Antagonistic Activity and the Mechanism of *Bacillus amyloliquefaciens* DH-4 Against Citrus Green Mold

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ABSTRACT

Citrus fruit usually suffer significant losses during the storage and transportation stages. Green mold, a postharvest rot of citrus fruit caused by *Penicillium digitatum*, is one of the most serious fungal diseases. In this study, the antagonist strain DH-4 was identified as *Bacillus amyloliquefaciens* according to morphological observation and 16S ribosomal DNA analysis. In addition, it showed broad antifungal activity, especially the suppression of *Penicillium* spp. The culture filtrate of strain DH-4 exhibited apparent activity against *P. digitatum* in vitro and in vivo. In storage, the culture filtrate with DH-4 in it showed a better antiseptic effect. The antifungal substances in the culture filtrate, produced by strain DH-4, displayed stable activity in various extreme conditions. In addition, the antifungal substances in the culture filtrate were identified as macrolactin, bacillaene, iturins, fengycin, and surfactin by ultraperformance liquid

chromatography (UPLC) electrospray ionization mass spectrometry analysis. The UPLC fractions containing these antifungal compounds were basically heat tolerant and all responsible for the antagonistic activity against *P. digitatum*. Transmission electron microscope observation indicated that the antifungal substances might cause abnormalities in the *P. digitatum* cellular ultrastructure, which could be the possible mode of action of *B. amyloliquefaciens* against *P. digitatum*. In addition, it was confirmed via scanning electron microscope analysis that the main way it inhibited *P. digitatum* was by secreting antimicrobial compounds without direct interaction. This study contributes to the understanding of the mechanism of *B. amyloliquefaciens* against citrus green mold as well as providing a potential application for the biocontrol of postharvest rot diseases in citrus fruit.

Citrus fruit is an economically important fruit and usually suffers significant losses during the storage and transportation stages. Green mold, caused by *Penicillium digitatum*, is one of the most serious postharvest fungal diseases in citrus (Arcas et al. 2000). This pathogenic necrotrophic fungus has attracted increasing attention around the world. Chemical control methods have been broadly used to control plant diseases for a long time. However, because chemical fungicides can threaten human health, potentially damage the environment, and induce drug resistance in microbes (Marcet-Houben et al. 2012), the development of novel control approaches is urgent, especially biological control approaches (Janisiewicz and Korsten 2002).

Yeast and *Bacillus* spp. were widely used for selecting antagonists. Yeasts played a very important role in postharvest biocontrol. Blue mold and green mold can be controlled by *Pichia membranifaciens* (Luo et al. 2012). Yeasts such as *Candida oleophila*, *Debaryomyces hansenii*, *Kloeckera apiculata*, *Metschnikowia fructicola*, and *Rhodotorula glutinis*, among others, have also been identified as antagonists (Sharma et al. 2009). The abilities of *Bacillus* spp. as biocontrol agents have been studied and reported for several years (Szczech and Shoda 2006; Yáñez-Mendizábal et al. 2012). *Bacillus amyloliquefaciens* has a great potential to control a variety of plant pathogens (Zhao et al. 2013). *B. pumilus* was reported to significantly

inhibit the infection of *Penicillium digitatum* in Valencia orange (Huang et al. 1992). *Paenibacillus polymyxa* demonstrated a suppression effect on the conidial germination of *Penicillium digitatum* in citrus (Lai et al. 2012).

The antimicrobial mechanisms of *Bacillus* spp. have been well analyzed. The most common discovery was the production of active antimicrobial compounds that directly inhibit diseases (Monteiro et al. 2005). These compounds have been purified and identified. The cyclic lipopeptides (CLP) (surfactin, fengycin, and iturin) were found to control the clubroot disease of crucifers, and the CLP were possibly one of the main antimicroorganism mechanisms of *B. subtilis* XF-1 (Li et al. 2014). *B. amyloliquefaciens* Q-426 generated bacillomycin D, fengycin A, and fengycin B to completely inhibit the spore germination of *Fusarium oxysporum* (Zhao et al. 2013). The antibacterial substances macrolactin A, 7-O-malonyl macrolactin A, and surfactin B were produced by *B. amyloliquefaciens* HR62 to prevent the growth of *Ralstonia solanacearum* (Huang et al. 2014). *B. amyloliquefaciens* ES-2 was also a coproducer of surfactins and fengycins (Sun et al. 2006). Additionally, the culture filtrate of *Bacillus* spp. had a positive effect in a previous study. For timber producers, sapstain was a major problem. The filtrate of *B. amyloliquefaciens* CGMCC 5569 showed growth inhibition activity against some sapstain fungi, which could be utilized as a bioresource (Yuan et al. 2012).

