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Effect of pyrimethanil on *Cryptococcus laurentii*, *Rhodosporidium paludigenum*, and *Rhodotorula glutinis* biocontrol of *Penicillium expansum* infection in pear fruit

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ABSTRACT

The effect of biocontrol yeasts and pyrimethanil at low concentration on inhibition of blue mold rot caused by *Penicillium expansum* in pear fruit was investigated. Pyrimethanil at low concentration (40 μg/mL) alone had little inhibitory activity against the *P. expansum* infection in pear fruit wounds although it was effective in inhibiting the survival of *P. expansum* on Asp-agar medium. Pyrimethanil at this low concentration significantly enhanced the efficacy of *Cryptococcus laurentii* at 1×10^7 CFU/mL in reducing blue mold rot *in vivo* compared with *C. laurentii* at 1×10^7 CFU/mL alone. However, there was no additive inhibitory activity when pyrimethanil was combined for application with biocontrol yeasts *Rhodosporidium paludigenum* or *Rhodotorula glutinis*. Combination of pyrimethanil and *C. laurentii* at low concentration also inhibited blue mold rot when *P. expansum* was inoculated into fruit wounds 12 h before treatment and fruit was stored at low temperature (4 °C). Pyrimethanil at 0.04 to 400 μg/mL did not influence the survival of *C. laurentii in vitro*, and it only slightly reduced the population growth of *C. laurentii* after 48 h of incubation in the pear fruit wounds. There was no significant difference in quality parameters including total soluble solids, titratable acidity and ascorbic acid of pear fruit wounds among all treatments after 5 days of treatment at 25 °C. Integration of *C. laurentii* and pyrimethanil at low concentration might be an effective and safe strategy to control *P. expansum* infection in pear fruit, especially in an integrated postharvest disease management strategy.

1. Introduction

Blue mold rot caused by *Penicillium expansum* Link is one of the major postharvest diseases of pear fruit world-wide (Rosenberger, 1990). Fungicide application is the most effective method to control postharvest mold rots of pear (Eckert and Sommer, 1967) and other fruit (Forster et al., 2007; Hao et al., 2010; Koh et al., 2005; Prusky et al., 2006). However, postharvest treatments with fungicides are increasingly being limited because of environmental and toxicological risks as well as the onset of fungicide-resistant strains of fungal pathogens (Lima et al., 2011). Therefore, great efforts have been made to exploit alternatives to synthetic chemicals in the past 20 years (Droby et al., 2009).

Strains of *Cryptococcus laurentii* are biocontrol yeasts which have been widely studied and shown antagonistic activity in reduction of mold rot in pear fruit when applied alone or with various additives, including low dose of the traditional fungicide thiabendazole (Benbow and Sugar, 1999; Lima et al., 1998; Roberts, 1990; Sugar and Spotts, 1999; Yao et al., 2004).

Pyrimethanil belongs to the anilinopyrimidine class of fungicides, which has been registered for postharvest application to pears in the United States since 2004 (Kanetis et al., 2007; Sugar and Basile, 2008, 2011; Xiao and Boal, 2009). The antifungal activity of pyrimethanil *in vitro* could result from a block in the excretion of hydrolytic enzymes involved in the pathogenesis in a site-specific manner and/or from an inhibition of methionine biosynthesis in fungal cells (Leroux and Gredt, 1996). This fungicide is highly effective in inhibiting conidial germination and germ-tube elongation of *P. expansum* (Li and Xiao, 2008a). Currently, pyrimethanil is increasingly being used as an alternative to thiabendazole for control of blue mold and other postharvest diseases in pome fruits, and particularly as a tool for control of blue mold caused by thiabendazole resistant strains of *P. expansum* (Li and Xiao, 2008a). However, to our knowledge, the sensitivity of antagonistic yeasts to pyrimethanil and the impacts of pyrimethanil on the efficacy of antagonistic yeasts have not been explored.

The objective of this study was to investigate the effect of pyrimethanil on inhibition of *P. expansum* and *C. laurentii in vitro* and *in vivo* and the potential of the combined use of pyrimethanil and *C. laurentii* in reducing the postharvest blue mold rots of pear fruit.

