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Combined treatment with *Rhodosporidium paludigenum* and ammonium molybdate for the management of green mold in satsuma mandarin (*Citrus unshiu* Marc.)

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ABSTRACT

This study evaluated the effectiveness of the combined treatment of ammonium molybdate and *Rhodosporidium paludigenum*, a yeast species with broad-spectrum antifungal effects, in controlling green mold caused by *Penicillium digitatum* in satsuma mandarin (*Citrus unshiu* Marc.). The addition of 0.1 mmol L^{-1} ammonium molybdate markedly improved the biological activity of *R. paludigenum* against green mold and decreased disease incidence by 89.3%, and simultaneously discontinued mold development within 0–12 h of infection. Although treatment with ammonium molybdate alone did not effectively reduce the incidence of green mold, treatment with high doses of ammonium molybdate attenuated disease severity. The use of *R. paludigenum* markedly reduced the dose of ammonium molybdate required to control green mold. Ammonium molybdate significantly depressed the ecto-phosphatase activity of *P. digitatum*, as well as interrupted the environmental acidification caused by this pathogen. These results suggested that ammonium molybdate contributes to the control of green mold infection by suppressing *P. digitatum* spore germination and proton-pump activity on the surface of *P. digitatum* membranes. Moreover, the results indicated that ammonium molybdate may be utilized as an environmentally-friendly additive that can enhance the performance of *R. paludigenum* against green mold rot in satsuma mandarin fruit.

1. Introduction

Green mold caused by *Penicillium digitatum* (Pers.: Fr.) is the leading cause of the postharvest rot of satsuma mandarin (*Citrus unshiu* Marc.), consequently resulting in severe economic losses to the global citrus industry (Palou, 2014; Louw and Korsten, 2014). *P. digitatum* has a relatively short disease cycle (3–5 days at 25 °C) and can produce 1–2 billion conidia on a single fruit; these conidia are efficiently dispersed via water or air currents (Holmes and Eckert, 1999). Dormant *Penicillium* spores on the surface of satsuma mandarin become active when the peel is injured (Droby et al., 2008). Although satsuma mandarin oranges have thick peels, minor bruising or damage may cause severe injury that may not be apparent during a typical cursory visual inspection. The presence of a single rotten fruit in a shipment box considerably increases the risk of mold infection during transportation or shipment (Tashiro and Nita, 2017).

The pathogenicity of *P. digitatum* may rely on the acidification of an infected fruit (Macarisin et al., 2007). During fruit decay, the pathogen acidifies the ambient environment of the fruit cytoplasm by producing organic acids, mainly citric and gluconic acids, and by sequestering ammonium ions in the cytoplasm. Low pH may facilitate the regulation of various gene-encoded pathogenic factors, such as polygalacturonase (Prusky et al., 2004). Ecto-phosphatases are surface-membrane-bound proteins with catalytic sites that face the extracellular environment and are likely among the first pathogenic proteins that come in contact with host cells (Gomes et al., 2011). Once activated, these enzymes hydrolyze phosphorylated substrates in the extracellular environment. The balance between the antagonistic activities of protein kinases and phosphatases is responsible for numerous cellular functions, including proliferation, differentiation, adhesion, virulence, and infection

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(Cohen, 2002). Biochemical characterization has revealed that these activities are differentially modulated by classical phosphatase inhibitors, divalent metals, and pH (Freitas-Mesquita and Meyer-Fernandes, 2014).

Biological control through the use microbial antagonists is an environmentally sound and effective means of reducing the incidence and severity of postharvest diseases. Microbial antagonists are either used alone or as a component of an integrated control strategy with low fungicide input (Lahlali et al., 2014). *Rhodosporidium paludigenum* Fell & Tallman, an antagonistic yeast originally isolated from the Southeast China Sea, inhibits the development of various postharvest fungal diseases of fruit (Wang et al., 2008, 2009; Lu et al., 2013, 2014; Zhu et al., 2015). Marine yeasts possess several unique and promising features, such as high tolerance for low-temperature and osmotic stresses; that are mediated by specific chemical adaptations; thus, marine yeasts have potential applications as adjuvants in the refrigerated transport of fruits (Wang et al., 2010, 2014). However, the single application of *R. paludigenum* is insufficient for the control of postharvest rot, particularly during the latter stage of infection after spore germination.

Ammonium molybdate is a molybdenum salt tetrahydrate that is used as a controlled-release biological fertilizer, foliar spray, and insecticide. It is also used to prevent and treat pest-related diseases. It exhibits superior effects in the control of the postharvest rot of cherry (Qin et al., 2006), peach (Cao et al., 2010), apple (Nunes et al., 2001), and citrus (Palou et al., 2002). Information on its mechanisms of action, however, remains limited. Thus, the present study examines the efficacy of a mixed application of *R. paludigenum* and ammonium molybdate against green mold on a laboratory scale model system, and the effect of ammonium molybdate on the in vivo population growth of *R. paludigenum,* in vitro spore germination as well as the ecto-phosphatase activity of *P. digitatum*.

2. Material and methods

2.1. Fruit sample collection

Satsuma mandarin fruits in the yellow mature stage and without mechanical wounding or damage were hand-picked from an experimental orchard in Chun'an City (Zhejiang Province, China) and directly transported to the Food Biotechnology Laboratory of Zhejiang University. The fruits were soaked in 0.1% aqueous solution of sodium hypochlorite for 1–2 min for surface disinfection, rinsed thrice with tap water, and dried at room temperature (20 °C–25 °C).

