</sub> values were calculated using ECX, an open-source R package developed by Torres-Londoño et al. (2016). The following parameters were entered into the software for calculation: plate size (15 mm in diameter), agar medium height (3.3 mm), molecular weight of MSB (276.24 g mol<sup>-1</sup>), stock MSB concentration (100,000 ppm), rad1 (the distance in mm from observed total inhibition point to the center of the plate), and rad2 (the distance in mm from the zero inhibition point to the center of the plate).

#### 2.4. Inhibition assay of fungal spore germination

The spore germination inhibition assay was performed as described by He et al. (2016) and Song et al. (2020) with modifications. MSB solutions at different concentrations (0.156, 0.625, 2.5, and 10 mg mL<sup>-1</sup>) were prepared in 2 × potato dextrose broth (PDB, Difco Laboratories, Detroit, MI, US). The 2 × PDB without MSB was used as the no-MSB control. For each concentration, 0.1 mL of MSB was mixed with 0.1 mL of spore suspension (~1 × 10<sup>6</sup> spore mL<sup>-1</sup>) in a 96-well plate well, in triplicate. The final spore concentration was around 2 × 10<sup>5</sup> spore mL<sup>-1</sup>, and the final MSB concentrations ranged from 0 to 5 mg mL<sup>-1</sup>. The inoculated plates were then incubated in the dark at ambient temperature for 30 min to allow adequate interactions between the PS and the spores before the exposure to 15 min of UV-A or simulated sunlight. Meanwhile, another plate prepared in identical conditions without light exposure was used as the dark control. Subsequently, all the prepared plates were incubated in the dark at ambient temperature for 16 h under shaking at 300 rpm. After incubation, at least 200 spores were examined per treatment under a light microscope (ZEISS standard 20, Carl Zeiss AG, Oberkochen, Germany) at × 40 magnification. Germlings were photographed using a microscope eyepiece digital camera (Thermo Fisher Scientific, Waltham, MA, U.S.). A spore was recorded as germinated when the germ tube was equal or longer than the spore diameter (Kłosowski, Castellar, Stammmer, & May De Mio, 2018). Spore germination (SG, %) was calculated with the following equation:

$$\text{Spore germination (SG, \%)} = \frac{N_g}{N_t} \times 100$$

where N<sub>g</sub> and N<sub>t</sub> are the numbers of germinated spores and the total numbers of spores inspected, respectively. This experiment was performed in triplicate and repeated twice independently with different batches of inoculum.

#### 2.5. Spore viability assay

MSB solutions were prepared in sterile Milli-Q water at concentrations of 0, 10, 20, 30, and 40 mg mL<sup>-1</sup>. Each MSB solution was then mixed 1:1 with the spore suspension (1 × 10<sup>6</sup> spore mL<sup>-1</sup>) in a 96-well plate well, in triplicate. Sterile Milli-Q water without MSB was used as the no-MSB control. The prepared plates were incubated in the dark for 30 min and subsequently subjected to UV-A or simulated sunlight for 15 or 30 min. Another plate used as the dark control was incubated in the dark for 1 h. After treatments, 100 µL of the sample from each well was 10-fold serially diluted, and appropriate dilutions were gently spread on PDA. All plates were then incubated in the dark for 72 h at ambient temperature before enumeration. Survival of spores was expressed in CFU (colony forming units). Three plates were prepared for each treatment, and two independent experiments were performed.

#### 2.6. Mycelium growth inhibition assay

The mycelium growth inhibition assay was adapted from Imada et al. (2014) with modifications. MSB was prepared in sterile Milli-Q water at 0, 5, 10, 20, and 40 mg mL<sup>-1</sup>. Mycelium agar plugs (3 mm in diameter), cut from the peripheral of a seven-day fungal mycelium on PDA plates using a sterile cork borer, were transferred to an empty petri dish, immediately covered with 100 µL of MSB, and incubated in the dark for 30 min. The plugs in the light groups were then exposed to simulated sunlight or UV-A for 15 or 30 min, while those used as dark controls (dark group) were continuously incubated in the dark for another 30 min. All the treated plugs were then placed in the center of each PDA plate (90 mm in diameter) and incubated in the dark for 7 day at ambient temperature. Photos were taken at the end of the incubation period to record the development status of the mycelium. The percentage of inhibition of mycelial growth (MGI) was calculated according to the following formula:

$$\text{MGI (\%)} = \frac{dc - dt}{dc - 0.3} \times 100$$

where dc (cm) is the mean radial growth diameter for the control sample in the dark group without MSB, dt (cm) is the mean radial growth diameter for the treatment sets and 0.3 cm is the initial diameter of the mycelium plug.

#### 2.7. In vivo antifungal assay using *P. digitatum*-inoculated oranges

The in vivo antifungal assay was conducted based on the methods of Vilanova et al. (2012) and Cai (2015), with modifications. Unwaxed Valencia oranges at harvest maturity were provided by the Lindcove Research & Extension Center, Exeter, CA, US. Oranges were washed thoroughly with tap water and disinfected for 30 s in 10% sodium hypochlorite solution before being rinsed twice with tap water and dried with paper towels. Each orange was wounded by a sterile nail (1 mm wide, 3 mm long, and 2 mm deep) at the equator and inoculated with 10 µL of *P. digitatum* spore suspension at a concentration of 1 × 10<sup>6</sup> spores mL<sup>-1</sup>. The inoculum was then allowed to dry overnight at room temperature. After that, a pipette was used to apply 10 µL of MSB solution at 40 mg mL<sup>-1</sup> to the wounded surface sites. For the no-MSB controls, the same volume of sterile water was substituted for the MSB solution. Oranges were then incubated in the dark for 30 min and illuminated by SS or UV-A for 15 or 30 min. All light-treated oranges, along with the controls kept in the dark, were stored in black plastic boxes at ambient temperature with 85–90% relative humidity (RH) for five days. Disease incidence and severity were assessed daily after two days of storage, when the first sign of rotting appeared. Disease incidence corresponded to the percentage of inoculated fruit displaying visual signs of tissue maceration or soft rot. Disease severity was determined as the average lesion diameter (in mm) of each inoculated fruit displaying signs of rot. Six oranges were used per treatment, and the experiment was performed twice independently.

