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Isolation and characterization of metabolites from *Bacillus licheniformis* MH48 with antifungal activity against plant pathogens

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ABSTRACT

In this study, we isolated *Bacillus licheniformis* MH48 from rhizosphere soil and demonstrated that this strain shows significant antifungal activity against *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, and *Phytophthora capsici*. Our results showed that a 50% concentration of bacterial cell-free culture filtrate of *B. licheniformis* MH48 shows strong activity against fungal pathogens. Benzoic acid produced by *B. licheniformis* MH48 was purified by various chromatographic techniques and identified by nuclear magnetic resonance and gas chromatography-mass spectrometry analysis. Benzoic acid displayed antifungal activity against *R. solani* and *C. gloeosporides* with minimum inhibitory concentration of 128 μg/mL against mycelial growth. Microscopic examination revealed that benzoic acid (50 μg/mL and 100 μg/mL) transformed *C. gloeosporioides* conidial morphology and inhibited conidial germination. In addition, benzoic acid (100 μg/mL and 200 μg/mL) degraded *R. solani* mycelia. Therefore, our results demonstrate that *B. licheniformis* MH48 strain shows potential for utility as a biological agent for the control of various fungal pathogens of plants.

1. Introduction

Fungal phytopathogens are known to cause serious loss of and damage to agricultural products. Plant diseases are mostly controlled by the use of pesticides and, in some cases, by cultural practices. However, the widespread use of chemicals has been a subject of public concern and scrutiny due to their potential harmful effects on the environment, unwanted effects on non-target organisms, and possible carcinogenicity [1–3]. Therefore, there is an urgent need for development of non-chemical alternative methods to control plant disease.

The difficulties in controlling fungal plant pathogens and the increasing consumer demand for fungicide-free products have emphasized the need for development of alternative disease control strategies such as biological control [4]. Biological control of plant diseases has been considered as a viable alternative for the management of plant diseases. The use of biological control agents (BCAs) and natural products (NPs) is environmentally safe and sustainable, and in some cases, the only available option for the protection of plants against pathogens [2,5,6]. NPs are broadly defined as active ingredients, derived from a plant, animal, or microbial source, that may be used to control disease development by stimulating plant defences or directly exerting antifungal activity [7]. Several fungi, yeasts, and bacteria have been found to be effective in controlling fungal plant pathogens due to the production of antibiotics as NPs [8–10]. Some of these antibiotics have been commercialized and used extensively in agricultural biocontrol [11]. However, to date, the mechanisms of action of BCAs-derived antibiotics against plant pathogens remain poorly understood [12].

Numerous *Bacillus* strains, including *B. licheniformis*, have been used for controlling plant disease, in particular fungal diseases such

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as *B. cinerea* and *P. capsici* [13–15]. *Bacillus* strains possess the advantage of sporulation, which confers heat-resistance, desiccation-tolerance, and the ability to successfully colonise the plant micro-environment, thereby restricting pathogen infection and inducing plant resistance. *Bacillus* species also produce a variety of antibiotic compounds such as volatiles, lipopeptides [16], peptides [17], and proteins [18,19], and are already widely used for agricultural biocontrol [20], industrial enzyme production [21], and antibiotic production [22].

B. licheniformis and its products inhibit the growth of numerous microorganisms in the environment. Secondary metabolites such as lytic enzymes and biosurfactants produced by *B. licheniformis* inhibit the growth of other microorganisms at low concentration [23,24]. *B. licheniformis* additionally produces antibiotics such as lichenysin [25], surfactin, and iturin [26]. *B. licheniformis* has been shown to inhibit the growth of various fungi, and its potential utility as a BCA against several fungal plant pathogens has recently been investigated.

Benzoic acid is used as a preservative in food, beverages, toothpaste, mouthwash, dentifrices, cosmetics, and pharmaceutical preparations. The inhibition of fungal growth by benzoic acid was first reported over 100 years [27]. Additionally, benzoic acid exerts antimicrobial activity against various bacteria, yeasts, and fungi involved in food poisoning and food spoilage, such as *Escherichia coli*, *Listeria monocytogenes*, *Aspergillus* sp., and *Penicillium* sp. [28]. The present study reports the isolation and characterisation of *B. licheniformis* MH48 as a BCA against various plant pathogens.

2. Materials and methods

2.1. Bacterial culture and fungal phytopathogens

B. licheniformis MH48 was isolated from soil samples obtained from Gunsan province, Republic of Korea and deposited in Genbank under accession No. KP099612. The strain was stored at -70 °C in sterilized distilled water with 25% glycerol solution until use. *B. licheniformis* MH48 was cultured on fertilizer medium containing ($(NH_2)_2CO$, 0.27%; KH_2PO_4, 0.24%; KCl, 0.06%; K_2SO_4, 0.01%, Ca-Mg fertilizer, 0.02%; sucrose, 0.8%; yeast extract, 0.1%; and crab shell powder, 0.1%) at 30 °C on a rotary shaker at 140 rpm for 7 days. Phytopathogens *R. solani* AG-2-2 (IV) KACC 40,132, *Phytophthora capsici* KACC 40,483, and *C. gloeosporioides* KACC 40,003 were purchased from Korea Agriculture Culture Collection (KACC; Suwon, Korea).