2. Materials and methods

2.1. Microorganisms and material

Pear fruit (*Pyrus pyrifolia* Nakai. cultivar "Shuijing") were selected for uniform size, ripeness and absence of mechanical damage. After

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being immersed in a solution of 0.1% sodium hypochlorite (actual concentration of available chlorine ≥ 52 μg/mL) for about 1–2 min, fruit were washed with tap water and were allowed to air dry at the room temperature (approximately 25 °C).

The yeast *C. laurentii* (Kufferath) Skinner was isolated from the surface of pear fruit, *Rhodosporidium paludigenum* Fell & Tallman was originally isolated from the south East China Sea, *Rhodotorula glutinis* (Fresenins) Harrison was obtained from the Institute of Microbiology, Chinese Academy of Science (Beijing, P.R. China), respectively. Previously these yeasts had been reported to have biocontrol activity against postharvest fungal diseases (Wang et al., 2008; Yu et al., 2007; Zheng et al., 2005). The culture of biocontrol yeasts and the preparation of their cell suspensions were carried out as previously reported (Wang et al., 2008; Yu et al., 2007; Zheng et al., 2005). The yeasts were cultured in 250 mL conical flasks containing 50 mL of nutrient yeast dextrose broth (NYDB, containing 8 g nutrient broth, 5 g yeast extract, and 10 g glucose in 1 L of distilled water) medium at 28 °C for 48 h on a rotary shaker at 200 rpm. Yeast cells were centrifuged at 3000 rpm for 10 min and washed twice to remove the growth medium. The yeasts were resuspended with sterile distilled water and adjusted to required concentration.

P. expansum was originally isolated from infected pear fruit and cultured on potato-dextrose agar (PDA) medium (containing the extract from 200 g potato, 20 g glucose and 20 g agar in 1 L of distilled water) at 25 °C in the dark. Spore suspensions were obtained by flooding 7-day-old culture medium of *P. expansum* with sterile distilled water. The number of spores was calculated with the aid of a hemacytometer and the spore concentration was adjusted with sterile distilled water as required.

Pyrimethanil (as Scala, 40%, active ingredient) was obtained from BayerCropScience, China.

2.2. Efficacy of antagonistic yeasts and pyrimethanil in reducing blue mold rot on pear fruit wounds

2.2.1. Effect of different antagonistic yeasts and pyrimethanil on blue mold rot

Wounds were made (5 mm diameter and 3 mm deep) on each pear fruit with the tip of a sterile borer and each wound was treated with 50 μL of one of the following: (1) pyrimethanil at 400 μg/mL of active ingredient (the maximum label rate), pyrimethanil at 40 μg/mL of active ingredient, (2) cell suspensions of *C. laurentii* at 1×10^8 CFU/mL, (3) cell suspensions of *C. laurentii* at 1×10^7 CFU/mL alone or with pyrimethanil in final concentration at 40 μg/mL of active ingredient, (4) cell suspensions of *R. paludigenum* at 1×10^7 CFU/mL alone or with pyrimethanil in final concentration at 40 μg/mL of active ingredient, (5) cell suspensions of *R. glutinis* at 1×10^7 CFU/mL alone or with pyrimethanil in final concentration at 40 μg/mL of active ingredient and (6) sterile distilled water as control. Two hours later, 30 μL of *P. expansum* spore suspension at 1×10^4 spores/mL was inoculated into each wound. The pears were then air dried and stored in the covered plastic trays to maintain a 90% relative humidity at 25 °C. The percentage of infected fruit wounds and the average lesion diameters of the treated fruit were examined daily after inoculation.There were three replicates per treatment with six pears per replicate. The data are from one individual experiment and are representative of two independent experiments with similar results.

