Inhibition on Conidial Germination of *Colletotrichum gloeosporiodes* and *Pestalotiopsis eugeniae* by *Bacillus subtilis* LB5

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Abstract—The effect of antifungal compound from *Bacillus subtilis* strain LB5 was tested against conidial germination of *Colletotrichum gloeosporioides* and *Pestalotiopsis eugeniae*, causal agent of anthracnose and fruit rot of wax apple, respectively. Observation under scanning electron microscope and light compound microscope revealed that conidial germination was completely inhibited when treated with culture broth, culture filtrate, or crude extract from strain LB5. Identification of purified antifungal compound produced by strain LB5 in cell-free supernatant by nuclear magnetic resonance and fast atom bombardment showed that the active compound was iturin A-2.

Keywords—Iturin A-2, *Bacillus subtilis* LB5, *Colleteotrichum gloeospporioides*, *Pestalotiopsis eugeniae*, wax apple

I. INTRODUCTION

WAX apple (*Syzygium samarangense* Merr. Et Perry) which is native to Southeast Asia, is an important fruit crop in Taiwan [7]. Anthracnose disease cause by *Colletotrichum gloeosporioides* (Penzig) Penzig et Sacc is one of the most important diseases in wax apple fruits [3]

Fungi of the genus *Colletotrichum* is one of the most common and important genera of plant pathogenic fungi. Virtually every crop grown throughout the world is susceptible to one or more species of *Colletotrichum* [9]. *C. gloeosporioides*. causes anthracnose disease on various temperate, subtropical, and tropical fruits worldwide [10]. A wide range of chemicals including copper compounds, dithiocarbamates, benzimidazole, chlorotharonyl, imazalil and prochloraz can be used to control *Colletotrichum* diseases. The systemic compounds are particularly effective because of their ability to penetrate host tissue and eradicate latent infection. However, the uses of systemic compounds are increasingly restricted because of the public concerns over toxic residues [14] and fungicide tolerance of phytopathogenic fungi [5].

Another important disease of wax apple is fruit rot caused by *Pestalotiopsis eugeniae* [2, 3]. To harvest a good quality crop with high commercial value it is necessary to control the disease before harvesting fruits. Fungicides can be used to control the disease. However, the effect of the fungicides on human health and environment is a big concern. Biological control is the alternative way which can be used to control plant disease.

Members of the *Bacillus* genus are often considered micro factories for the production of a vast array of biologically active molecules with the potential to inhibit growth of phytopathogens [7].

B. subtilis strains are well known for their synthesis of diverse antimicrobial peptides with high potential for biotechnological applications [13]. These peptides include a prominent class of membrane-active lipopeptides, such as surfactin, fengycin, iturin, and plipastatin which are surfactants and antibiotics with certain specificity [11].

B. subtilis strain LB5 was isolated from the wax apple orchard in the Pingtung County in Taiwan. The objectives of this study were to investigate the effect of antifungal compound produced by *B. subtilis* strain LB5 on conidial germination of *C. gloeosporioides* and *P .eugeniae*, and to identify the compound responsible for the inhibition.

II. MATERIALS AND METHODS

A. Fungal Pathogens

Pathogenic fungus *C. gloeosporioides* was cultured on oatmeal agar (OMA; 72.5 g/l of oatmeal agar: Difco Laboratories, Augsburg, Germany) for 7-10 days at 25°C under continuous fluorescent light to produce conidia. *P. eugeniae* was cultured on potato sucrose agar (PSA; 200 g/l of potato, 20 g of sucrose, and 18 g of agar) for 10-20 days at 25°C under continuous fluorescent light.

B. Antagonistic bacteria

B. subtilis strain LB5 was cultivated on PSA at 25°C for 2 days. The suspension of strain LB5 at 10^8 cfu/ml was added into 1 L of potato sucrose broth (PSB) and cultured for 10 days under shacking (100 rpm) at 25 °C. Culture filtrate was prepared by passing the culture broth through 0.2 μm of Milipore filter membrane.

C. Conidial germination after treated by strain LB5

Conidia of each pathogenic fungus were harvested by gentle scraping into sterile distilled water and filtering the suspension through two layers of cheesecloth to remove mycelia. The conidial suspension was centrifuged (AllegraTM X-22R Centrifuge, BECKMAN COULTER TM) at 5,000 rpm for 5 min and conidia were resuspended in sterile distilled water.

