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Effect of Crude Extract from *Bacillus Subtilis* LB5 Cultivated Broth on Conidial Germination of *Colletotrichum Gloeosporioides*

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Abstract*—Bacillus subtilis* strain LB5 produced lipopeptide antibiotic iturin A-2 in liquid medium. Crude extract from cell-free supernatant of *B. subtilis* cultivated broth extracted with *n*-butanol showed antifungal activity to conidial germination of *Colletotrichum gloeosporioides*. The germination of conidia was completely inhibited by crude extract. The ultrastructure of conidia after treated with crude extract was found an accumulation of vesicle-like material between cell wall and plasma membrane while this accumulation was not observed in untreated and germinated conidia. Besides, the cell wall was not affected by crude extract.

Keywords*—Bacillus subtilis* strain LB5, iturin A-2, *Colletotrichum gloeosporioides*, TEM, vesicle-like material.

I. Introduction

THE filamentous fungus *Colletotrichum gloeosporioides* (Penzig) Penzig et Sacc is one of the most important pathogens in wax apple fruits (*Syzygium samarangense* Merr. Et Perry) in Taiwan [3]. Besides, it caused anthracnose disease on various temperate, subtropical, and tropical fruits worldwide [13] and it is one of the most common and widely distributed plant pathogens in the world [2], [10]. To control the disease, agricultural chemicals have been used for a long time [11]. These chemicals including copper compounds, dithiocarbamates, benzimidazole, chlorotharonyl, imazalil and prochloraz can be used to control *Colletotrichum* diseases. However, these chemicals were increasingly restricted because of the public concerns over toxic residues [14] and fungicide tolerance of phytopathogenic fungi [7].

Biological control offers an environmentally friendly alternative to the use of pesticides for controlling plant diseases [5]. The using microorganisms to suppress plant disease, offers a powerful alternative to the use of synthetic chemicals. *Bacillus subtilis* is a gram-positive and spore forming bacterium and shows antagonistic activities against several plant pathogens [6] because they have a well-developed secretory system producing diverse secondary metabolites with a wide spectrum of antibiotic activity. Therefore they are widely used in biocontrol of plant diseases and become very valuable for medical and agricultural applications [15].

In previous study, *B. subtilis* strain LB5 was isolated from the wax apple orchard in the Pingtung County in Taiwan (ROC). The strain LB5 was successfully used as biological control agent on mango (Jingkwang cultivar) [1]. This LB5 was found to produce lipopeptide antibiotic iturin A-2 in liquid culture medium [9]. The objective of this study is to analyses the effect of iturin A-2 in crude extract on the conidial germination of *C. gloeosporioides*, causal agent of anthracnose of wax apple.

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II. Materials and Methods

A. Antagonistic Bacteria

B. subtilis strain LB5 was cultivated on PSA at 25°C for 2 days. The bacterial suspension at 10^8 cfu/ml was prepared and adding 10 ml of suspension into 1 L of peptone potato dextrose medium (peptone PD) and cultured for 10 days under shacking (100 rpm) at 25 °C. Cell-free supernatant was prepared by centrifugation at 8,000 rpm for 20 min at 4°C.

B. Conidial Suspension of Fungal Pathogens

The anthracnose 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. Conidia suspension was prepared by harvesting conidia on the media with gentle scraping into sterilize 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 re-suspended in sterilize distilled water which added with 0.1 % of Lecithin (Taiwan Sugar Cooperation) to improve germination rate and adjusted to 10^5 conidia/ml.

C. Crude Extract from Cell-Free Supernatant

Cell-free supernatant from liquid culture of strain LB5 was prepared as previous described. Cell-free supernatant was concentrated in vacuum and then extracted with an equal volume of *n*-butanol (*n*-BuOH). The *n*-BuOH soluble fraction was concentrated and added with final equal volume of water and mixing by hand shacking. This *n*-BuOH portion was evaporated in vacuum. After the *n*-BuOH in the solution was completely evaporated, the residue portion was used to test antifungal activity.