2.2.2. Effect of time between biocontrol treatment and pathogen inoculation on blue mold rot

Four wounds were made on each pear fruit as above and inoculated with 30 μL of *P. expansum* spore suspension at 1×10^4 spores/mL. Twelve hours later, each fruit wound was treated with 50 μL of the one of the following: (1) sterile distilled water as the control; (2) pyrimethanil at 40 μg/mL of active ingredient; (3) *C. laurentii* at 1×10^7 CFU/mL; (4) *C. laurentii* at 1×10^7 CFU/mL plus pyrimethanil in final concentration at 40 μg/mL of active ingredient. The pears were then air dried and stored in the covered plastic trays to maintain a 90% relative humidity at 25 °C. The percentage of infected fruit wounds and the average lesion diameters of the treated fruit were examined daily after inoculation. There were three replicates per treatment with six pears per replicate. The data are from one individual experiment and are representative of two independent experiments with similar results.

2.2.3. Effect of C. laurentii and pyrimethanil on blue mold rot at low temperature

Six wounds were made on each pear fruit as above and each wound was treated with 50 μL of one of the following: (1) sterile distilled water as the control; (2) *C. laurentii* at 1×10^8 CFU/mL; (3) pyrimethanil at 400 μg/mL of active ingredient; (4) pyrimethanil at 40 μg/mL of active ingredient; (5) *C. laurentii* at 1×10^7 CFU/mL; (6) *C. laurentii* at 1×10^7 CFU/mL + pyrimethanil in final concentration at 40 μg/mL of active ingredient. Two hours later, 30 μL of *P. expansum* spore suspension at 1×10^4 spores/mL was inoculated into each wound. The pears were then air dried and stored in the covered plastic trays to maintain a high relative humidity at 4 °C. The percentage of infected fruit wounds and the average lesion diameters of the treated fruit were examined daily after inoculation. There were three replicates per treatment with six pears per replicate. The data are from one individual experiment and are representative of two independent experiments with similar results.

In the above three experiments, when the visible rot zone beyond the wound area on each fruit was more than 1 mm wide, it was counted as an infected fruit and lesion diameter included wound diameter. Disease incidence and lesion diameter were measured according to the following formulas:

$$\text{Disease incidence } (\%) = \frac{\Sigma \text{ Numeber of infected pear fruit}}{\text{Total number of treated fruit}} \times 100$$

$$\text{Lesion diameter } (\text{mm}) = \frac{\Sigma \text{ Lesion diameter of infected pear fruit}}{\text{Total number of treated fruit}}$$

2.3. In vitro effect of pyrimethanil on the colonization of P. expansum and C. laurentii

The effect of pyrimethanil on the colonization of *P. expansum* and *C. laurentii* was determined on Anilinopyrimidines (Aps) agar medium (1 g K_2HPO_4 and 1 g $MgSO_4 \cdot 7H_2O$ were each dissolved in 30 mL water (stocks I and II), 0.5 g KC1 and 0.01 g $FeSO_4 \cdot 7H_2O$ were dissolved in 40 mL distilled water (stock III), 2 g Lasparagine and 15 g agar were dissolved in 400 mL water (stock IV) and 22 g glucose$\cdot H_2O$ was dissolved in 490 mL water (stock V)). Stocks I and II were pooled. The precipitate that was formed was dissolved by adding 10 M hydrochloric acid dropwise, and then stock III was added. Again, the formation of a precipitate was observed, which dissolved after adding stock IV and autoclaving at 1.013 bar for 20 min. Stock V was autoclaved separately and then pooled with the rest. The pH values of all components ranged between 6.5 and 7.0. Pyrimethanil was dissolved in acetone before mixing with the agar that was cooled to 50 °C according to the method described by Hilber and Schuepp (1996). Pyrimethanil was added to the Aps-agar medium at the final concentration of 0, 0.04, 0.4, 4, 40 and 400 μg/mL of active ingredient, respectively. The concentration of yeast cells or the pathogen spores was diluted to 1×10^3 yeast CFU/mL or the pathogen spores/mL with sterile distilled water and 100 μL of each suspension was spread on Asp-agar. After the plates were incubated for 48 h–72 h at 28 °C, the colonies per plate were counted and the results are expressed as the mean number of colony forming units (CFU) per plate. Each treatment was replicated three times with three plates per replicate. The data are from