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Antifungal Activity of Hypothemycin against *Peronophythora Litchii* In Vitro And In Vivo

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ABSTRACT: The antifungal activity of a natural resorcylic acid lactone, hypothemycin (HPM), against *Peronophythora litchii* in vitro and in vivo was investigated. HPM treatment substantially suppressed spore germination of *P. litchi*, with the inhibition rate of 100% when 0.78 μg/mL HPM was applied. Similarly, mycelial growth of *P. litchii* was efficiently inhibited. Furthermore, HPM caused the ultrastructural modifications of *P. litchii*, including the disruption of the cell wall and the endomembrane system, especially the plasma membrane, mitochondria, and vacuoles, which led to the destruction of the cellular integrity. Moreover, application of HPM significantly reduced decay and suppressed peel browning of postharvest litchi fruit inoculated with *P. litchii* during storage at 28 °C. Overall, these findings suggested that HPM exhibited excellent antifungal activity against *P. litchii* both in vitro and in vivo, which could be helpful for the storage of harvest litchi fruit.

KEYWORDS: *Peronophythora litchii, β-resorcylic acid lactones, hypothemycin, antifungal activity*

INTRODUCTION

Litchi (*Litchi chinensis* Sonn.) is one of the important fruit crops of Southern China. However, the fruit deteriorate quickly after harvest because of peel browning and decay development.[1,2] *Peronophythora litchii* is the major fungus causing the decay of harvested litchi fruit.[2] Currently, chemicals such as sulfur dioxide and benomyl are successfully used to control the disease of postharvest litchi.[2] However, considering pathogen resistance, carcinogenic risk, and environmental pollution, some alternative means to control the decay of postharvest litchi are required.[2]

Microorganisms are rich in bioactive secondary metabolites, some of which may have antifungal, antibacterial, antioxidant, and anticarcinogenic properties.[3−5] Resorcylic acid lactones (RALs) are secondary metabolites produced in some fungal species, with a variety of biological activities.[6] Hypothemycin (HPM, Figure 1), an RAL containing a *cis*-enone moiety, was originally

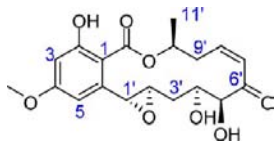

Figure 1. Structure of hypothemycin (HPM).

isolated from the metabolites of *Hypomyces trichothecoides*.[7] Research results have shown that HPM could inhibit cis-transformation and T cell activation.[8−10] Furthermore, Fukazawa et al. reported HPM controlled cell proliferation via selectively inhibiting the mitogen-activated protein kinase kinase-extracellular signal-regulated kinase (MAPKK/ERK) pathway.[11] Recently, we isolated HPM from *Paecilomyces* sp. SC0924 on solid culture.[12] Preliminary investigations revealed that HMP exhibited antifungal activity against *Peronophythora litchii*, the major postharvest disease in litchi fruit. However, there is currently a deficit of information on the antifungal activity of HPM.

In this study, we have investigated the antifungal activity of HPM against *P. litchii* in vitro, and evaluated the potential of HPM in controlling the postharvest litchi fruit decay caused by *P. litchii*. The antifungal mechanism of HPM against *P. litchii* was first explored.

MATERIALS AND METHODS

Preparation of Hypothemycin. Hypothemycin (HPM) was isolated from the metabolites of *Paecilomyces* sp. SC0924 and identified by nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry (ESI-MS).[13] The fermentation and extraction procedures was conducted according to the method previously reported.[12] The $CHCl_3$-soluble extract was separated on silica gel column and eluted with $CHCl_3$−MeOH mixtures (100:0 to 80:20). The resulting fraction, obtained by eluting with $CHCl_3$−MeOH (95:5) was further purified by silica gel column using petroleum ether-acetone (90:10 to 50:50) as mobile phase. The subfraction obtained from the ether/acetone (75:25) eluent was finally recrystallized from MeOH to obtain HPM (20.0 g).