D. Antifungal Activity observed with Light Compound Microscope

A 10 µl of crude extract mixed with 10 µl conidial suspension. The mixture was dropped on glass slide and incubated for 12 h at 25 °C in moist petri dish. Sterilize distilled water similarly treated was used as the control.

E. Observation with Transmission Electron Microscopy (TEM) Analysis

Conidial suspension at 10 µl was mixed with 10 µl of crude extract and dropped on cellophane surface which placed on surface of 2 % water agar medium (WA) and incubated for 3 h at 25°C. The conidia on the cellophane were freeze in liquid nitrogen and keep in low temperature (-20°C or 4°C) before used. Crude extract treated and control conidia were removed from cellophane by sterilized distilled water. The conidia in water were collected by centrifuge at 8,000 rpm for 15 min at 4 °C. The pellets of conidia were fixed overnight with 2.5% glutraldehyde in 0.1 M phosphate buffer saline (PBS), pH 7.2 and rinse with PBS for 15 min. Then the conidia were suspended in low temperature melted 2% agarose gel. The samples were fixed with 1% osmium tetroxide (OsO_4) for 2 h at room temperature and rinse with PBS and followed by dehydration in ethanol series from 30% to 100%. The samples were embedded in Spurr's resin and polymerized at 70°C for 48 h. To select the resin sample with *C. gloeosporioides*, the polymerized block were trimmed and semithin tissue sections were obtained and stained with 1% toluidine blue and then observed under a light microscope. For TEM observations, the trimmed blocks were sectioned at 80 nm with microtome, mounted on copper grids and with uranyl acetate and lead citrate. The samples were examined with a H-7500 transmission electron microscope (Hitachi, Ltd., Japan) at an accelerating voltage of 80 kV.

III. Results

A. The Activity of Antifungal Crude Extract Observed with Light Microscope

In treatment of crude extract, conidial germination was completely inhibited since 2 h until 12 h of incubation (Figs. 1b, d, f, h, and j) when compared with control treatment. In contrast, conidia began to germinate germ tube after 2 h of incubation with sterilize distill water mixed with Lecithin on the glass slide (Fig. 1a; arrows). Appressorium formation started to differentiate after 3 h (Fig. 2b). The appressorium with black melanin formation were observed around 8 h of incubation (Fig. 1g; arrowheads).

In addition, at 3 h of conidial germination was selected to study for TEM. The conidia could geminate by producing germ tube (Fig. 2b; arrow) and some conidia start to differentiate an appressorium formation on the cellophane which put on surface of 2% WA (Fig. 2b; arrowhead). The treatment of crude extract from bacterium cell-free supernatant, conidia can be inhibited by crude extracts which containing iturin A-2 (Fig. 2c). The conidia without treatment were showed in Fig. 2a. The result from light microscope was difficult to observe the differences of structure change between conidia of non-treated and conidia treated with crude extract.

B. The Activity of Antifungal Crude Extract Observed with TEM

To analyze the effect of crude extract containing iturin A-2 from cultivated media of *B. subtilis* strain LB5 on conidial ultrastructure of *C. gloeosporioides* were investigated. The ultrathin sections were obtained and visualized by TEM. Ultrathin sections of normal conidia (Fig. 3a and b) were prepared from conidia which were produced on OMA. Ultrastructure of conidia which incubated with sterile distilled water for 3 h was showed with normal germination (Fig. 3c and d). While conidia treated with crude extract incubated for 3 h could not germinate (Fig. 3e-g). The ultrastructure of conidia cytoplasm between normal and treated with crude extract conidia was not different. However, the conidia treated with crude extract was found an accumulation of vesicle-like materials (VE) appeared between cell wall and plasma membrane (Fig. 3e-g; arrow) but could not found this vesicle in untreated conidia or incubated conidia at 3 h with sterile distilled water. In addition, no visible alteration of cell wall was observed.

