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Growth inhibitory properties of *Bacillus subtilis* strains and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of citrus fruit

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Abstract

Twenty three strains of *Bacillus* spp. screened from 205 *Bacillus* spp. isolated from soil, showed antagonistic activities *in vitro* towards the *Penicillium digitatum* pathogen, a cause of citrus fruit rot disease. Culture supernatants from nine strains caused >80% inhibition of *P. digitatum* growth when they were serially diluted to 1:32. Volatile compounds produced by these strains also caused 30–70% inhibition of fungal growth. An ethanol extract from a *Bacillus subtilis* 155 cell-free supernatant referred to as secondary metabolites (SMs) produced the best inhibitory effect on mycelial growth and spore germination of the fungus with EC_{50} values of 77.26 and 82.10 $\mu g m L^{-1}$, respectively. Inhibitory compounds, separated from the SMs by preparative thin-layer chromatography ($CHCl_3$/MeOH/H_2O: 65/25/4, v/v/v), had R_f values of 0.14, 0.28, 0.31, 0.49, and 0.64 with EC_{50} values of 95.73, 14.07, 15.19, 108.59, and 99.98 $\mu g mL^{-1}$, respectively. Protein precipitated with 80% saturated ammonium sulphate, from the culture supernatant, had an EC_{50} of 288 $\mu g mL^{-1}$. After native polyacrylamide gel electrophoresis of this protein the antifungal protein activity was detected only in the lowest band. Inoculation of a suspension of *P. digitatum* conidia (10^4 conidia mL^{-1}) onto wounded citrus fruit induced disease symptoms at day 3 and decay at day 5. Inoculation with 20 μL of a 10^8 CFU mL^{-1} *B. subtilis* endospore suspension 24 h prior to fungal spore inoculation decreased disease incidences by 86.7%, and disease symptoms were delayed by 6 days and decay symptoms to day 9. Addition of the SMs (10 mg mL^{-1}), simultaneously with the fungus decreased disease incidence by 72.5%, delayed disease symptoms up to 5 days after inoculation, and no sign of decay was observed up to 9 days. The average lesion diameters observed from treatments with bacterial endospores, SMs and a control fungicide, imazalil were significantly different from the size of the wounds in the control set treated only with fungal conidia. *B. subtilis* 155 and its antibiotics are considered to be potent biological control agents to suppress growth of *P. digitatum* in the postharvest protection of citrus.

Keywords: *Bacillus subtilis*; *Peniillium digitatum*; Antifungal; Antibiotic; Citrus; Postharvest disease

1. Introduction

Up to 25% of the total production of harvested fruit is subject to fungal attack in both industrialized and developing countries; damage is often higher, exceeding 50% (Spadaro and Gullino, 2004). Green mold, caused by *Penicillium digtatum* Sacc., is generally the most serious postharvest disease of citrus. Infection occurs through injuries made during picking or handling and results in decay during storage or marketing.

Synthetic fungicides are the primary means to control postharvest diseases (Eckert, 1990). They are used alone, combined in mixtures, or applied separately in sequence (Ismail and Zhang, 2004). However, several fungicides have been removed from the market due to possible toxicological risks. In addition, repeated use of certain systemic fungicides in packinghouses has led to the appearance of fungicide-resistant populations of *Penicillium digitatum*. For example, in citrus packing facilities in California although thiabendazole, imazalil, and *O*-phenylphenol are still widely used for postharvest treatments, some resistance has developed (Holmes and Eckert, 1999; Kinay et al., 2007). The need for alternative methods of control has encouraged research on possible biological control

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methods, and this has led to some biofungicides being registered for use (Mercier and Jiménez, 2004). Biocontrol, or the use of microorganisms or their secretions to prevent plant disease, is eco-friendly, normally safe, and may provide long-term protection to the crop (San-Lang et al., 2002; Fernando et al., 2005). Some saprotrophic bacteria like *Bacillus* spp., can serve as excellent biocontrol agents against plant pathogens.