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Conidial suspension was adjusted to 10^5 conidia/ml. Conidial germination was evaluated on glass slide and wax apple fruit surface. Conidial suspension (1 μl) was mixed with 1 μl of bacterial culture filtrate and placed on glass slide. Sterile distilled water mixed with conidial suspension was used as a control. Slides were incubated at 25°C for 12 h under high relative humidity. After 12 h, germination of 100 conidia was determined under light compound microscope and conidia with germ tubes longer than the conidia length were counted as germinated.

Conidial suspension of pathogenic fungi at 10 μl (10^5 conidia/ml) was mixed with 10 μl of culture broth on wax apple fruit surface and incubated in a moist chamber box for 12 h at room temperature. Sterilized distilled water similarly treated was used as the control. The fruit surface were cut off and prefixed with 2.5% glutaraldehyde for 2 h and post fixed with 1% osmium tetroxide (OsO_4) for 2 h. After rinsing in 0.1 M of cacodylate buffer for 10 min, the samples were dehydrated in a graded series of ethanol solutions and with 100% iso-amyl acetate for 2 h followed by critical point drying method using liquid CO_2 (Hitachi model HCP-2). The entire samples were coated with ion coater (Hitachi E-1010) and examined under SEM (Hitachi, S-3000).

D. Crude extract of antifungal compound from B. subtilis strain LB5

Bacterial cultured broth prepared as previous described, was centrifuged at 8,000 rpm for 20 min at 4 °C to precipitate bacterial cells. Cell-free supernatant was concentrated in vacuum and then partitioned with an equal volume of *n*-butanol (*n*-BuOH) to separate into two fractions. The *n*-BuOH soluble fraction was concentrated and added with equal volume of water. After mixing by shacking, the *n*-BuOH portion was evaporated and the antifungal compound in water was used for further experiment.

E. Extraction, purification and identification of antifungal compound

Cell-free supernatant, prepared as previous described, was concentrated in vacuum and was then partitioned with an equal volume of *n*-BuOH to separate into two fractions. The active *n*-BuOH fraction was passed through a reverse phase C-18 column (7x60 cm) and eluted with H_2O: MeOH (2:8, 4:6, and 5:5). Twenty fractions were obtained and active fraction was further passed through C-18 column (5x45 cm) and eluted with H_2O: CAN (1:9). The fractions with antifungal activity were purified with reversed phase high-performance liquid chromatography (RP-HPLC), with elution solvent H_2O: CAN (60: 40, and 45: 55), 2 ml/min.

Identification of antifungal compound was done by nuclear magnetic resonance (NMR) and fast atom bombardment-mass spectrophotometer (FAB-MS). NMR spectra were obtained at room temperature on a Varian Mercury 400 spectrometer. FAB was recorded on a Finnigan/Thermo Quest MAT 95XL (Merk).

III. RESULTS

A. The effect of culture broth, culture filtrate, and crude extract on conidia morphology

The inhibitory effect of antifungal compound from LB5 on *C. gloeosporioides* was investigated by light compound microscope and SEM. Conidial germination of *C. gloeosporioides* was completely inhibited on glass slide and wax apple fruit surface after 12 h of incubation with culture filtrate (Fig. 1b) or crude extract (Fig. 2b) from *B. subtilis* strain LB5. After treatment with culture filtrate some conidia showed swollen germ tube (Fig.1c). Normal germination of this pathogen was found in sterilized distilled water after 12 h of incubation on the glass slide and fruit surface (Fig. 1a, 2a, and 3a). Under SEM, on the fruit surface after treatment with culture broth, conidia did not germinate. These conidia were surrounded by bacterial cells of strain LB5 and appeared swollen or broken (Fig. 3b).

The inhibitory phenomenon on *P. eugeniae* was similar to *C. gloeosporioide*. Antifungal compound in culture filtrate completely inhibited conidial germination of *P. eugeniae* (Fig. 1e). In the treatment without culture filtrate, after 12 h of incubation with sterilize distilled water, all conidia had germinated on glass slide or wax apple fruit surface (Fig. 1d and 3c). Nongerminated conidia of *P. eugeniae* had balloon form conidia on glass slide (Fig. 1e) and swollen or broken cell on wax apple surface (Fig. 3d) after being treated with culture broth from strain LB5.