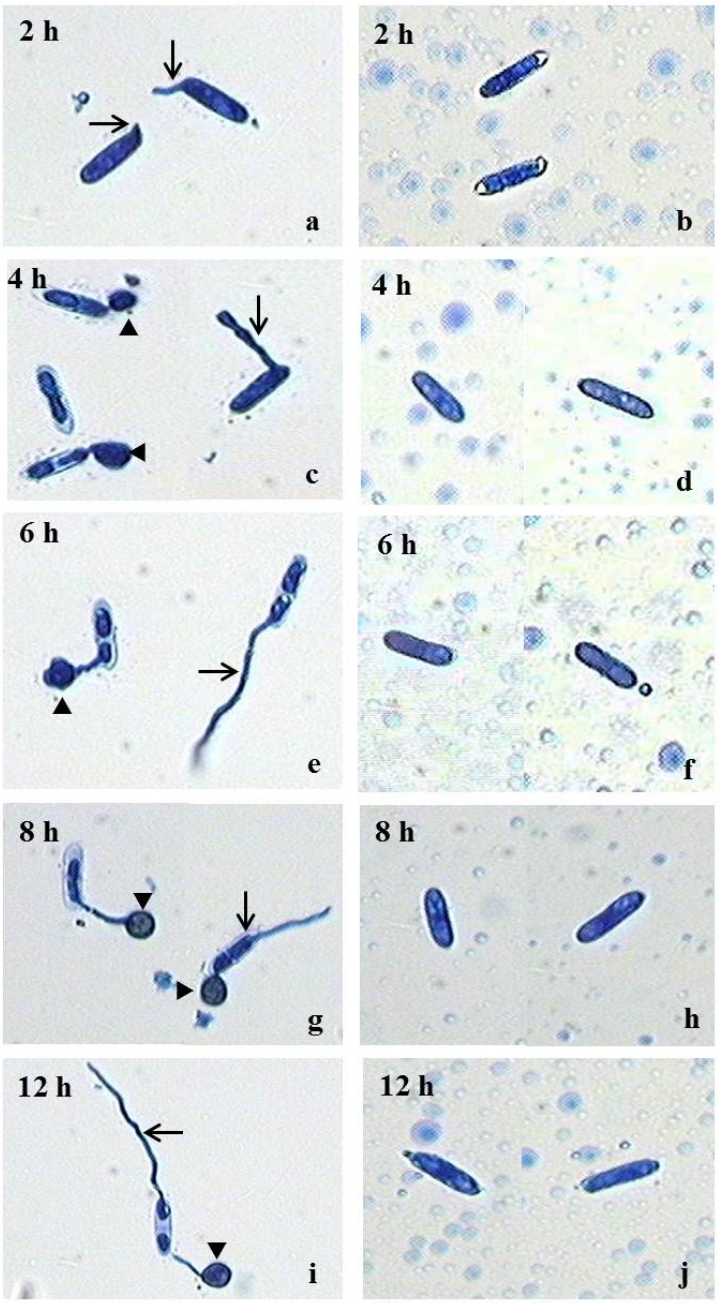

Fig. 1 Conidia of *Colletotrichum gloeosporioides* at a different time of incubation in sterilize distilled water and crude extract of *Bacillus subtilis* cultivated broth on glass slide under light compound microscope. a, c, e, g, and i represent conidial germination with germ tube (arrows) and appressorium formation (arrowheads) incubated in sterilize distilled water for 2, 4, 6, 8, and 12 h respectively. b, d, f, h, and j represent ungerminated conidia incubated in crude extract for 2, 4, 6, 8, and 12 h respectively