The aim of this study was to analyze the antagonistic characteristics and the antifungal mechanism of *B. amyloliquefaciens* strain DH-4 and its culture filtrate against citrus green mold. This will facilitate the application of strain DH-4 in the horticultural field and promote the exploration of the potential of the culture filtrate as a biofungicide.

MATERIALS AND METHODS

Strains and culture media. The strain DH-4 was isolated from rhizosphere soil of citrus at Huazhong Agricultural University,

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China. The antagonist and the 18 fungal strains (used in this study) were cultured in potato dextrose agar (PDA) medium (20% potato extract, 2% dextrose, and 2% agar) at 28°C (Table 1).

Citrus fruit. FuBen navel orange fruit and Newhall navel orange fruit were harvested from the orchard of Huazhong Agricultural University, China. After being disinfected with 2% NaClO, the fruit were washed with distilled water and then air dried for use.

Screening antagonist. The candidate strains used for the antifungal ability assay were cultured in PDA media at 28°C and shaken at 180 rpm. After incubating for 36 h, the supernatants were collected by centrifugation and filtered through a 0.22-µm micro-filtration membrane to remove the microbes. The culture filtrates were used for the antifungal activity assay.

The 100-µl spore suspension of *P. digitatum* (1×10^6 CFU/ml) was mixed with 100 ml of melting PDA media (at a moderate temperature that did not kill *P. digitatum*) and poured into the petri dishes. First, agar lawns with the candidate strains were pasted on the center of the petri dishes. Second, a 0.5-cm well in the center of the petri dishes was created using a hole puncher, and 20 µl of culture filtrate was added to the hole. Sterile potato dextrose broth (PDB) solution was used as the control. After being cultured for 3 days, the inhibitory zone of each strain appeared. This experiment was carried out in triplicate. Strain DH-4 showed significant inhibitive activity against *P. digitatum* and could be used as an antagonist.

Identification of strain DH-4. Morphological observation. First, 1×10^6 CFU/ml of the strain DH-4 was diluted using a gradient, and 50 µl of diluent was coated on a PDA plate and cultured at 28°C for 36 h. The morphology of the cells and a single colony were observed.

Molecular identification and phylogenetic tree construction. Strain DH-4 cultured in PDB at 28°C for 36 h was collected by centrifugation at 10,000 rpm for 10 min. Total DNA was extracted following the method described in a previous study (Xia et al. 2005). The 16S ribosomal DNA (rDNA) was amplified by using a universal primer pair: forward 8f: 5'-AGAGTTTGATC CTGGCTCAG-3' and reverse rp2: 5'-ACGGCTACCTTGTTACGA CTT-3'. The polymerase chain reaction (PCR) conditions were as follows: initial denaturation at 94°C for 5 min; denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and an extension at 72°C for 2 min for 35 cycles; and a terminal extension at 72°C for 10 min. The PCR product was sequenced. The sequence was submitted to the National Center for Biotechnology Information website to perform BLAST for a homologous search against microbial genomes. In total, 12 sequences from the matched results were used to construct the phylogenetic tree of strain DH-4 with the software MEGA 6.

Antifungal effect of *B. amyloliquefaciens* DH-4 in vitro. For the antifungal spectrum assay, 17 fungal strains (*P. digitatum* N1, *P. digitatum* P44, *P. italicum* B3, *P. italicum* A10, *P. italicum* P6,

P. chrysogenum B5, *P. chrysogenum* Q, *P. polonicum* A1, *P. ulaiense* Pu, *P. expansum* L, *P. crustosum* B1, *Geotrichum citri-aurantii* AY-1, *Alternaria citri* A31-2-1, *Phomopsis citri* MT, *Botryosphaeria dothidea* Ahau, *Colletotrichum gloeosporioides* AISH, and *Aspergillus niger* Fen) were evaluated using the procedures for screening antagonists, and 20 µl of sterile PDB was used as the control. The diameter of each inhibition zone was measured after cultivation for 5 days. Each treatment was carried out for three replications.

Inhibition testing in vivo. Two wounds at a 5-mm depth were made at the waist of citrus fruit by using a bodkin. The wounds were inoculated with the following treatments: (i) control, 20 µl of *Penicillium digitatum* spore suspension (1×10^6 CFU/ml); (ii) 20 µl of *P. digitatum* spore suspension and 20 µl of DH-4 cell suspension (without culture filtrate, 1×10^6 CFU/ml); and (iii) 20 µl of

Fig. 1. Screening and identification of the strain DH-4. **A**, Inhibition effect of the strain DH-4 against *Penicillium digitatum* N1; **B**, single colony; **C**, phylogenetic tree based on 16S ribosomal DNA of strain DH-4.