#### 2.8. Statistical analysis

Data was analyzed using general linear models (GLM) from JMP (SAS, Cary, NC). Mean values were compared by least significant difference (LSD) multiple-comparison test. P values of less than 0.05 were considered significant. Results were reported as mean ± standard deviation (SD).

### 3. Results

#### 3.1. Antifungal activity of MSB with or without light

The spiral gradient dilution method was applied to assess the

effective concentration range of MSB needed to inhibit growth of the fungicide-resistant *P. digitatum* isolate 3189. MSB was able to inhibit the growth of the fungal culture on plates incubated in the dark when compared to the no-MSB control under the same incubation conditions (Fig. 1A). The fungicide-resistant *P. digitatum* isolate 3189 is sensitive to MSB and the EC<sub>50</sub> and EC<sub>99</sub> of MSB under dark conditions were  $0.05 \pm 0.003$  and  $0.26 \pm 0.030 \text{ mg mL}^{-1}$ , respectively (Fig. 1B). The inhibition effect of MSB was in general improved by exposing the inoculated plates with MSB to UV-A or SS. Although the 15-min UV-A exposure did not lead to any significant changes in EC<sub>50</sub> and EC<sub>99</sub> (UV-A15 vs. dark), the antifungal effect of MSB was significantly enhanced when UV-A irradiation time increased to 30 min (decreasing the EC<sub>50</sub> to  $\sim 0.04 \text{ mg mL}^{-1}$  and EC<sub>99</sub> to  $\sim 0.19 \text{ mg mL}^{-1}$ ).

The exposure to SS generated a more pronounced impact on the control of *P. digitatum* by MSB. Under a shorter exposure time, the inhibitory effect of MSB + SS15 was significantly better than MSB + UV-A15 ( $p < 0.05$ ). Moreover, increasing the exposure time to 30 min further improved the antifungal activity of MSB (SS30 vs. SS15), with the EC<sub>50</sub> and EC<sub>99</sub> decreased from 0.03 to 0.02 mg mL<sup>-1</sup> and 0.14 to 0.06 mg mL<sup>-1</sup>, respectively.

### 3.2. Effect of MSB the germination of *P. digitatum* spores

The impact of MSB on the spore germination of the fungicide-resistant *P. digitatum* isolate was assessed under dark and light conditions (Fig. 2). In the dark, the spore germination rate decreased as MSB concentrations increased from 0.078 to 5 mg mL<sup>-1</sup>. The germination rates were significantly lower ( $p < 0.05$ ), with MSB concentrations of 1.25 mg mL<sup>-1</sup> (56.8% spore germinated) or 5 mg mL<sup>-1</sup> (0% spore germinated) compared with the no MSB control (83.1% spore germinated). The inhibitory effect of MSB on spore germination was significantly promoted when UV-A or simulated sunlight was applied. In the presence of 0.78 mg mL<sup>-1</sup> MSB, UV-A and simulated sunlight reduced the germination rates of *P. digitatum* by 15.3% and 38.6% respectively, compared to 76.7% in the dark (Fig. 2B). When MSB concentrations

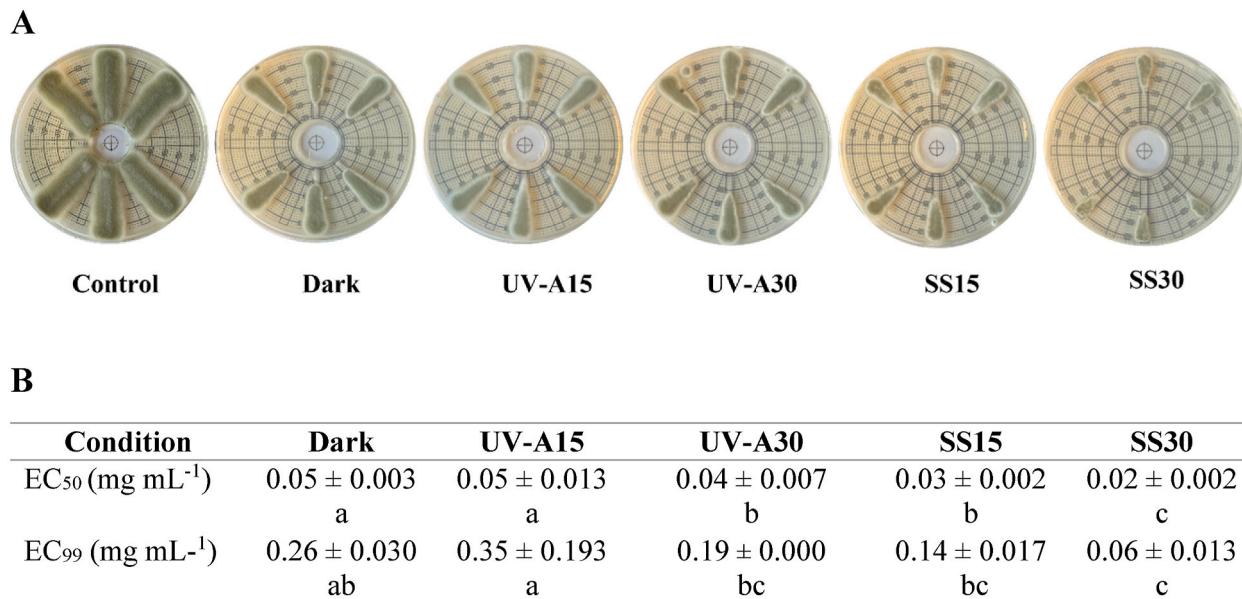
increased to 1.25 and 5 mg mL<sup>-1</sup>, the presence of light completely inhibited spore germination regardless of the light type. Exposure to UV-A or SS alone also led to a small reduction in the spore germination rate ( $p > 0.05$ ).