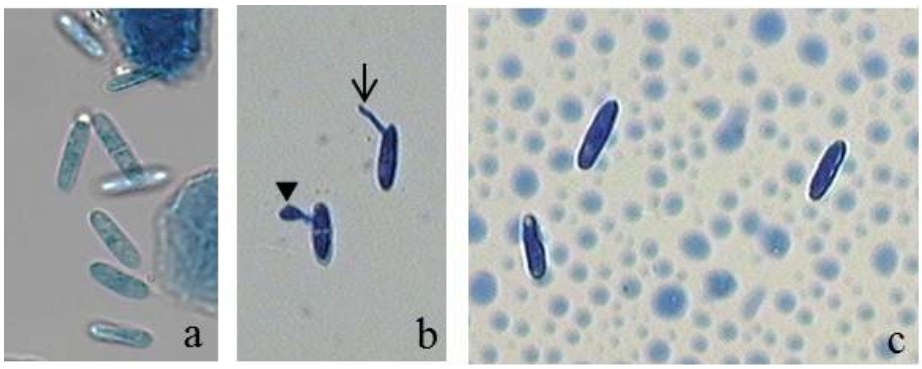

Fig. 2 Effect of crude extract containing iturin A-2 from liquid culture of *Bacillus subtilis* strain LB5 on conidia of *Colletotrichum gloeosporioides*. Normal conidia without any treatment (a). Normal conidial germination after incubate with sterilize distilled water for 3 h, arrow represent germ tube and arrowhead represent appresorium formation (b). Ungerminated conidia after incubated with crude extract for 3 h (c)