Fruit Material. Litchi fruit were harvested at commercial maturity from an orchard in Conghua, Guangzhou, P. R. China. The fruit with uniform shape, color, and size, and free of disease were selected for the experiment in vivo.

Fungal Culture. The tested microorganism, *Peronophythora litchii*, was provided by Dr. Ruqian Pan from the Laboratory of Fungicides & Bacteria, South China Agricultural University, Guangzhou, P. R. China. The fungus was revived on potato dextrose agar (PDA) at 28 °C for

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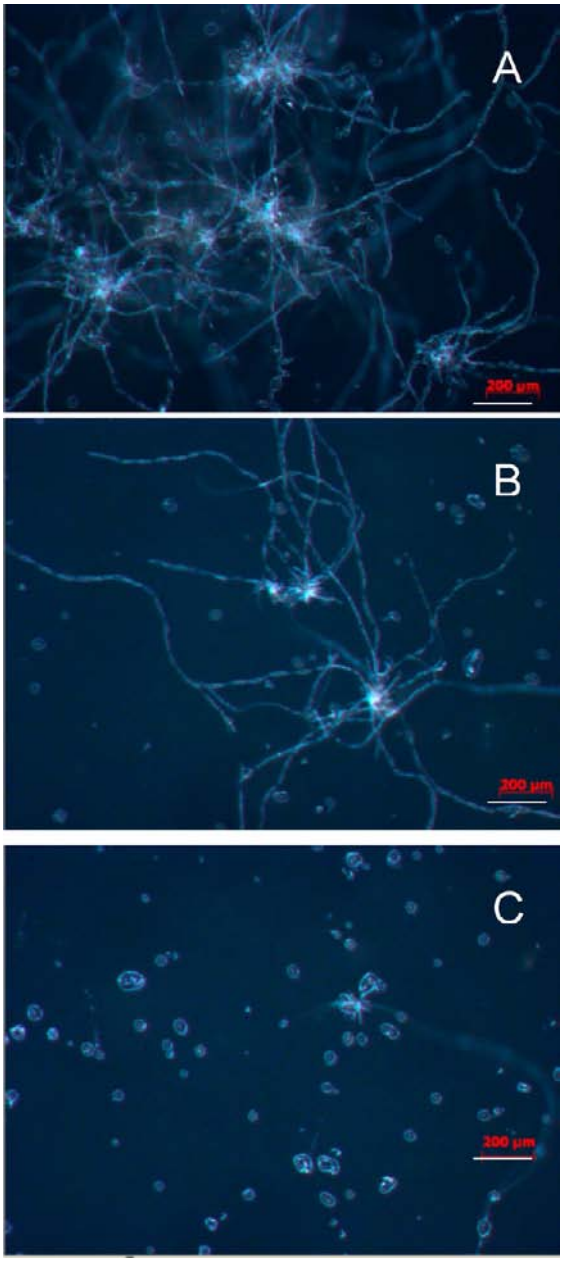

Figure 2. Inhibitory effect of HPM on spore germination of *P. litchi* in vitro. A, Control; B, 0.39 mg/L HPM; and C, 0.78 mg/L HPM.