2.2. Chemical products

Ammonium molybdate [$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (PubChem CID: 16211167)], sodium molybdate [$Na_2MoO_4{\cdot}2H_2O$ (PubChem CID: 16211258)], and ammonium chloride [NH_4Cl (PubChem CID: 25517)] were purchased from Sigma-Aldrich.

2.3. Biocontrol agent and fungal cultures

The isolate of *R. paludigenum* Fell & Tallman used in this experiment was identified by CABI Bioscience Identification Services (IMI 394084). Yeast cells were incubated in 250 mL Erlenmeyer flasks containing 50 mL of nutrient yeast dextrose broth medium (NYDB), 8 g of nutrient broth, 5 g of yeast extract, and 10 g of glucose in 1 L of distilled water) on a rotary shaker (3.3 s^{-1}) for 36 h at 28 °C. Yeast cells were collected by centrifugation at 50 s^{-1} for 15 min and resuspended twice in sterile water. The final cell suspension concentration was adjusted to 1×10^7 cells mL^{-1}.

P. digitatum strains were isolated from mold-infected citrus fruit and cultured on potato dextrose agar (PDA) at 4 °C in the dark. A spore suspension was prepared by flooding 7-day-old sporulating cultures with sterile distilled water. Spore concentrations were determined through microscopic counting using a hemocytometer and adjusted to 1×10^4 spore mL^{-1}. The spore suspension used for inoculation was freshly prepared to ensure viability.

2.4. Treatments with ammonium molybdate and antagonistic yeast

2.4.1. Preventive activity of ammonium molybdate and disease suppression activity of antagonistic R. paludigenum against green mold in satsuma mandarin

Satsuma mandarin fruits were wounded (2 mm in depth and 5 mm in diameter) using a sterile borer as previously described (Lu et al., 2013). Each fruit was treated with 30 µL of one of following agents: (1) sterile distilled water as the control; (2) different concentrations of ammonium molybdate solution (0.01, 0.1, 1, or 5 mmol L^{-1}); (3) cell suspension of *R. paludigenum* (1×10^7 cells mL^{-1}); or (4) cell suspension of *R. paludigenum* (1×10^7 cells mL^{-1}) in combination with the above concentrations of ammonium molybdate solutions. Two hours after inoculation, each wound was inoculated with 20 µL of *P. digitatum* spore suspension (1×10^4 spore mL^{-1}). Each treatment was performed twice with three replications, with 12 fruits per each replicate. The percentage of infected wounds and average diameters of lesions were measured at 60, 72, and 84 h after storage at 90%–95% (relative humidity) and 25 °C.

2.4.2. Synergistic activity of ammonium molybdate and R. paludigenum in the suppression of green mold in satsuma mandarin

To determine the synergistic activity of ammonium molybdate and antagonistic *R. paludigenum* in the inhibition of green mold, each wound was pretreated with 20 µL of *P. digitatum* or *P. italicum* spore suspension at 1×10^4 spore mL^{-1}. At 0, 6, and 12 h after treatment, 30 µL of one of four following agents was applied: (1) sterile distilled water as the control; (2) cell suspension of *R. paludigenum* (1×10^7 cells mL^{-1}); (3) ammonium molybdate solution (0.1 mmol L^{-1}); (4) or cell suspension of *R. paludigenum* combined with ammonium molybdate solution (0.1 mmol L^{-1}). Each treatment was performed twice with three replications, with 12 fruits per each replicate. The percentage of infected wounds and average diameters of lesions were determined 3 days after inoculation.

2.4.3. Effect of ammonium molybdate on the in vitro spore germination of P. digitatum and the in vivo population growth of R. paludigenum

The effect of ammonium molybdate on the spore growth of *P. digitatum* was evaluated. Conidial suspensions were mixed with 0.1, 1, or 5 mmol L^{-1} ammonium molybdate solution in PDB medium. Sodium molybdate (7 mmol L^{-1}), ammonium chloride (18.7 mmol L^{-1}), and imazalil (20 mmol L^{-1}) at the recommended concentration for standard postharvest treatments were used as negative controls. Spore growth was evaluated under a microscope (BX-60, OLYMPUS) following 18 h of incubation at 25 °C. Each treatment was performed with three replicates.

To determine the effect of ammonium molybdate on the colonization ability of *R. paludigenum*, the population dynamics of this yeast in the surface wounds of satsuma mandarin fruits was investigated. Peel tissue samples were collected from fruit at 0, 24, 48, 72, and 96 h after inoculation using a cork borer. Cylinders of excised tissue (2 mm deep × 1 cm wide) were acquired from three fruits, placed in a mortar with 15 mL of sterile distilled water and ground with a pestle. Serial 10-fold dilutions were subsequently prepared and sprayed on glass plates containing nutrient yeast dextrose agar (20 g of agar in 1 L of NYDB). Yeast colonies were counted after 2 days of inoculation at 25 °C.

2.4.4. Ecto-phosphatase and acidification activities of P. digitatum

To determine the effect of inorganic salts on the ecto-phosphatase activity of *P. digitatum*, *P. digitatum* spores were inoculated at the rate of 1×10^5 spores mL^{-1} in 2.5 mL of a mixture containing 116.0 mmol L^{-1} NaCl; 5.4 mmol L^{-1} KCl; 5.5 mmol L^{-1} D-glucose; 50.0 mmol L^{-1} PBS