### 3.3. Effect of MSB on spore viability

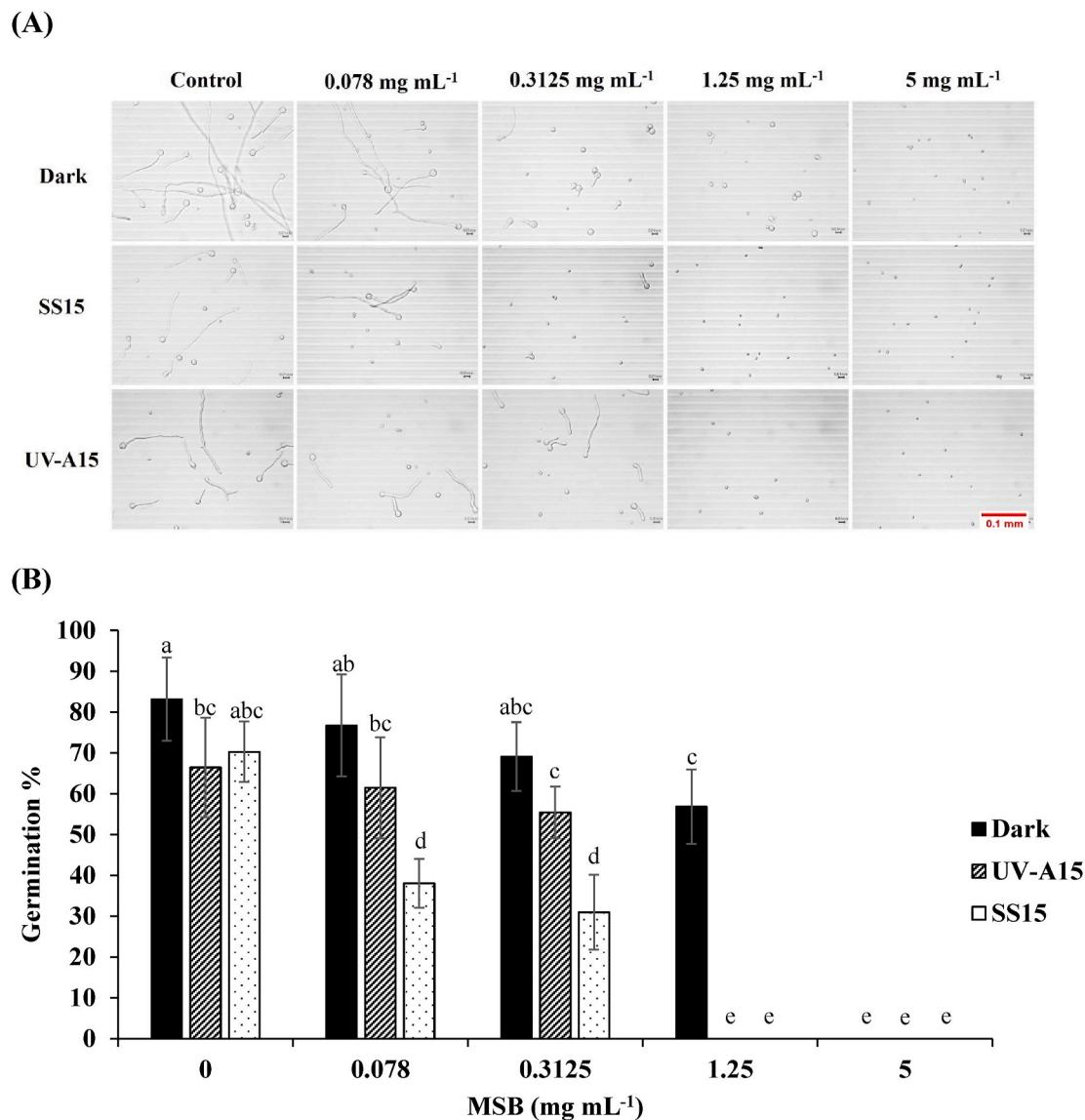
To test the effect of MSB on spore viability of the *P. digitatum* 3189 isolate, a suspension of  $1 \times 10^6$  spore mL<sup>-1</sup> was 1:1 (v:v) mixed with different concentrations of MSB. After 30 min of pre-incubation in the dark, the mixture was subjected to SS or UV-A for 15 or 30 min. Fig. 3 shows the survival of *P. digitatum* spores right after MSB treatments with or without light exposure. In the dark, MSB concentrations up to 40 mg mL<sup>-1</sup> did not show significant change on spore viability counts. Exposure to light alone was also unable to inactivate *P. digitatum* spores; the spore populations' post-light exposures were  $4.8 \pm 0.13$ ,  $4.9 \pm 0.23$ ,  $4.9 \pm 0.08$ , and  $5.0 \pm 0.09 \log \text{CFU mL}^{-1}$  for UV-A15, UV-A30, SS15, and SS30, respectively. When light was used in combination with MSB, the overall inactivation efficiency increased proportionally to the exposure time and the MSB concentration. At 40 mg mL<sup>-1</sup> MSB, approximately 0.5-log reductions in spore populations were achieved regardless of the light type. Increasing the irradiation time from 15 to 30 min did not lead to any apparent loss in spore viability with 10 mg mL<sup>-1</sup> of MSB. However, in the presence of 20 mg mL<sup>-1</sup> of MSB, the number of spores was reduced significantly by 1.2 and 3.2 log after UV-A30 and SS30 treatments, respectively. Further increasing the MSB concentration to 30 mg mL<sup>-1</sup> or higher and combining with UV-A30 or SS30 exposure reduced the number of culturable spores below the detection limit on the PDA plates.

### 3.4. Effect of MSB on the control of mycelial growth of *P. digitatum*

Mycelium plugs of *P. digitatum* 3189 isolate were treated with MSB solutions at different concentrations and kept in the dark for 30 min before being subjected to UV-A or SS. After light treatment, each was



**Fig. 1.** Antifungal activity of menadione sodium bisulfate (MSB) against fungicide-resistant *P. digitatum* 3189 under different treatment conditions. (A) Images of 3189 on spiral gradient plates after being exposed to UV-A or simulated sunlight (SS) different length of time and then incubated at ambient temperature for 4 days in dark. The MSB solution applied from the center of each plate is 100 mg mL<sup>-1</sup> and the concentrations of MSB decreases as it gets spiral plated toward the edge of the plate. "UV-A15" and "UV-A30" represent UV-A treatment for 15 and 30 min, respectively. "SS15" and "SS30" represent simulated sunlight treatment for 15 and 30 min, respectively. The dark control (Dark) had no light exposure treatment before the 4-day incubation. For the control plates (Control), sterile Milli-Q water instead of MSB solution was spiral plated. (B) Calculation of the EC<sub>50</sub> and EC<sub>99</sub> of MSB under different light exposure conditions. EC<sub>50</sub> and EC<sub>99</sub> represent the concentrations of MSB at which the growth of 3189 was inhibited by 50% and 99% under each light exposure condition, respectively. Data are presented as means ( $n = 6$ )  $\pm$  standard deviations. Different lowercases in each row indicate significant differences between different treatment combinations ( $p < 0.05$ ).