2.2. Antifungal activity of bacterial culture filtrate (BCF) against fungal plant pathogens

B. licheniformis MH48 was cultured on fertilizer medium (as mentioned above) on a rotary shaker at 140 rpm for 5 days. Supernatant was collected and centrifuged at 8000 rpm for 20 min at 4 °C followed by filtration through a sterile membrane with 0.45-μm pore size to obtain BCF. BCF was added to potato dextrose agar (PDA) at around 55 °C to the final concentrations of 0, 10, 30, and 50% (v/v). PDA plates without BCF (0%) were used as controls. A mycelial plug from a culture of *R. solani*, *P. capsici*, and *C. gloeosporioides* was placed at the centre of the PDA plate and incubated at 28 °C in the dark. Mycelial growth was measured over 3 days for *R. solani* and over 7 days for *P. capsici* and *C. gloeosporides*. Growth inhibition percentage was calculated as (R - r)/R * 100, where 'R' is the radius of the fungal colony in the control plates and 'r' is the radius of the fungal colony in the treatment plates.

2.3. Fractionation and purification of antifungal compound

For separation of antifungal metabolites, BCF was acidified with concentrated HCl to pH 2.0 and extracted with an equal volume of chloroform. The soluble organic fraction was concentrated by rotary evaporator (Büchi Labortechnik, Flawil, Switzerland) and 3.3 g crude extract was obtained. The antifungal metabolites were dissolved in 3 mL methanol (MeOH) and subjected to silica gel column chromatography (Kieselgel 60, 70-230 mesh; Merk, Darmstadt, Germany) with stepwise elution of $CHCl_3$:MeOH (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 0:100; v/v). All eluted fractions were separately collected and concentrated using evaporator. Each fraction dissolved in MeOH was tested for antifungal activity against *R. solani* and *C. gloeosporioides* by paper disc method. The fraction (100:0, 1.87 g) showing the highest antifungal activity was purified by gel filtration on Sephadex LH-20 column (1.5 × 30 cm, 25-100 mesh; Sigma-Aldrich, Steinheim, Germany) using running phase of 100% MeOH (100 mL). Fractions (2 mL) were collected separately and tested for antifungal activity by paper disc method. Fractions (710 mg) showing strong antifungal activity were further purified by ODS column chromatography, and eluted with a stepwise gradient of H_2O:MeOH with a difference 5 mL between each (100:0, 95:5, 90:10, …, 10:90, 5:95, 0:100; v/v). One fraction (30:70, 52 mg) showing strong antifungal activity was further purified by C18 column chromatography (Inopak C18, RS tech, Korea) by the stepwise elution of H_2O:MeOH with a difference of 0.25 mL between each (5:0, 4.75:0.25, 4.5:0.5, …, 0.5:4.5, 0.25:4.75, 0:5; v/v). Each fraction was separately collected and tested for antifungal activity against *R. solani*. Finally, one major peak was observed in high-performance liquid chromatography (HPLC) analysis using a C18 reversed-phase column, confirming the purity of the antifungal compound (2.25:2.75, 6 mg). The mobile phase of H_2O:MeOH (35:65; v/v) at a flow rate of 0.5 mL/min was used and the peak was detected at 254 nm using a SPD-10 UV-VIS detector (Shimadzu, Japan).

2.4. Identification of the purified antifungal compound

The chemical structure of the purified antifungal compound was determined by ^{1}H-nuclear magnetic resonance (NMR) analysis. Briefly, the purified antifungal compound was dissolved in a 0.5 mL volume of methanol-d4 (CD_3OD) and subjected to spectral analysis. NMR spectra were recorded on a Bruker (Rheinstetten, Germany) DRX 500 NMR instrument operating at 600 MHz for ^{1}H at room temperature. Chemical shifts were reported in ppm (δ) using CD_3OD as a solvent (unless otherwise indicated), tetramethylsilane $(CH_3)_4Si$ as an internal standard, and certain specific values (s = singlet; d = doublet; t = triplet; J = value in Hz). The molecular weight of the purified compound was determined by gas chromatography/mass spectrometry (GC/MS; GCMS-QP2010, Shimadzu, Japan) and confirmed by comparing with that of the standard compound. GC/MS equipped with HP-5 MS column (30 mm × 0.25 mm id. film thickness 0.25 μm) was used, with helium as carrier gas, at a flow rate of 1 mL/min and an injector temperature of 220 °C. Mass spectrometry (Hewlett Packard 5973N, Austria) conditions were as follows: ionization voltage of 70 eV, ion source temperature of 220 °C, mass range of 30–300 mass units, MS quadrupole temperature of 190 °C, and interface temperature of 220 °C.

2.5. Antifungal properties of the purified antifungal compound

2.5.1. Paper disc assay

Benzoic acid used in the antifungal activity assays was purchased from Sigma Aldrich Korea Cooperation, Gyunggi province,