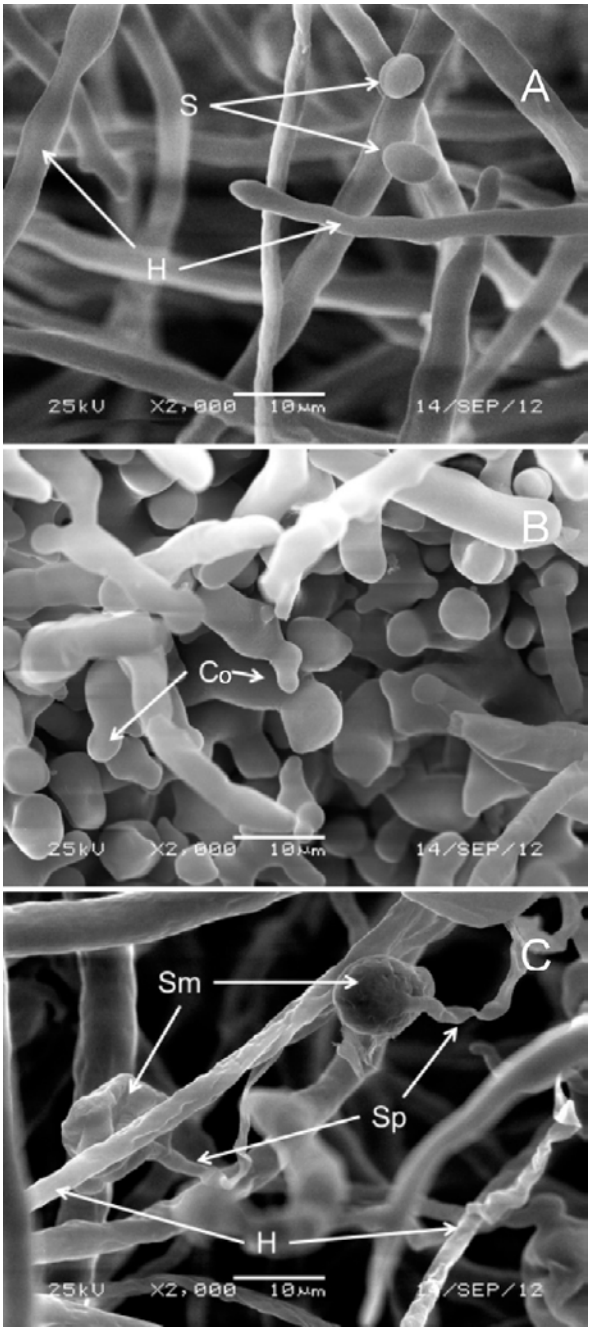

Figure 3. Scanning electron micrographs of the hyphae or sporangia of *P. litchii* grown for 4 days on PDA plates with or without HPM at 28 °C. A and B, control; C, HPM. Co, Conidia; H, hyphae; S, spores; Sm, sporangia; Sp, sporangiophore.

3 days, and cultured in yeast extract, malt extract, glucose (YMG) medium at 28 °C for 2 days to obtain the culture.

Spore Germination Assay. Conidia of *P. litchii* were collected from 4-day-old culture on PDA media at 28 °C in the dark according to the method described by Feng et al.[14] The influence of HPM on spore germination of *P. litchii* was evaluated on PDA. HPM was dissolved and diluted with DMSO. HPM solutions were then added into warm PDA to obtain Petri dishes containing different concentrations of HPM from 0.098 to 200 μg/mL. A 100 μL aliquot of conidia suspension (1×10^6 conidia per mL) of *P. litchii* was spread to each Petri dish. After 2 days of incubation at 28 °C, the plates were observed under light stereoscope (Axioplan 2 imaging, ZEISS) and stereo microscope (Stemisv 11, ZEISS) to count both germinated and nongerminated conidia. Each concentration comprised three duplicates and about 150 spores per duplicate were observed. The spore germination percentage was calculated.

Mycelial Growth Assay. The inhibitory activity of HPM on mycelia growth of *P. litchii* was evaluated according to Xu et al.[12] Thiram was used as positive control. A 100 μg HPM or thiram disk was applied in per paper disk. The sizes of inhibition zones were recorded to assess the inhibitive effect of HPM on *P. litchii* mycelia growth.