TABLE 1. Inhibition effect of *Bacillus amyloliquefaciens* DH-4 against 17 fungi

Strain	Diameter of inhibition zone(cm) ± SE	Source
<i>Penicillium digitatum</i> N1	5.56 ± 0.05	Zhejiang, China
<i>P. digitatum</i> P44	6.07 ± 0.11	Italy
<i>P. italicum</i> B3	6.19 ± 0.07	Hubei, China
<i>P. italicum</i> A10	4.74 ± 0.21	Hubei, China
<i>P. italicum</i> P6	3.22 ± 0.07	Italy
<i>P. chrysogenum</i> B5	2.32 ± 0.06	Hubei, China
<i>P. chrysogenum</i> Q	2.32 ± 0.1	Hubei, China
<i>P. polonicum</i> A1	2.65 ± 0.08	Hubei, China
<i>P. ulaiense</i> Pu	6.35 ± 0.07	Italy
<i>P. expansum</i> L	2.02 ± 0.05	Hubei, China
<i>P. crustosum</i> B1	2.53 ± 0.1	Hubei, China
<i>Geotrichum citri-aurantii</i> AY-1	1.89 ± 0.02	Hubei, China
<i>Alternaria citri</i> A31-2-1	2.4 ± 0.02	Hubei, China
<i>Phomopsis citri</i> MT	2.77 ± 0.05	Hubei, China
<i>Botryosphaeria dothidea</i> Ahau	3.04 ± 0.12	Anhui, China
<i>Colletotrichum gloeosporioides</i> AISH	4.28 ± 1.1	Hubei, China
<i>Aspergillus niger</i> Fen	3.17 ± 0.42	Hubei, China

P. digitatum spore suspension and 20 µl of DH-4 culture filtrate. These fruit were stored in a plastic fresh-keeping box (15 by 25 cm) at 26°C with a relative humidity of approximately 90%. Each treatment was done in three replications, and six fruit were used for each replication.

Storage experiment. Newhall navel orange fruit were soaked for 2 min with the following treatments: (i) DH-4 cell suspension (noncentrifuged fermentation, 1×10^6 CFU/ml); (ii) DH-4 culture filtrate; (iii) control (distilled water). Then, the fruit were air dried and each fruit was put in a storage bag. The fruit were directly stored in a ventilated warehouse (approximately 5 to 10°C) for a natural storage test. Visual decay was evaluated after 2 months of storage. Each treatment was replicated 10 times, and 100 fruit were used for each replication.

Antifungal stability analysis of the culture filtrate. The agar diffusion assays were carried out to evaluate the antifungal stability of the culture filtrate and included a heat treatment, a light treatment, volatility testing, and different pH values, with an untreated filtrate as the control. Each treatment was done in three replications.

For heat tolerance, 1 ml of sterile filtrate was subjected to 100°C for 30 min and then cooled to 20°C for bioassay.

For the light treatment, 100 µl of culture filtrate was put into one well of a 24-well cell culture plate, and the experiment was performed three times. One plate was exposed to direct sunlight for 30 min and another to 365-nm UV light for 30 min at a distance of 10 cm.

For volatility testing, three 100-µl plates of filtrate were exposed at room temperature for 1 h.

For different pH values, filtrates were adjusted to pH 3, 5, 7, 9, and 11 using 0.5 M HCl and NaOH and placed at room temperature for 24 h. Next, the samples and the pH control group (no pH adjustment) were adjusted to pH 7 for bioassay.

Purification and ultraperformance liquid chromatography/electrospray ionization mass spectrometry analysis of antifungal substances. The culture filtrate of *Bacillus amyloliquefaciens* DH-4 (200 ml) was extracted three times in an equal volume by ethyl acetate. The ethyl acetate phase was concentrated using a rotary evaporator, and the residues were dissolved in 10 ml methanol for bioassay and analysis of antifungal substances.