B. Identification of antifungal compound

For identification of the antifungal compound in cell-free supernatant of *B. subtilis* strain LB5, the FAB-MS spectrum showed a sodinated molecular ion peak $[M+Na]^+$ at 1,065, indicating that the molecular weight of this compound is 1,042. From NMR spectrum, the ^{1}H-NMR spectrum showed signals for N-binding protons at δ 8.70-6.86 and α-protons at δ 4.48-4.00 of peptide, one set of A2B2 coupling pattern protons of benzene ring at δ 7.01 and 6.64 (each d, J=8.4 Hz, 2H), methylene protons of long aliphatic chain at δ 1.30-1.06 and one terminal methyl protons at δ 0.83 (t, J=6.4 Hz, 3H). The ^{13}C-NMR and DEPT spectra revealed signals for 12 carbonyl groups at δ 174.3-170.4, one para-substitued benzene at δ 155.9 (1C), 129.9 (2C), 128.0 (1C), and 115.2 (2C), oxymethylene carbon at δ 61.5, and one methyl carbon at δ 14.1. Based on the results from FAB-MS and NMR, this major antifungal compound produced by *B. subtilis* LB5 was identified as iturin A-2.

Molecular analysis data from NMR
[α] 24D 3.1° (c 0.5, MeOH). ^{1}H NMR (400 MHz, DMSO-d6) δH: 8.70 (d, J = 6.8 Hz, 2H, Tyr and Asn-6 NH), 8.05 (d, J = 7.6 Hz, 1H, Asn-3 NH), 7.71 (d, J = 6.4 Hz, 1H, Asn-1 NH), 7.39 (s, 1H, Asn-6 NH2), 7.34 (s, 1H, Asn-1 NH2), 7.33 (d, 1H, Ser NH, overlapped by δ 7.34), 7.24 (s, 1H, Asn-3 NH2), 7.17 (s, 1H, Gln NH2), 7.13 (d, J = 9.6 Hz, 1H, β-amino acid NH), 7.01 (d, J = 8.4 Hz, 2H, Tyr δ), 6.98 (d, 1H,