**Fig. 2.** Effect of menadione sodium bisulfate (MSB) on the germination of *P. digitatum* 3189 spores with or without light exposure. The germination images and rates were taken and calculated 16 h after treatments. (A) microscopic images of *P. digitatum* spores and germinated spores after being treated with different concentrations of MSB with or without light exposure. Dark, no light exposure control; SS15, simulated sunlight exposure for 15 min; UV-A15, UV-A light exposure for 15 min. (B) calculated spore germination rates after different MSB × light exposure treatments. Data are presented as means ( $n = 6$ )  $\pm$  standard deviations. Different lowercases indicate significant differences between different treatment combinations ( $p < 0.05$ ).

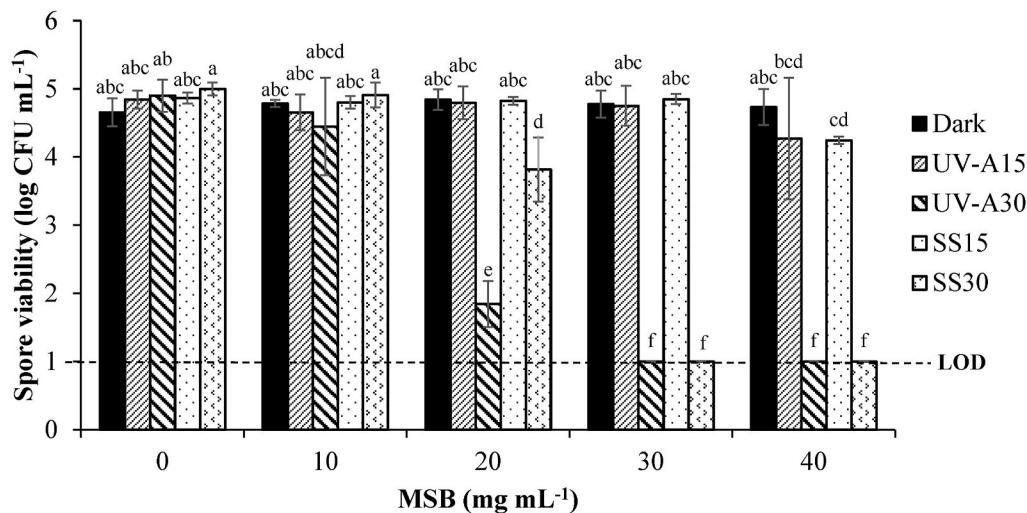
transferred to a sterile PDA plate to observe their growth (Fig. 4). The light treatments without MSB reduced the mycelium radial growth by 14.8–23.9%. In general, the inhibitory effect was increased when MSB was applied to the mycelium plugs prior to the light exposure. At lower MSB concentration (5 or 10 mg mL<sup>-1</sup>), the treatment of UV-A15 significantly ( $p < 0.05$ ) reduced the mycelium diameters by 42.1 and 54.5%, respectively. However, subsequent increase of the UV-A exposure time to 30 min did not lead to significant decreases in the mycelium diameters. On the other hand, the SS15 in the presence of 5 or 10 mg mL<sup>-1</sup> of MSB resulted in 30.9 and 43.3% reduction of the mycelial growth, respectively. Significant ( $p < 0.05$ ) enhancement of the inhibition effect of MSB on mycelial growth was observed when the SS exposure time increased to 30 min. The combinations of 5 and 10 mg mL<sup>-1</sup> MSB with SS30 reduced the radial growth of mycelium by 41.2 and 58.8%, respectively. MSB alone also exhibited inhibitory effect in a concentration-dependent manner (Fig. 4B). MSB at 5, 10, 20, and 40 mg mL<sup>-1</sup> reduced the radial growth of mycelium by 25.8, 46.1, 70.0, and 100%, respectively, when compared to the no-MSB control in the dark.

Nevertheless, the minimum MSB concentration required to completely inhibit *P. digitatum* mycelium growth was 40 mg mL<sup>-1</sup> under dark conditions compared to only 20 mg mL<sup>-1</sup> under light conditions regardless of the light type or exposure time (Fig. 4B).

### 3.5. Effect of MSB on the control of orange decay

Unwaxed oranges that had been wound-inoculated with a spore suspension ( $1 \times 10^6$  spore mL<sup>-1</sup>) of *P. digitatum* 3189 isolate and were treated with various concentrations of MSB with or without light exposure. In our preliminary trial, all the oranges treated with lower MSB concentrations (2.5, 5, 10, 20 mg mL<sup>-1</sup>) from the dark or UV-A30 groups were spoiled three days after inoculation. The 30-min SS alone also showed a 100% fungicidal effect (Table S1). Therefore, 40 mg mL<sup>-1</sup> of MSB and two exposure durations including a shorter irradiation time of 15 min were chosen in the *in vivo* study.