The extract was subjected to an ultraperformance liquid chromatography (UPLC) (Waters Acquity UPLC) electrospray ionization mass spectrometry (ESI-MS) (Waters XEVO TQD) system with a C18 column (1.7 µm, 2.1 by 100 mm; Waters Acquity UPLC BEH). The temperature of the column was 40°C. The sample was eluted by the mobile phase, consisting of 0.2% acetic acid in water (A) and 0.2% acetic acid in acetonitrile (B), with a flow rate at 0.45 ml/min. The gradient program was as follows: 0 to 0.2 min,

95% A and 5% B; 0.2 to 8.2 min, 95 to 0% A and 5 to 100% B; 8.2 to 9.7 min, 100% B; 9.7 to 11.30 min, 100 to 5% B and 0 to 95% A; and 11.30 to 13.00 min, 95% A and 5% B. For the MS analysis, the eluted fractions were measured in both positive- and negative-ion modes to determine the molecular weights. The electrospray source for ESI⁺ was operated at a capillary voltage of 3.5 kV, a cone voltage of 46 V, a desolvation temperature of 450°C, and a gas flow of 1,000 liters/h. The different part for ESI⁻ was the capillary voltage at 2.5 kV.

Bioassay of different fractions in culture filtrate. The extract of the culture filtrate was subjected to the semipreparative UPLC system. The antimicrobial compounds were separated, and 17 fractions at the peaks were collected and dried. Then, the residues were dissolved in 500 µl of methanol for inhibition assay against *P. digitatum*.

Antifungal role of the heat-killed fractions. The 17 fractions were treated at 100°C for 30 min. Then these heat-killed fractions were used to test the antifungal role against *P. digitatum*.

Antagonistic modes of action analysis. *Scanning electron microscope observation in vivo.* Citrus tissue samples (5 by 5 mm) were collected from the orange lesion (treated as such in an inhibition test in vivo) with a scalpel at the time points of 3, 8, 24, and 36 h after inoculation. These samples were fixed in 2.5% glutaraldehyde at 4°C overnight. The samples were washed and fixed in 1% OsO₄ for 2 h, and the samples were dehydrated with concentration-increasing alcohol and dried using the method of critical-point drying (Zhang et al. 2013). The specimens were immediately gold-palladium sputtered and photographed with the scanning electron microscope (SEM) platform (JSM-6390 LV; JEOL) of HZAU, China (Liu et al. 2014b).

Action site detection. First, 200 µl of *P. digitatum* spore suspension (1×10^6 CFU/ml) was inoculated into 50 ml of sterile PDB media and shaken at 180 rpm for 24 h. Next, 200 µl of culture filtrate of DH-4 was added to the flask (200 µl of sterile water was used as the control). After being cultured for 0, 0.5, 1, and 2 h, the cells of *P. digitatum* were observed by a transmission electron microscope (TEM) (JEOL H-7650; Hitachi High-Technologies Co.). The method of preparing a TEM section was completed following a previous report (Liu et al. 2014a). We performed this experiment twice.

Statistical analysis. The statistical analysis was conducted using the Statistical Program SAS V8 for Windows. Data were expressed as mean ± standard error. Analysis of variance was performed and groups were compared by *t* test to identify significance of difference between groups. *P* value = 0.05 was considered statistically significant.

Fig. 2. Inhibition effect of DH-4 against green mold in vivo. The citrus fruit treated with *Penicillium digitatum* strain N1, *P. digitatum* strain N1 and the strain of DH-4, and *P. digitatum* strain N1 and the culture filtrate of DH-4 are indicated as N1, DH-4, and Ext, respectively.

Fig. 3. Antiseptic effect in storage. Fruit treated with DH-4 cell fermentation, DH-4 supernatant, and distilled water are indicated as Cell, Supernatant, and CK, respectively.

RESULTS

Screening and identification of the antagonist. The strain DH-4 was selected as an antagonist. Strain DH-4 and its culture filtrate showed apparent inhibition activity against *P. digitatum* N1

(Fig. 1A). A clear inhibitory zone appeared between strain DH-4 and N1. A single colony of the strain DH-4 was milky in color, with a circular, wrinkled protuberance around the edge on the PDA plates (Fig. 1B). The 16S rDNA sequence (1,432 bp) was deposited in GenBank with accession number KP313602. The comparison

Fig. 4. Stability of the culture filtrate of DH-4. Culture filtrates of strain DH-4 were subjected to various stresses for the stability assay of the antifungal activity. **A**, Control without any treatment; **B**, heat treatment; **C**, UV irradiation; **D**, sunlight irradiation; **E**, volatilization; **F**, pH control group; **G**, pH 3; **H**, pH 5; **I**, pH 7; **J**, pH 9; and **K**, pH 11.