Gln NH2, overlapped by δ 7.01), 6.92 (s, 1H, Asn-1 NH2), 6.88 (s, 1H, Asn-3 NH2), 6.86 (s, 2H, Asn-6 and Gln NH2), 6.64 (d, J = 8.4 Hz, 2H, Tyr ε), 4.89 (br t, J = 6.0 Hz, 1H, Ser OH), 4.48 (m, 1H, Gln α), 4.43 (m, 3H, Asn-3 α, Asn-1 and Asn-6 α), 4.15 (m, 2H, Pro and Ser α), 4.00 (m, 1H, Tyr α), 3.98 (m, 1H, β-amino acid C3H), 3.75 (m, 2H, Pro δ), 3.64 (m, 2H, Ser β), 2.95 (br d, J =14.6 Hz, 1H, Tyr β), 2.73 (br d, J = 14.2 Hz, 1H, Tyr β), 2.71 (m, 1H, Asn-6 β), 2.57 (dd, J = 9.2, 15.4 Hz, 1H, Asn-3 β), 2.47 (m, 2H, Asn-3 β and Asn-6 β), 2.32 (m, 2H, β-amino acid C2H2), 2.26 (m, 1H, Asn-1 β), 2.16 (m, 2H, Asn-1 β and Pro β), 2.08 (m, 3H, Gln β and Gln γ), 1.99 (m, 1H, Pro γ), 1.86 (m, 1H, Pro γ), 1.74 (m, 2H, Gln β and Pro β), 1.39 (m, 2H, β-amino acid C4H2), 1.30–1.06 (m, β-amino acid aliphatic CH2), 0.83 (t, J = 6.4 Hz, 3H, β-amino acid C14H3); ^{13}C NMR (100 MHz, DMSO-d6) δC: 174.3 (C=O), 173.4 (C=O), 172.9 (C=O), 172.0 (C=O), 171.6 (C=O), 171.5 (C=O), 171.4 (C=O), 171.2 (C=O), 171.0 (C=O), 170.9 (C=O), 170.8 (C=O), 170.4 (C=O), 155.9 (Tyr, ζ), 129.9 (2C, Tyr, δ), 128.0 (Tyr, γ), 115.2 (2C, Tyr, ε), 61.5 (Ser, β), 60.9 (Pro, α), 56.5 (Tyr, α), 56.3 (Ser, α), 50.9 (2C, Asn-6, α and Asn-1, α), 49.7 (2C, Asn-3, α and Gln, α), 47.4 (Pro, δ), 45.6 (β-amino acid, C3), 41.9 (β-amino acid, C2), 36.4 (Asn-1, β), 36.1 (Asn-3, β), 35.3 (Asn-6, β), 35.0 (Tyr, β), 34.7 (β-amino acid, C4), 31.4 (β-amino acid, C12), 30.7 (Gln, γ), 29.3-29.2 (5C, β-amino acid and Pro, β), 28.9 (β-amino acid), 28.7 (β-amino acid), 26.6 (Gln, β), 25.5 (β-amino acid, C5), 24.8 (Pro, γ), 22.2, (β-amino acid, C13), 14.1 (β-amino acid, C14). FAB-MS m/z 1065 [M+Na]$^+$.
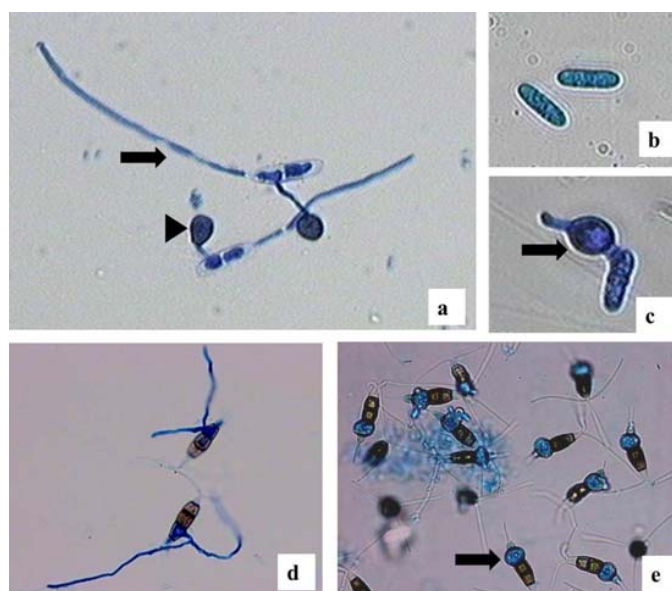

Fig. 1 Effect of culture filtrate of *Bacillus subtilis* strain LB5 on conidial germination of *Colletotrichum gloeosporioides* and *Pestalotiopsis eugeniae* on the glass slide observed under light compound microscope after 12 h of incubation. *C. gloeosporioides* with normal conidial germination with germ tube (arrow) and appressorium (arrowhead) (a), nongerminated conidia (b), and conidia with swollen germ tube (c). *P. eugeniae* with normal germination (d) and nongerminated with balloon formation (arrow) (e)
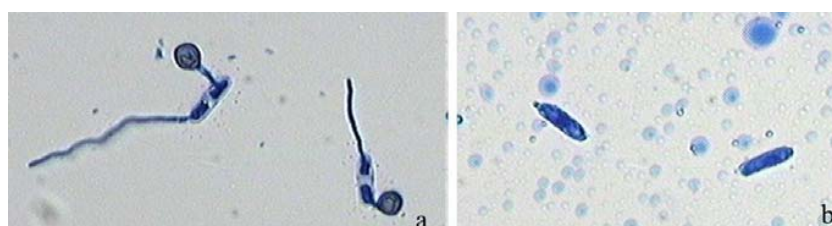
Fig. 2 Effect of crude extract of *Bacillus subtilis* strain LB5 on conidial germination of *Colletotrichum gloeosporioides*
on the glass slide observed under light compound microscope after 12 h of incubation.
Conidial germination with normal germination (a) and nongerminated conidia (b).
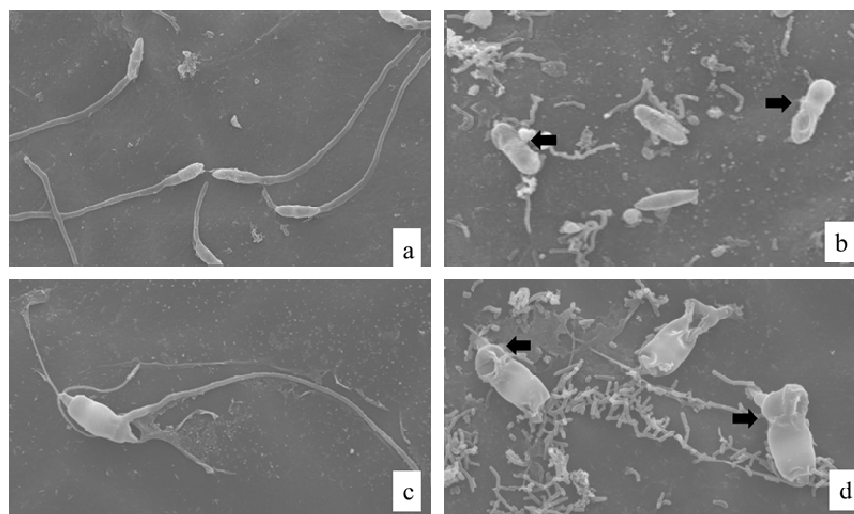