After five days of storage, 100% of the no-MSB control oranges incubated in the dark were fully covered with green mold. The



**Fig. 3.** Effect of menadione sodium bisulfate (MSB) on the spore viability of *P. digitatum* 3189. “UV-A15” and “UV-A30” represent UV-A exposure for 15 and 30 min, respectively. “SS15” and “SS30” represent simulated sunlight exposure for 15 and 30 min, respectively. The limit of detection (LOD) by direct plating is 1 log CFU mL<sup>-1</sup>. The initial inoculation levels for “Dark”, “UV-A15”, “UV-A30”, “SS15” and “SS30” were 4.7 ± 0.20, 4.8 ± 0.13, 4.9 ± 0.23, 4.9 ± 0.08 and 5.0 ± 0.09 log CFU mL<sup>-1</sup>, respectively. Dark represents the no light exposure control. Data are presented as means ( $n = 6$ ) ± standard deviations. Different lowercases indicate significant differences between different treatment combinations ( $p < 0.05$ ).

application of 40 mg mL<sup>-1</sup> MSB alone without any light exposure decreased the disease incidence to 17%. Among the spoiled oranges, the average lesion diameter was reduced to 41.0 ± 26.87 mm from 74.7 ± 4.37 mm (no-MSB controls in the dark). UV-A irradiation alone failed to control fungal infection on oranges (Disease incidence = 100%). However, the combination of MSB application and UV-A not only lowered the disease incidence to 33% but also reduced the lesion diameters of the infected oranges from 74.7 ± 4.37 mm (no MSB control in dark) to 32.8 ± 14.69 (UV-A15 + 40 mg mL<sup>-1</sup> MSB) and 29.8 ± 12.45 mm (UV-A30 + 40 mg mL<sup>-1</sup> MSB), respectively. The synergistic effect between SS and MSB became apparent with a shortened irradiation time. Compared to the oranges treated with 40 mg mL<sup>-1</sup> MSB (Disease incidence = 17%, Severity = 41.0 ± 26.87 mm) or 15 min of SS (Disease incidence = 50%, Severity = 56.1 ± 19.34 mm), only 8% of the oranges in the SS15 + 40 mg mL<sup>-1</sup> MSB treatment group showed disease symptoms, with an average lesion diameter at 69.7 ± 0.00 mm (Fig. 5B).

#### 4. Discussion

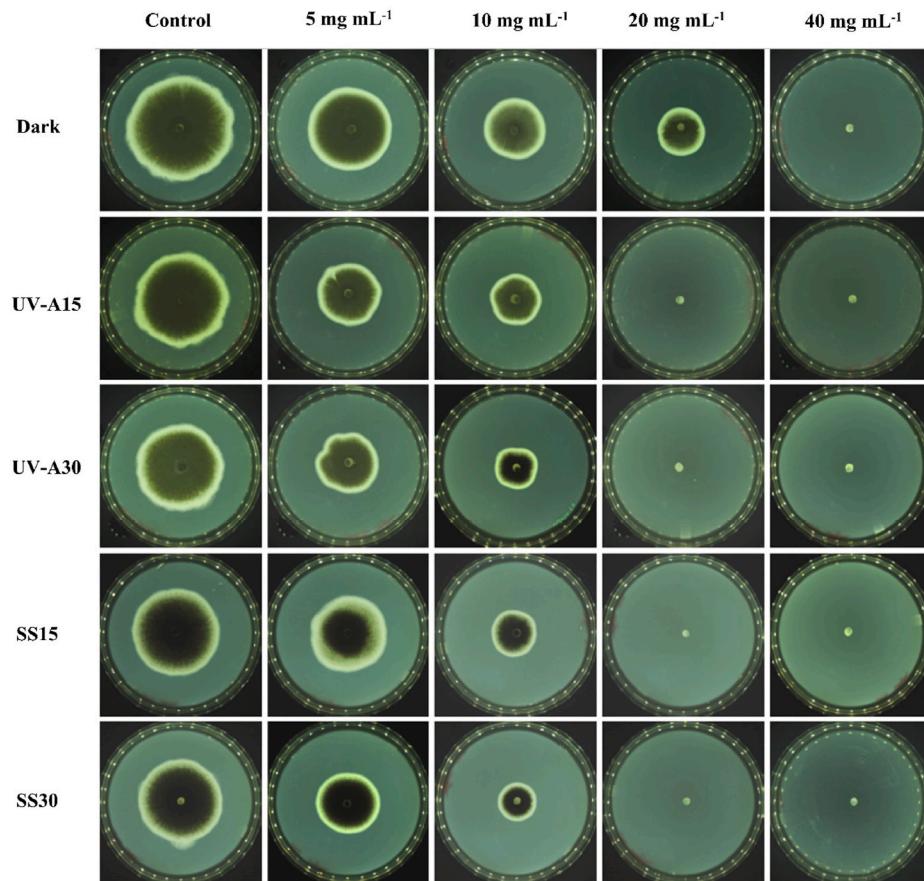
Green mold caused by *P. digitatum* is one of the major sources of postharvest decay in citrus fruits (Poppe et al., 2003). In addition to food loss and waste caused by spoilage, fruit tissues damaged by mold growth might serve as suitable substrates for the growth of foodborne pathogens, leading to serious food safety concerns (Tournas, 2005). Although the use of chemical fungicides plays an important role in limiting losses in commercial fruit shipment and storage, their use has led to the emergence of fungicide-resistant strains, which makes postharvest control of fungal decay increasingly difficult (Ballester et al., 2010). The use of photodynamic technology is emerging as an alternative to control fungicide-resistant strains in postharvest. Upon light irradiation, photoactive PS generates ROS, which non-selectively targets multiple essential cellular components and results in rapid cell death, reducing chance of resistance development (Ghate et al., 2019). Recently, MSB was demonstrated to have excellent photoactivity under UV irradiation with absorption peaks at both UV-A and UV-B regions and to generate ROS, including hydroxyl radical (-OH), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and singlet oxygen (<sup>1</sup>O<sub>2</sub>), through type I and type II photoreactions (Zhang et al., 2021). ROS can cause lethal damage to microorganisms by oxidizing cellular components such as lipid bilayer membranes, proteins, and nucleic acids (Ezraty et al., 2017; Lovell et al., 2010; Luksiene & Zukauskas, 2009; Zaid & Mohammad, 2018). Therefore, in this study we systematically evaluated the efficacy of MSB against a fungicide-resistant isolate of the postharvest pathogen *P. digitatum* both *in vitro* and *in vivo*.