TABLE 2. Active compound production by *Bacillus amyloliquefaciens* DH-4 detected by ultraperformance liquid chromatography/electrospray ionization mass spectrometry in positive or negative modes^a

Sample	Retention (min)	m/z		Product ions		MW	Compound type
		Pos	Neg	Pos	Neg		
BA1-1	4.48	ND	623.4	ND	[M-H] ⁻	624	Macrolactin
BA1-2	5.21	ND	433.4	ND	[M-H] ⁻	434	Macrolactin
BA1-3	5.32	ND	433.4	ND	[M-H] ⁻	434	Macrolactin
BA1-4	5.38	ND	501.4	ND	[M-H] ⁻	502	Macrolactin
BA2-1	4.82	ND	741.4	ND	[M-H] ⁻	742	Bacillaene
BA2-2	4.9	ND	741.4	ND	[M-H] ⁻	742	Bacillaene
BA2-3	5.49	ND	579.4	ND	[M-H] ⁻	580	Bacillaene
BA2-4	5.57	ND	581.5	ND	[M-H] ⁻	582	Bacillaene
BA3-1	5.83	1,052.1	1,050.5	[M+H] ⁺	[M-H] ⁻	1,051	Iturins
BA3-2	6.17	1,080.4	1,078.4	[M+H] ⁺	[M-H] ⁻	1,079	Iturins
BA4-1	7.21	1,534.2	ND	[M+H] ⁺	ND	1,533	Fengycin
BA4-2	7.45	1,562.4	ND	[M+H] ⁺	ND	1,561	Fengycin
BA5-1	8.15	994.7	992.5	[M+H] ⁺	[M-H] ⁻	993	Surfactin
BA5-2	8.37	1,008.6	1,006.6	[M+H] ⁺	[M-H] ⁻	1,007	Surfactin
BA5-3	8.65	1,022.7	1,020.8	[M+H] ⁺	[M-H] ⁻	1,021	Surfactin
BA5-4	8.72	1,022.6	1,020.4	[M+H] ⁺	[M-H] ⁻	1,021	Surfactin
BA5-5	8.82	1,022.5	1,020.5	[M+H] ⁺	[M-H] ⁻	1,021	Surfactin
BA5-6	8.91	1,036.7	1,034.5	[M+H] ⁺	[M-H] ⁻	1,035	Surfactin

^a Abbreviations: Pos = positive, Neg = negative, MW = molecular weight, and ND = not determined.

showed a 99% homologous identity with *B. amyloliquefaciens* (accession number NC_023073). The phylogenetic analysis revealed that strain DH-4's closest genetic relationship was *B. amyloliquefaciens* (Fig. 1C).

Antifungal spectrum of *B. amyloliquefaciens* DH-4. *B. amyloliquefaciens* DH-4 demonstrated broad antifungal activity, with the appearance of inhibitory zones. The fungi used were mainly horticultural pathogens, including several citrus *Penicillium* spp. diseases, *P. expansum* (apple blue mold), *G. citri-aurantii* (citrus sour rot), *Alternaria citri* (citrus or tomato black rot), *Phomopsis citri* (citrus stem-end rot), *C. gloeosporioides* (citrus anthracnose), *B. dothidea* (kiwi fruit soft rot), and the typical food pathogen *Aspergillus niger* (Table 1). In addition, strain DH-4 showed prominent suppression of *Penicillium digitatum*, *P. italicum*, and *P. ulaiense* compared with the others. This indicated that *B. amyloliquefaciens* was very suitable as a significant antagonist in the horticultural field.

Inhibition tests in vivo. The treatment with the culture filtrate strongly suppressed the growth of *P. digitatum* and inhibited the diameter of the lesions (Fig. 2). However, the treatment with the

DH-4 suspension did not have an obvious inhibition effect on citrus green mold. The storage experiments showed that DH-4 cell suspension (noncentrifuged fermentation) and DH-4 supernatant (culture filtrate) could clearly reduce the rot incidence of the fruit. Also, the effect of the DH-4 cell suspension was better than DH-4 supernatant (Fig. 3; Supplementary Table S1).

The stability of culture filtrate. The culture filtrate retained apparent stability in the inhibition assay. The results showed that the activity of the filtrate against *P. digitatum* was not reduced when treated with either 100°C heat or sunlight and UV light. In addition, it maintained excellent antifungal activities under different pH values. When exposing the filtrate to air for enough time, it still suppressed *P. digitatum* considerably. Therefore, the antifungal compounds were very stable (Fig. 4).