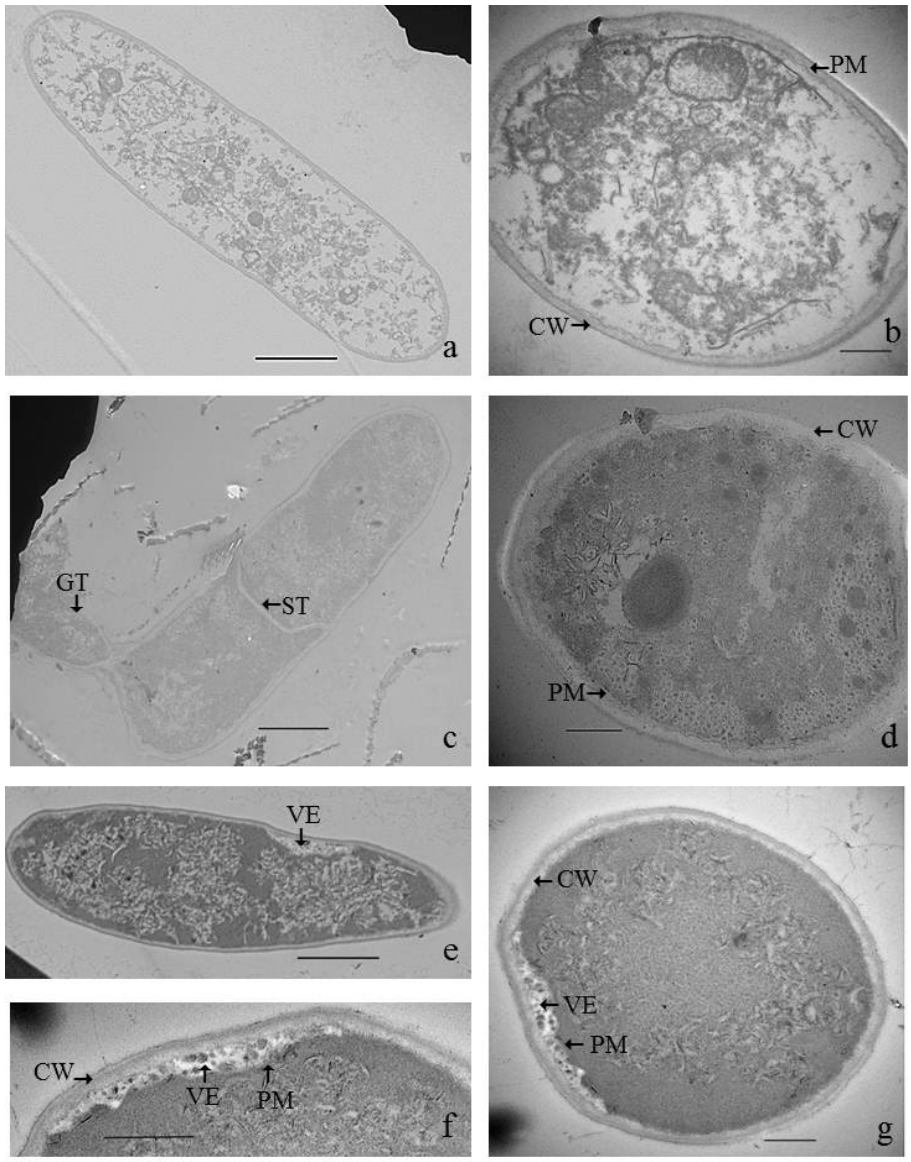

Fig. 3 Ultrastructure of conidia of *Colletotrichum gloeosporioide.* Normal conidium without any treatment, longitudinal section of conidium (a) and transverse of conidium (b). Normal conidial germination after incubate with sterilize distilled water for 3 h, longitudinal of germinated conidium with germ tube (GT) and septum (ST) (c) and transverse of germinated conidium (d). Ungerminated conidium after incubated with crude extract containing iturin A-2 for 3 h showing vesicle-like material (VE) (e-g), longitudinal section of treated conidium (e), transverse of non-germinated conidium (g) and at higher magnification of vesicle-like material (f)

IV. Discussion

Bacillus subtilis LB5 produces lipopeptide antibiotic iturin A-2 in liquid culture [9]. Iturins are family of lipopeptides extracted from culture media of various strain of *Bacillus subtilis* [12]. These compounds are well known for their broad spectrum of antifungal activity [14]. The amphiphilic structure of these lipopeptides allows them to antagonistically interact with biological membranes including the formation of pores [12]. The ultrastructure of conidia of *C. gloeosporioides* after treated with crude extract for 3 h was observed with TEM. The accumulation of vesicle-like materials (VE) was found in conidia after treated the conidia with crude extract. Our result was similar with those induced by iturin A in the plasma membrane of yeast cell [8]. The researcher demonstrated that iturin A could affect morphology and membrane ultrastructure of yeast cell (*Candia albican* and *Sacharomyces cerevisiae*). Iturin A could pass through the cell wall and disrupts the plasma membrane with the formation of small vesicle-like structure but iturin A did not affect to cell wall structure. Besides, iturin A disrupt the nuclear membrane in small fragments which adopted a circular form. In addition, our finding also was similar with the results of Remoro et al. [4]. They used culture media extract from *B. subtilis* strains which contained lipopeptide antibiotics fengycin, iturin/bacillomycin and surfactin to test for their ability to *Podosphaera fusca* conidial germination. After treated with culture extract, the cell was increased in vacuolization and responding disorganization of the cytoplasm which becomes more granulated and less electro-dense. Besides, they also found that no visible changes in the cell wall but the plasma membrane was disrupted by the occurrence of small vesicle-like structure and divided into small fragments spread over the cytoplasm. The changing in plasma membrane represent the first step in which promote internal osmotic imbalance and wide cytoplasmic distortion, increasing the aggregation of cytoplasm and loss of characteristic organelles which result in the plasmolysis in cells of *P. fusca* and produce the morphological damage visible in conidia [4].

V. Conclusion

In conclusion, our finding demonstrated that crude extract containing lipopeptide antibiotic iturin A-2 inhibited conidial germination of *C. gloeosporioides in vitro*. The possible mechanism in the conidial germination inhibition of *C. gloeosporioides* by iturin A-2 in crude extract might involve the disruption of plasma membrane of conidia.

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