Dormant spores germinate when favorable conditions are met

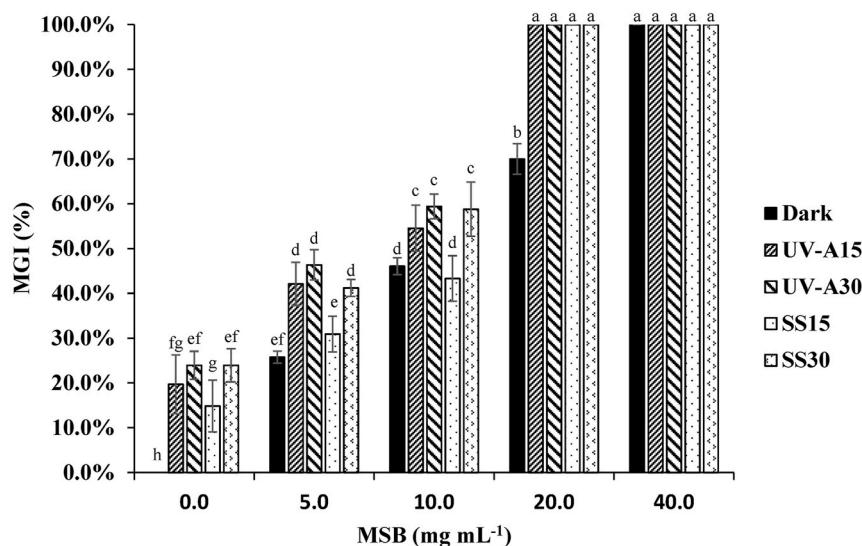
(Sephton-Clark & Voelz, 2018). The spores of *P. digitatum* can germinate and grow rapidly after coming into contact with the wound tissue (Droby et al., 2008), and completes its growth cycle within two days, resulting in citrus soft rot (Yang et al., 2019). As shown in Fig. 6, MSB can control the fungal spoilage by targeting different developmental stages of fungi. In this study, MSB alone was able to reduce the spore germination rate of *P. digitatum* isolate 3189 by 26% when its concentration reached 1.25 mg mL<sup>-1</sup>. Further increase in the MSB concentration to 5 mg mL<sup>-1</sup> led to complete inhibition of fungal spore germination. Similarly, retarded spore germination was observed under a light microscope when the fungal pathogen *Botrytis cinerea* was inoculated on tomato leaves at  $2 \times 10^5$  spores mL<sup>-1</sup> after they were pretreated with 10 mM of MSB (Jo et al., 2020). The efficacy of MSB over the control of spore germination was significantly improved by the addition of light exposure. For example, only a quarter of the MSB (0.3125 mg mL<sup>-1</sup>) was required to reduce the spore germination level by the same amount (26%) in UV-A treated samples when compared to samples kept in the dark (Fig. 2). The synergistic effect of PS and light has also been reported by Gonzales et al. (2017). In this report, 25 μM methylene blue in combination with 30 min of full-spectra solar irradiation reduced the germination rate of *Colletotrichum abscissum* spores ( $2 \times 10^5$  spores mL<sup>-1</sup>) by 87–95%, while light alone had little impact on spore germination. The photo-induced inhibitory effect of curcumin against *P. expansum*, a fungal pathogen species closely related to *P. digitatum*, was observed by Song et al. (2020). With an inoculation level at  $1 \times 10^5$  spores mL<sup>-1</sup>, the authors found that the treatment of 100 μmol L<sup>-1</sup> curcumin alone had little effect on spore germination, while exposure to LED (420 nm) for 30 min together with curcumin reduced the spore germination rate to approximately 0.2% (Song et al., 2020). In the same study, it was observed that the treated *P. expansum* spores displayed abnormal cell morphology, including broken cell walls, vacuolation, and obscured nuclear envelope accompanied by intracellular materials efflux after treatments (Song et al., 2020). It has been reported that PDI can induce DNA damage that prevents the cell cycle progression in fungi (Dardalhon et al., 2008; Goldman et al., 2002). Notably, although the treatment of 5 mg mL<sup>-1</sup> MSB and 15-min light completely inhibited spore germination, this effect is likely to be fungistatic rather than fungicidal, as the population of the spores remained unchanged until the MSB concentration reached to 20 mg mL<sup>-1</sup> and the irradiation time increased to 30 min (Fig. 3).

Compared to the vegetative cells, the spores of *Penicillium* spp. are more resistant to adverse environments, including relatively high temperature, drought, and UV radiation (Wyatt et al., 2013). Light treatment only or MSB alone did not significantly impact spore viability. Combining MSB with light exposure, on the other hand, significantly

(A)



(B)

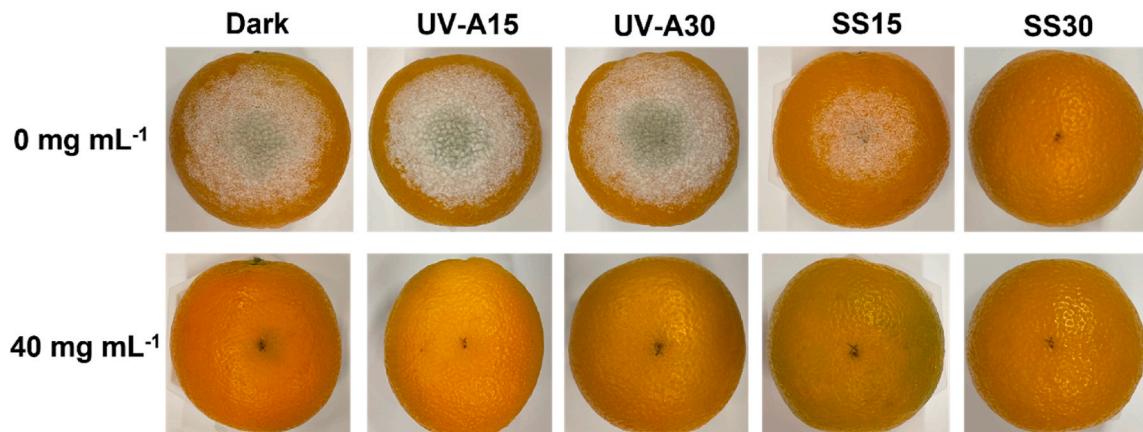


**Fig. 4.** Effect of menadione sodium bisulfite (MSB) on the radial growth of *P. digitatum* 3189 mycelium after different light exposures. The growth of *P. digitatum* mycelium after MSB × light treatments was followed for 7 days at ambient temperature in dark. (A) Images of *P. digitatum* mycelium (colony morphology) during the 7-day dark incubation after treatments. (B) Percentage inhibition of mycelial growth of *P. digitatum* after treatments. Radial mycelium growth was measured after 7-d incubation in dark at room temperature. Data are presented as means ( $n = 6$ )  $\pm$  standard deviations. Different lowercases indicate significant differences between different treatment groups ( $p < 0.05$ ). MGI % represents the percentage of inhibition of mycelial growth. “UV-A15” and “UV-A30” represent UV-A treatment for 15 and 30 min, respectively. “SS15” and “SS30” represent simulated sunlight treatment for 15 and 30 min, respectively.