Characterization of the antifungal compounds. The methanol extract showed distinct antifungal activity, which indicated that the antifungal compounds could exist in the methanol fraction. After further purification by UPLC, the fractions at each peak were subjected to ESI-MS analysis. The UPLC/ESI-MS analysis revealed that five types of antimicrobial substances were

Fig. 5. Ultraperformance liquid chromatography chromatograms of antibiotics produced by *Bacillus amyloliquefaciens* DH-4.

detected in the culture filtrate, including macrolactin, bacillaene, iturins, fengycin, and surfactin (Table 2; Figs. 5 and 6). The spectra of the series of BA1 revealed four macrolactin homologs with the molecular masses of 623.4 [M-H]⁻, 433.4 [M-H]⁻, 433.4 [M-H]⁻, and 501.4 [M-H]⁻ in the negative-ion mode (Huang et al. 2014; Nagao et al. 2001). According to ESI-MS data and the previous study, the fractions with 741.4 [M-H]⁻, 741.4 [M-H]⁻, 579.4 [M-H]⁻, and 581.5 [M-H]⁻ were identified as bacillaene, and the nominal molecular weight was 580 (Butcher et al. 2007; Patel et al. 1995). In addition, the members of the lipopeptide family (iturins, fengycin, and surfactin) corresponded to previous reports (Huang et al. 2014; Song et al. 2013; Sun et al. 2006; Yu et al. 2002). The samples BA5-3, BA5-4, and BA5-5 had the same molecular weight of 1,021 and were identified as surfactin geometric isomers (Fig. 6).

Antifungal role of different UPLC fractions. The 17 fractions isolated from the semipreparative UPLC all showed obvious anti-*P. digitatum* effects of different levels. According to the compound identification above, the antifungal compounds in these 17 fractions were preliminarily classified, including the fractions 1, 2, and 4 (macrolactin), 3, 5, and 6 (bacillaene), 7 to 13 (iturins and fengycin), and 14 to 17 (surfactin) (Fig. 7). However, the effect of fractions 8, 10, 12, 13, 16, and 17 was less than that of the others. This test indicated that the macrolactin, bacillaene, and lipopeptide families produced by *B. amyloliquefaciens* were all responsible for the antagonistic activity against *P. digitatum*. In addition, because the antifungal compounds in the culture filtrate were produced in vitro, all of the antifungal compounds in DH-4 were triggered, not after the exposure with *P. digitatum*. These compounds were constitutive in the synthesis of *B. amyloliquefaciens*.

Fig. 6. Mass spectrometric analysis of antifungal compounds observed in peaks eluted at **A**, 4.48 min; **B**, 5.38 min; **C**, 4.82 min; **D**, 5.49 min; **E**, 5.83 min; **F**, 7.21 min; **G**, 8.15 min; and **H**, 8.65 min by ultraperformance liquid chromatography.

Antifungal role of the heat-killed fractions. After heat-killed treatment, the 17 fractions still showed antifungal activity against *P. digitatum*, which indicated that there heat-tolerant antifungal compounds existed in each of the 17 fractions (Fig. 8). However, the inhibition level changed somewhat. The effect of the fractions 1, 5, 14, and 15 decreased. It was possible that there was some unknown antifungal protein in these three fractions. This test also indicated that most of the antifungal compounds in the culture filtrate were heat tolerant, including macrolactin, bacillaene, and lipopeptide. This result corresponded with the antifungal stability analysis.

SEM observation. Compared with the control group, the treatments with both strain DH-4 and the culture filtrate inhibited mycelia generation at 8 h. The culture filtrate of strain DH-4 showed excellent anti-*P. digitatum* activity, in that the whole growth process of *P. digitatum* was strongly inhibited. From 3 to 8 h, *P. digitatum* spores were sparse and did not produce mycelia; spores started to form bits of short mycelia at 24 h, and other spores collapsed and some mycelia became fractured and incomplete. At 36 h, many

mycelia developed in the wounds, which was certainly related to the quantity and concentration of culture filtrate. The DH-4 suspension showed relatively weaker suppression of *P. digitatum* than the culture filtrate. The presence of mycelia appeared at 24 h. At 36 h, the mycelia displayed a wrinkled property, which was possibly associated with the antifungal metabolites of strain DH-4 (Fig. 9). It was possible that the citrus peel environment was not suitable for the growth of strain DH-4, and few antifungal metabolites were produced. In addition, there was no direct interaction observed between strain DH-4 and *P. digitatum*.