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). SEM and TEM observations of the hyphae or conidia of *P. litchii* were conducted according to the method described by Feng et al.[14]

Inoculation with *P. litchii* and Treatment with HPM. Litchi fruit were pretreated according to the method described by Feng et al.[14] The fruit were then infiltrated in 100, 200, and 300 μg/mL of HPM in 0.25% ethanol under a pressure of 75 kPa for 3 min, respectively. The fruit infiltrated with 0.25% ethanol without HPM were used as control. A 5-mm wide and 2-mm deep cross-like wound

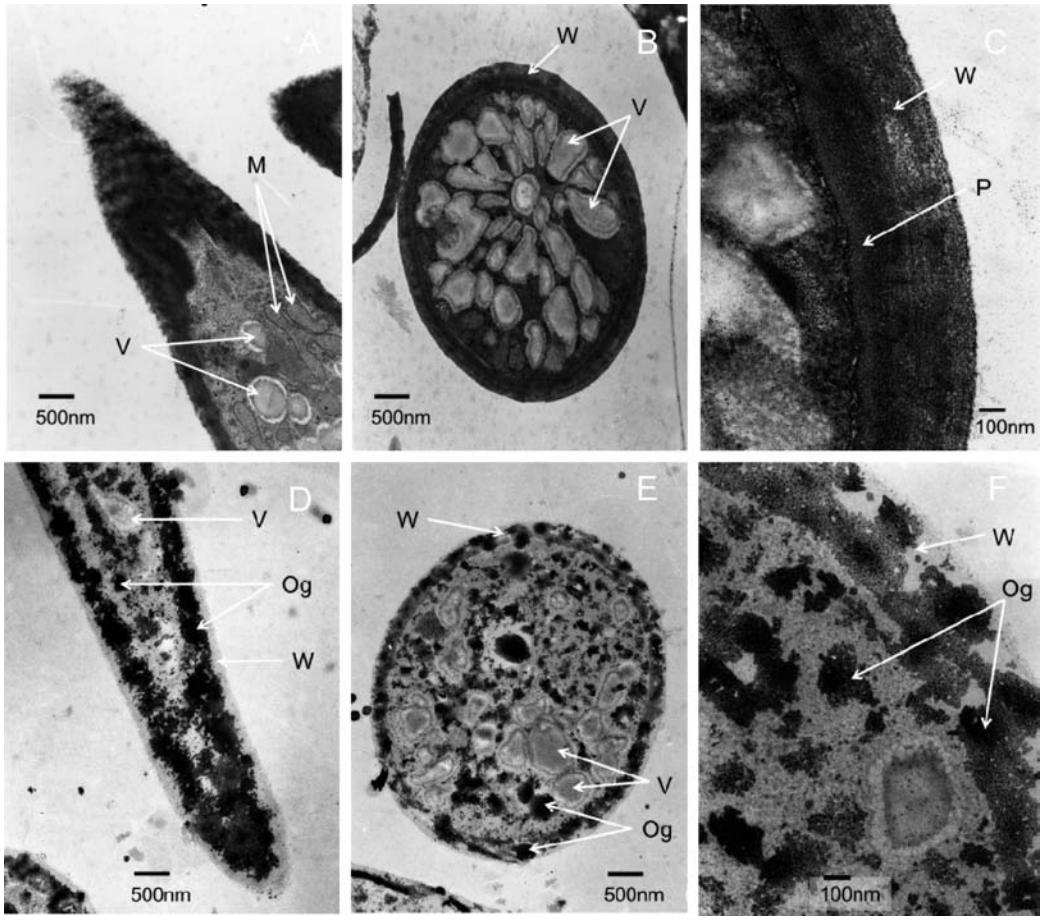

Figure 4. Transmission electron micrographs for the hyphae of *P. litchii* grown for 4 days on PDA plates with or without HPM at 28 °C . A−C, control; D−F, HPM; A and D, longitudinal section through thehyphae of *P. litchii* (×12 K); B and E, tangential section through the hyphae (×12 K); and C,F, cell wall and plasma membrane of the hyphae (×50 K). M, mitochondria; Og, osmiophilic globuli; P, plasma membrane; V, vacuoles; and W, cell wall.

was cut on the equatorial area of each fruit peel with a sterile blade. Finally, 10 μL of spore suspension of *P. litchii* (1×10^6 spores/ml) was added into the wound with a pipet. Each concentration comprised three duplicates and 72 fruit were used for each duplicate. Following package with 0.02 mm polyethylene plastic bags, the fruit were stored at 28 °C.