improved inactivation of *P. digitatum* spores (Fig. 3). Similar results were reported by Zhang et al. (2021). A 5-log-reduction of the *Listeria innocua* population was achieved after 60 min of UV-A irradiation in the presence of 2 mM MSB, whereas the UV-A or MSB treatments alone showed little to no inactivation effect. In the same study, the ROS production

from MSB in various solvent system was determined and both UV-A and UV-B irradiation were shown to be effective in generating ROS during the photoreaction of MSB. Therefore, based on the observations from both Zhang et al.’s study and the current study, the ROS produced by photo-activated MSB were probably the main contributors to the

(A)



(B)

Light type	Irradiation time (min)	MSB conc. (mg mL⁻¹)	Disease incidence (%)	Severity (mm)
Dark	/	0	100	74.7 ± 4.37 <sup>1</sup> a <sup>2</sup>
Dark	/	40	17	41.0 ± 26.87 c
UV-A	15	0	100	75.5 ± 6.63 a
UV-A	15	40	33	32.8 ± 14.69 c
UV-A	30	0	100	74.0 ± 5.53 a
UV-A	30	40	33	29.8 ± 12.45 c
SS	15	0	50	56.1 ± 19.34 b
SS	15	40	8	69.7 ± 0.00 ab
SS	30	0	0	0.0 d
SS	30	40	0	0.0 d

1. The average rotting diameters are calculated based on fruits with signs of spoilage.

2. Different letters represent significant different in the column ( $p < 0.05$ ).

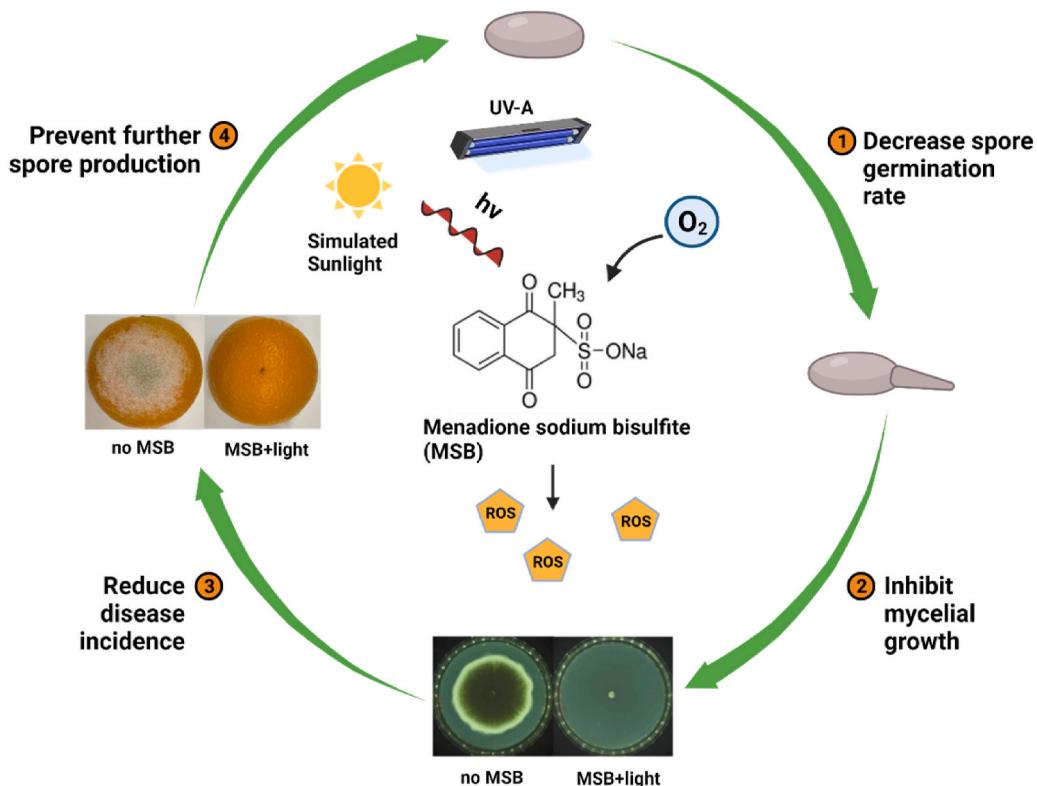
**Fig. 5.** The control of orange decay caused by *P. digitatum* 3189 by MSB in combined with different light treatments. (A) Representative pictures of oranges showing disease signs during storage after treatments. (B) Calculated disease incidence and severity of infected oranges after treatments during storage. Six oranges were used per treatment. The experiment was performed twice. The average lesion diameters were calculated based on fruits with signs of spoilage. The calculated data are presented as mean ( $n = 12$ ) ± standard deviations. Different lowercases indicate significant differences between different treatment groups ( $p < 0.05$ ). “UV-A15” and “UV-A30” represent UV-A treatment for 15 and 30 min, respectively. “SS15” and “SS30” represent simulated sunlight treatment for 15 and 30 min, respectively. Dark represents that MSB was applied but no light exposure was given.

observed fungicidal effects observed in the current study instead of the direct absorption of UV-A or UV-B photons by fungal cellular biomolecules or the toxicity of MSB. In support of our findings, Olmedo et al. (2017) reported that the combination of 30-min UV-A (365 nm) and 0.5 mM of harmol reduced the viability of *P. digitatum* to 1.00%, while UV-A alone had no impact on spore viability and harmol alone reduced spore viability to 8.30%. Similarly, the viability of *B. cinerea* spores was reduced to 63.6% with 0.1 mM of harmol, while the combination of the same concentration of harmol and 30 min of UV-A lowered the spore viability to 6.8%. In the experiment conducted by Gonzales et al. (2017), solar irradiation did not lead to significant reduction in the viability of *C. abscissum* spores, while the 30 min solar irradiation applied with 50  $\mu$ M methylene blue caused a 1.9–3.1 log-reduction, determined by plating. Results obtained from flow cytometry showed that there was no difference in the viability among untreated spores, spores treated with 50  $\mu$ M methylene blue only, and spores treated with 30-min solar irradiation only, while almost 100% of spores in the methylene blue + solar-treated samples showed plasma membrane damage (Gonzales et al., 2017).