Action site of the antifungal compounds in *P. digitatum*. TEM detection revealed that the addition of the culture filtrate from *B. amyloliquefaciens* DH-4 caused mitochondrial abnormalities in the mycelia of *P. digitatum* (Fig. 10). The mitochondria in *P. digitatum* degraded, and mitochondrial cristae became gradually disorganized as time progressed. Meanwhile, massive mitochondria became vacuolated along with the leakage of the outer membrane. A prominent feature was vacuole-mediated organelle degradation that

Fig. 7. Antifungal assay of the ultraperformance liquid chromatography (UPLC) fractions. In all, 17 fractions were collected from the semipreparative UPLC system and a bioassay was performed. Numbers 1 to 17 represent the fraction number. Abbreviations M, B, I/F, and S represent the macrolactin, bacillaene, iturins/fengycin, and surfactin families, respectively.

appeared in mycelia (Fig. 10) after 2 h of treatment. These indicated that the antifungal compounds in the culture filtrate came into the cytoplasm of *P. digitatum* and acted on the mitochondria and vacuole, thus damaging the growth of *P. digitatum*.

DISCUSSION

Strain DH-4 was screened as an antagonist to inhibit postharvest green mold of citrus fruit. Based on morphological observation and molecular identification, strain DH-4 was identified as *B. amyloliquefaciens*. Because it showed higher antifungal activity against *P. digitatum* compared with many other biocontrol yeasts, *B. amyloliquefaciens* was given sufficient attention (Arrebola et al. 2010).

B. amyloliquefaciens demonstrated antifungal and antibacterial activities (Sun et al. 2006). The findings of this study further explored the antifungal effects and antifungal spectrum of *B. amyloliquefaciens* against several plant fungal pathogens. Perhaps because *B. amyloliquefaciens* used the same culture media (PDA), it produced

the same antimicrobial compounds. Therefore, these substances were certain to exhibit different activities against the 17 fungi (Table 1). In addition, the culture filtrate exhibited better antifungal activity than the cell suspension of DH-4 (without culture filtrate) (Fig. 2). This might be because the conditions on the citrus peel were not suitable for the growth and production of the antimicrobial substance by DH-4. However, the storage experiments showed that DH-4 cell suspension (noncentrifuged fermentation) was slightly better than DH-4 supernatant. This might be because the DH-4 cell suspension contained both DH-4 cells and the culture filtrate. The findings suggest that the culture filtrate with DH-4 in it might be more suitable for controlling green mold in the application. Previous study showed that spore suspension of *Paecilomyces lilacinus* (noncentrifuged fermentation) achieved higher yield than its cell-free filtrate against *Sclerotinia sclerotiorum*. Cell suspension of biocontrol agents may continuously produce antifungal substances on plant surfaces, maintaining better suppression efficacy (Bhuiyan et al. 2003; Yang et al. 2015).

The culture filtrate maintained stable antifungal ability against *Penicillium digitatum* after exposure to UV light, sunlight,

Fig. 8. Antifungal role of the heat-killed fractions. Heat-killed treatment was performed on the 17 fractions for substance property investigation. Numbers 1 to 17 represent the fraction number. Abbreviations M, B, I/F, and S represent the macrolactin, bacillaene, iturins/fengycin, and surfactin families, respectively.

volatilization, high temperatures, and extreme pH values (Fig. 4). This indicated that the anti-*P. digitatum* metabolites in the filtrate showed stable antagonists, which were similar to previous reports (Zhao et al. 2013). In addition, once the culture filtrate was applied in the natural environment, it would not be influenced by a change in environmental conditions.

Bacillus spp. usually inhibit microorganisms by producing antimicrobial secondary metabolites, and the antimicrobial substances further cause the death of microorganisms (Hu et al. 2010). These substances are secreted into the culture filtrate, and the technology for identification has been successfully developed. Some of antimicrobial metabolites were determined to be CLP. The CLP family mainly contains surfactins, fengycins, and iturins, which consist of a polypeptide ring connected with a lipid tail of altering length (Falardeau et al. 2013). Two CLP from *B. amyloliquefaciens* BO5A were tested for their antifungal activities against *F. oxysporum*, *A. niger*, *Botrytis cinerea*, and *P. italicum*. In the results, only compound 2 inhibited all pathogens (Romano et al. 2013). Therefore, we can see that every kind of active compound is possibly effective against different pathogens, which probably explains why the filtrate can inhibit *P. digitatum* considerably but *P. polonicum* A1 and others are weakly inhibited in our study. The UPLC/ESI-MS analysis revealed that strain DH-4 could be a coproducer of macrolactin, bacillaene, iturins, fengycin, and surfactin. Also, each of these families was responsible for the

inhibition effect against *P. digitatum*. These antifungal compounds were constitutive in the synthesis of *Bacillus amyloliquefaciens*. The m/z ratios and molecular weights all corresponded to the previous study. According to the spectra and the available information, the type of the antimicrobial compounds could be deduced.