Assessment of Fruit Decay and Peel Browning. Decay development was monitored on 24 fruit each duplicate. The extent of decay was evaluated according to the decayed area on the peel: 1 = no decay; 2 = slight decay; 3 = 25% decay; 4 = 25%−50% decay; 5 ≥ 50% decay. The decay index was calculated with the following formula: Σ(decay scale × proportion of fruit corresponding to each scale)/5 × 100%. The extent of peel browning was evaluated and expressed as browning index according to the method described by Duan et al.[15]

Statistical Analysis. Data were expressed as mean ± standard error. Differences among different treatments were analyzed using SPSS version 7.5 at the 5% level.

RESULTS

Inhibition of Spore Germination. HPM inhibited spore germination of *P. litchii*. At 0.39 μg/mL, the germination of about 90% spore was inhibited by HPM, while application of 0.78 μg/mL HPM completely inhibited spore germination (Figure 2).

Similarly, the mycelia growth of *P. litchii* was suppressed by HPM. After 3 days of incubation, the inhibition zones in the medium containing HPM and thiram, the positive control, were 15 mm and 20 mm, respectively, indicating that the suppressive efficiency of HPM on mycelia growth of *P. litchii* was close to that of thiram.

Changes in the Ultrastructure of *P. litchii*. The control mycelia showed a regular and linear shape, and the surfaces of both mycelia and growing apexes were smooth (Figure 3A). Moreover, the apical cells of the hyphae swelled to form plenty of conidia (Figure 3B). However, HPM resulted in the hyphae deformed and the formation of evident craters on the surface (Figure 3C).

TEM observation showed that the cell wall and the plasma membrane of untreated hyphae of *P. litchii* was intact and unfolded with a uniform shape (Figure 4A−C). The mitochondria and vacuoles also appeared normal morphology. Application of HPM resulted in the damage of cell wall and plasma membranes (Figure 4E,F). The membrane of some organelles also seemed to be blurred (Figure 4D,E). Moreover, a strong electron density was observed in the hyphae treated with HPM (Figure 3D,F).

Efficacy of HPM for Reducing Litchi Disease Development in Artificially Inoculated *P. litchii*. The decay index in the litchi fruit inoculated with *P. litchii* rapidly increased during storage at 28 °C. HPM treatment significantly inhibited the increase of decay index. After 3 days and 5 days of storage, the decay indices of fruit treated with 300 μg/mL HPM were 15.1% and 51.5%, respectively, while those of the control were

35.5% and 95.9%, respectively, indicating that HPM treatment suppressed the decay development caused by *P. litchii*.

Decay is one of the major factors leading to peel browning of postharvest litchi fruit. Application of HPM significantly reduced peel browning. After storage for 5 days, browning indices of control and fruit treated with 300 μg/mL HPM were 5.0 and 3.1, respectively (Figure 5B), which were consistent with the inhibitory effect of HPM on the decay of postharvest litchi fruit.