Fig. 3 Scanning electron microscopy (SEM) micrograph of *Colletotrichum gloeosporioides* and *Pestalotiopsis eugeniae* conidia
morphology on wax apple fruit surface after 12 h of incubation with culture broth of *Bacillus subtilis* strain LB5. *C. gloeosporioides* conidia
with normal germinated conidia (a) and non-geminated conidia which showed broken swollen cell (b). *P. eugeniae* with normal germination
(c) and nongeminated conidia with swollen or broken swollen cell (d)

IV. DISCUSSIONS

The *Bacillus* strains have been reported to produce lipopeptides that are suppressive to growth of phytopathogenic fungi [11]. In this study, antagonistic *B. subtilis* strain LB5 was found to produce antifungal iturin A-2 strongly inhibitory to conidial germination of *C. gloeosporioides* and *P. eugeniae*. This antifungal compound belongs to a family of lipopeptides which can be extracted from the culture media of various strains of *B. subtilis* [6]. The lipopeptide iturin family consists of iturin A-E, bacillomycin D,F,and L, and mycosubulin.

The iturin family is amphiphilic cyclic peptides with seven α-amino acids (A1-A7) and one unique β-amino acid [1]. Chemical analysis from NMR and FAB-MS showed that Iturin A-2 compound is cyclic peptides with following sequences: L-Asn, D-Try, D-Asn, L-Gln, L-Pro, D-Asn, and L-Ser. The molecular formula of iturin A-2 is $C_{48}H_{74}N_{12}O_{14}$ with molecular mass of 1042.

Iturins are very active against phytopathogenic fungi and appears to be a good candidate to minimize the use of chemical fungicides [4]. They have limited antibacterial activity and no antiviral effect [7].

A has low toxicity and low allergic effect on human and animal [6]. The antifungal activity is related to the interaction of iturin with the cytoplasmic membrane of target cells [6]. Observation under light compound microscope and SEM revealed that all conidia could not germinate after being treated with culture broth or culture filtrate or crude extract from strain LB5. The phenomenon of inhibition on conidial germination of two pathogens under SEM was similar, showing swollen or broken swollen conidia and nongerminated conidia. It is well known that both sporulation and conidial germination are important preconditions for pathogenic microorganism survival and host infection. Inhibition of processes of fungal spore germination can protect host plants from infection by fungal pathogens. Our results show that iturin A-2 produced by strain LB5 is the major compound responsible for inhibition of conidial germination of *C. gloeosporioides* and *P. eugeniae*.

V. CONCLUSION

The main goal of commercial cultivation of wax apple in Taiwan is the production of fresh fruit for local consumption and several of wax apple orchards also are used for agricultural tourism.

B. subtilis strain LB5 which produces Iturin A-2 inhibitory to conidial germination of *C. gloeosporioides* and *P. eugeniae* can be applied to control anthracnose and fruit rot disease in wax apple orchards in Taiwan. In addition, the use of *B. subtilis* to control plant disease is another strategy to reduce the use of fungicide, safe for human health and environment, and also for sustainable agriculture.

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