The antifungal assay in vivo showed that strain DH-4 possibly could produce antifungal substances in citrus fruit but with few quantities suppressing green mold at 8 h. Then, the environment of the citrus peel limited the growth of *B. amyloliquefaciens*. Thus, strain DH-4 could not continue to inhibit *P. digitatum* (Figs. 2 and 9).

In the analysis of the antagonistic mode of action, the antimicrobial substance produced by DH-4 first caused pathological changes in the mitochondria of the mycelia of *P. digitatum*, including mitochondria abnormalities, vacuolation, leakage of the outer membrane of the mitochondria, and vacuole-mediated organelle degradation (Fig. 10). In a previous report, Kim and Chung (2004) observed that the mode of action of the examined antifungal protein was the disruption of the cell wall. Moreover, several discoveries have demonstrated that an antifungal protein from *B. amyloliquefaciens* (baciainin) can induce membrane permeabilization in fungi (Wong et al. 2008). The main lipopeptide product from *B. amyloliquefaciens* was found to damage the membranes and the whole cells of *Vibrio anguillarum* (Xu et al. 2014) and *Candida albicans* (Song et al. 2013). Analogously, we found that the filtrate from the strain DH-4 could restrain the pathogenic effect of *P. digitatum* in citrus fruit. It adequately suppressed the elongation of the mycelia of *P. digitatum*, even breaking them in the early stage. In addition, the experiment (in vivo) showed that the treatment with DH-4 suspension left mycelia of *P. digitatum* completely wilted in 36 h (Fig. 9). It is probable that the culture filtrate or the antifungal metabolites produced by *B. amyloliquefaciens* DH-4 damages the cell membrane structure of *P. digitatum*. Combining these results above, we can form a hypothesis for the antagonistic mode of action of *B. amyloliquefaciens* DH-4. The antifungal compounds coming from strain DH-4 probably induce membrane permeabilization in *P. digitatum*, damage cell membranes, and get into the cytoplasm and destroy mitochondria and vacuoles. Ultimately, strain DH-4 achieves the goal of inhibiting the growth and development of *P. digitatum*, even killing it, so that the postharvest green mold of citrus can be effectively controlled.

Further work might evaluate the application or development of strain DH-4 and its filtrate. Several members of the genus *Bacillus*

Fig. 9. Scanning electron microscope observation of the inhibitory mechanism of the DH-4 culture filtrate against citrus green mold. Citrus fruit treated with *Penicillium digitatum* strain N1, *P. digitatum* strain N1 and the strain of DH-4, and *P. digitatum* strain N1 and the culture filtrate of DH-4 are indicated as N1, N1+Ba, and N1+Ext, respectively.

Fig. 10. Transmission electron microscope observation of the ultrastructure of *Penicillium digitatum* treated with the culture filtrate of DH-4. *P. digitatum* was incubated in potato dextrose broth for 24 h and the culture filtrate of DH-4 was added into the cultures. Mycelia were sampled after treatment at 0, 0.5, 1, and 2 h. Symbols: m = mitochondria, N = nucleus, cw = cell wall, cm = cell membrane, er = endoplasmic reticulum, 1 = mitochondrial cristae, 2 = mitochondria abnormalities and vacuolation, 3 = leakage of the outer membrane of mitochondria, and 4 = organelle degradation.

are generally considered safe and have the status of “Generally Recognized As Safe” (Osman et al. 2011). Many products based on *Bacillus* spp. such as fungicides, bactericides, pesticides, and fertilizers have been marketed. In addition, even *Bacillus*-based biopesticides have been utilized on a large scale in conventional agriculture (Pérez-García et al. 2011). Applying the filtrate alone might not facilitate the best effect. The combination of *B. amyloliquefaciens* HF-01 with tea saponin can apparently improve the control efficacy against sour rot and blue and green mold of citrus (Hao et al. 2011). Additionally, the combination of sodium bicarbonate and hot water with HF-01 could significantly inhibit the main postharvest diseases in mandarin fruit and improve the quality of mandarin fruit (Hong et al. 2014). Therefore, the combination of the culture filtrate of strain DH-4 with other additives may be a better approach to control citrus green mold (Yang et al. 2015).

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