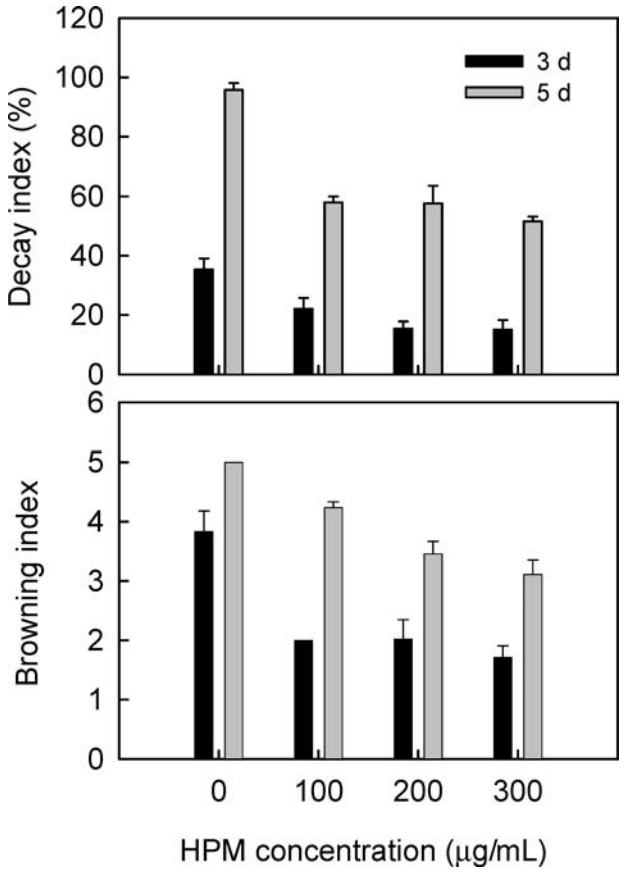

Figure 5. Effect of different concentrations of HPM on decay index (A) and browning index (B) of harvested litchi fruit inoculated with *P. litchii* after 3 and 5 days of storage at 28 °C. Each value represents a mean + standard error (n = 3).

DISCUSSION

Fruit decay is the major factor limiting the storage life of postharvest litchi fruit. The fruit are susceptible to postharvest molds, especially *Peronophythora litchii*.[2] Some chemical fungicides can control efficiently postharvest diseases of the fruit.[2] However, the safety issue and increased resistance to the fungi limit their use. Our study showed that HPM treatment effectively suppressed the rot of postharvest litchi fruit caused by *P. litchii*. Moreover, peel browning was also immensely reduced by HPM treatment. Until now, there was no report of the inhibitory effect of HPM on postharvest decay. In this study, HPM showed potential to control the decay caused by *P. litchii* in litchi fruit as an alternative.

The presence of the *cis*-enone moiety within the macrolactone endues HPM with a variety of biological functions.[6] Currently, the studies on HPM function are mainly focused on the antitumor activity.[8−11] Tanaka et al. found that daily HPM treatment was effective in inhibiting the tumor growth.[10] Moreover, Isaka et al. reported that HPM from *Aigialus parvus* BCC 5311 exhibited strong antimalarial activity in vitro, with the IC_{50} of 2.2 mg/mL.[16] However, few studies on effects of HPM on fungi, especially those involved in the decay of harvested horticultural products, were reported. Our study showed that HPM considerably suppressed spore germination and mycelia growth of *P. litchii* of litchi fruit in vitro. A concentration of 0.78 μg/mL HPM resulted in the entire inhibition of spore germination, indicating that the inhibition of HPM on *P. litchii* was effective.

The antitumor activity of HPM could be associated with its ability to irreversibly inhibit certain protein kinase.[11] The *cis*-enone moiety of HPM targets the cysteine residues of protein kinase to form stable Michael addition. Moreover, Tanaka et al. reported that HPM reduced the expression of Ras-inducible genes as a signaling inhibitor.[10] In the present study, SEM images showed that HPM treatments caused distortion and concave collapses of the hypha of *P. litchii*. Furthermore, the cellular wall, plasma membrane, and endomembrane of the spore of *P. litchii* on PDA-containing HPM broke down severely, resulting in the loss of the integrity of the cell. Therefore, these findings suggest that HPM inhibited spore germination and mycelium growth of *P. litchii* by disrupting the cell wall and plasma membrane.

In conclusion, HPM showed excellent antifungal activity against *P. litchii*. HPM inhibited the mycelia growth of *P. litchii* and resulted in deleterious morphological modifications in vitro. Moreover, HPM treatment efficiently reduced the decay of postharvest litchi fruit caused by *P. litchii*. However, the effects of HPM on the decay of different varieties of litchi fruits require further investigations for commercial application.

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Notes

The authors declare no competing financial interest.

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