Bacillus species, including *Bacillus subtilis*, a ubiquitous soil bacterium, play a role in the degradation of organic polymers in soil (Emmert and Handelsman, 1999). They produce spores that are resistant to desiccation, heat, UV irradiation, and organic solvents. *Bacillus* spp. have shown promise for controlling a wide range of fungi that cause decay, operating as an antagonist to plant pathogen growth through their production of antibiotics (e.g. iturin, surfactin, fengycin), enzymes that degrade fungal structural polymers (e.g. chitinase, β-1,3 glucanase), and antifungal volatiles (Fiddaman and Rossall, 1993; Knox et al., 2000; Jiang et al., 2001; Pinchuk et al., 2002; Leelasuphakul et al., 2006). In some situations, volatile organic compounds (VOCs) secreted by *B. subtilis* have been associated with increased plant growth and the induction of plant systemic resistance mechanisms (reviewed in Compant et al., 2005). The potential of *B. subtilis* for controlling postharvest decay was first introduced by the work of Pusey and Wilson (1984) on brown rot of stonefruit. In addition, *B. subtilis* has been recommended by the United States Food and Drug Administration (US FDA) as one of the GRAS (generally recognized as safe) organisms (Denner and Gillanders, 1996) for use in the food industry. *B. subtilis* endospores and its active vegetative products have shown promising activities against *P. digitatum*. In order to assess its potential use in postharvest treatments, it will be necessary to evaluate its activities and to compare these effects with that of commercial fungicides.

In this study, we had the following objectives: (1) to determine the mode of action of a *B. subtilis* isolated from soil, against *P. digitatum*, *in vitro*; (2) to partially purify the active antifungal substances and estimate their EC_{50} values against green mold mycelial growth and conidial germination; (3) to evaluate the effectiveness of *B. subtilis* endospores and metabolites in reducing postharvest green mold decay of wounded citrus fruit after a 24 h period of storage when used as either co-inoculant or post-inoculant with the pathogen.

2. Materials and methods

2.1. Fungal isolates

P. digitatum was isolated from decaying citrus fruit. It was maintained as a spore culture on potato dextrose agar (PDA) with periodic transfers through citrus fruit to maintain pathogenicity. For inoculating fruit, conidia from the isolate grown on PDA at 24 °C for 14 days, were harvested by adding a small amount of sterile distilled water and gently rubbing the sporulating mycelial with a bent glass rod. The conidial concentration was adjusted with the aid of a haemacytometer.

2.2. Bacterial strains

Soil samples were collected from citrus orchards around the south of Thailand. Each sample (5 g) was suspended in 95 mL of sterile distilled water, shaken vigorously and incubated on a rotary water bath shaker at 100 rpm for 30 min at 80 °C to destroy all vegetative microbial cells. Aliquots (0.1 mL) of 10-fold serial dilutions were spread onto nutrient agar (NA), and then incubated for 24 h at 37 °C. All the single colonies were sub-cultured onto fresh plates of the same medium and were characterized by Gram-staining, cell shape and presence of spores. *Bacillus* spp. isolates (Gram positive rods with spores) were kept on 1% NA slants at 4 °C.

2.3. Antagonism assay

The dual culture technique was conducted to test for the antagonistic activity of the *Bacillus* spp. on the growth of *P. digitatum*. A 0.1 cm agar plug, from the margin of a growing fungal culture on a PDA plate, was incubated centrally on a fresh PDA plate (4.5 cm) for 48 h at 24 °C. The bacterium test culture was grown with potato dextrose broth (PDB), shaken at 250 rpm for 24 h at 30 °C, then centrifuged at 8000 × *g* for 15 min and the pellet was streaked on the PDA plate 1 cm away from the fungal plug. Observations of the fungal reactions were recorded after 2 days and the radii of any zones of inhibition of the fungus were measured in two perpendicular directions by a vernia-caliper. The percentage of hyphal growth inhibition was calculated using the formula: $100 - [(\text{radius of treatment}^2/\text{radius of control}^2) \times 100]$ (Gamliel et al., 1989). Four replicates were used for each bacillus isolate tested.

2.4. Bacterial–fungal interactions from bacterial culture supernatants

Double strength PDA was mixed (1:1) with an equal volume of a cell-free supernatant of *Bacillus* spp. and the medium mixtures were sterilized at 121 °C for 10 min, then poured into a plate (4.5 cm). Experiments were performed in triplicate. A 0.1 cm agar plug of an actively growing fungal mycelium of *P. digitatum* was placed on the center of the test agars. The fungal cultures were incubated at 24 °C for 4 days. The diameter of the fungal colony on the supernatant bacterial mixed agar and control plates was measured. The percentage of hyphal growth inhibition was calculated as described earlier (Section 2.3).

2.5. Bacterial–fungal interactions via volatile organic compounds (VOCs)

An overnight bacterial culture in PDB broth was spread onto a PDA plate. The lid was replaced by a base plate of PDA containing a 0.1 mm diameter agar plug from an actively growing fungal mycelium of *P. digitatum*. The two base plates were sealed together with Parafilm. Control sets were prepared without bacteria in the bottom plate. The diameters of the fungal colonies, on three replicates, were measured after 4 days incubation at 24 °C. The measurement and the percentage of mycelial growth