Under favorable conditions, germinated spores form hyphal threads, and the extension of the hyphae ultimately results in the formation and

growth of mycelia (Sephton-Clark & Voelz, 2018). MSB alone at a concentration of 40 mg mL⁻¹ fully repressed radial growth of the *P. digitatum* mycelium. With the addition of 15-min UV-A or SS, the concentration required to completely inhibit the mycelium growth decreased to 20 mg mL⁻¹. Similarly, it was reported by Dibona-Villanueva and Fuentealba (2021) that visible light irradiation effectively potentiated the fungicidal ability of chitosan-riboflavin conjugate (CH-RF) against *P. digitatum* mycelia. Although CH-RF presented some fungicidal effects in the dark, the inhibition of mycelial growth reached almost 100% in the presence of light. In another study conducted by Ambrosini et al. (2020), the efficacy of tetra-4-sulfonatophenyl porphyrin tetra-ammonium (TPPS) against the growth of mycelium plugs of *B. cinerea* was tested using white light. The mycelium discs pretreated with 50  $\mu$ M ( $\approx$ 0.008 mg mL⁻¹) TPPS and exposed to at least 72 h of white light were unable to infect the detached leaves of grapevines, contrary to the untreated control, which caused rotting on the leaf samples.

*In vitro* studies, the efficacy of MSB was enhanced by light irradiation in general. In MSB + SS15 treated fruit, the disease incidence decreased from 100% to 0.08%. Increasing the light exposure time to 30 min completely repressed the growth of the *P. digitatum* during a five-day



**Fig. 6.** The overall scheme of the antimicrobial effects of water soluble VK3 (MSB) on *P. digitatum* 3189. MSB controls the fungal spoilage by 1, decreasing the spore germination rates; 2, inhibiting the mycelial growth; 3, reducing the disease incidences in fruits; and 4, preventing further spore production and spreading. This figure was created with BioRender.com.

storage period. One thing to point out is that SS30 alone also completely controlled the decay of inoculated oranges. This could be due to interactions between compounds present in the orange peel after light treatment or induced responses of the fruit to the SS treatment. It has been reported that the UV-B radiation present in the SS could lead to the accumulation of phenylpropanoids in the epithelial cells of quinoa cotyledons and lemon peels (Hilal et al., 2004; Ruiz et al., 2016). Phenylpropanoid flavones (PMFs) act as plant defense elicitors and contribute to cell-wall thickening (Lattanzio et al., 2012). In addition, some PMFs such as nobiletin isolated from *C. paradisi* and *C. sinensis* fruits possess antifungal properties themselves and were proved to be effective in inhibiting the mycelial growth of *P. digitatum* on PDA plates (Ortuño et al., 2006). Ortuño et al. (2011) compared the degree of fungal development on artificially inoculated *Citrus* fruits and concluded that the susceptibility of different *Citrus* species to *P. digitatum* infection was inversely proportional to the level of PMF accumulation inside the fruits. The major PMFs found in citrus fruit are tangeretin, sinensetin, and heptamethoxyflavone (Nogata et al., 2006), which are able to induce natural resistance against *P. digitatum* infection in oranges (Ballester et al., 2010; Kim et al., 2011; Ortuño et al., 2006, 2011).

Traditionally, fungicides are applied to citrus fruit through immersion or drenching (spraying) (Erasmus et al., 2011). Another application method is through waxing with the benefits of simplifying packinghouse operations and controlling sporulation (Altieri et al., 2013; Smilanick et al., 1997). Therefore, MSB can be potentially used as a novel wax supplement, and it does not add any additional steps to existing processing lines. Most importantly, such application approach can better utilize the light energies during the display and storage of fruits at the grocery stores as well as in consumers' kitchens. Currently, although MSB is considered as a low toxic compound in general, there is insufficient data to establish a safe upper limit for vitamin K in human (EFSA Panel on Additives Products or Substances used in Animal Feed, 2014). Additional research on consumer safety exposure assessment,

interactions between MSB and commercial wax components, and the interactions between MSB, light, and innate fruit immunity will be helpful for developing long-lasting strategies to protect fruit against other postharvest pathogens.

## 5. Conclusion

In summary, this study demonstrated that MSB mediated photodynamic technology has great antifungal activity against the fungicide-resistant *P. digitatum* isolate 3189. The synergistic effect of MSB and light irradiation effectively suppressed spore germination, inactivated spores, inhibited mycelial growth *in vitro*, and successfully protected oranges from fungal infections. Due to its low cost and low toxicity, MSB has a great potential to be used as a green biocide for controlling post-harvest fungal decay in the produce industry. This study serves as the foundation for future research on how to incorporate MSB into the current fruit processing line for achieving protection against fungal decay during storage, transportation, and retailing, thus ensuring fruit quality and safety.

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## CRediT authorship contribution statement

**Xiran Li:** conducted the experiments, prepared the manuscript. **Lina Sheng:** conducted the experiments, prepared the manuscript. **Adrian Oscar Sbodio:** conducted the experiments. **Zheng Zhang:** conducted the experiments. **Gang Sun:** reviewed and edited the manuscript. **Bárbara Blanco-Ulate:** developed the concept and experimental plan and, Funding acquisition, reviewed and edited the manuscript. **Luxin**

**Wang:** developed the concept and experimental plan and, Funding acquisition, reviewed and edited the manuscript. All authors read and approved the final draft.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2021.108807>.

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