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Master Thesis

利用液化澱粉芽孢桿菌 Ba01 防治馬鈴薯瘡痂病菌

Biological control of potato common scab by *Bacillus amyloliquefaciens* Ba01

林 芝

Chih Lin

指導教授：陳穎練 博士

Advisor: Ying-Lien Chen, Ph.D.

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國立臺灣大學碩士學位論文
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利用液化澱粉芽孢桿菌 Ba01

防治馬鈴薯瘡痂病菌

Biological control of potato common scab

by *Bacillus amyloliquefaciens* Ba01

本論文係林 芝君（R04633011）在國立臺灣大學植物病理與微生物學所完成之碩士學位論文，於民國 106 年 10 月 31 日承下列考試委員審查通過及口試及格，特此證明

口試委員：陳穎練（簽名）

（指導教授）

謝奉家　　楊元良

蔡佳欣　　曾敏南

系主任、所長　沈偉強（簽名）

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中文摘要

馬鈴薯瘡痂病(Potato common scab)主要由土傳性放線菌馬鈴薯瘡痂病菌(*Streptomyces scabies*)引起，是近年來影響台灣馬鈴薯產業甚鉅的細菌性病害。然而目前尚無有效殺菌劑及缺乏抗病植株，而輪作等方法亦無法有效降低病害發生，因此希望使用生物製劑成為替代的防治方法。本研究利用分離自台灣田間健康馬鈴薯之液化澱粉芽孢桿菌 (*Bacillus amyloliquefaciens*) Ba01 來拮抗馬鈴薯瘡痂病菌。在培養基對峙培養及掃描式電子顯微鏡的實驗中，發現 Ba01 能有效抑制馬鈴薯瘡痂病菌之生長分化及產孢。經由影像質譜儀分析，發現三種可能的抑菌物質，分別為 surfactin、iturin A 和 fengycin。在溫室的盆栽實驗中，馬鈴薯的罹病度由 55.6 ± 11.1% (只接種馬鈴薯瘡痂病菌) 降至 4.2 ± 1.4% (同時接種馬鈴薯瘡痂病菌和澆灌 Ba01)。而在田間試驗中，馬鈴薯的罹病度也由 14.4 ± 2.9% 降至 5.6 ± 1.1%，顯示 Ba01 有望發展成抗馬鈴薯瘡痂病之生物防治製劑。

關鍵字：液化澱粉芽孢桿菌、Ba01、生物製劑、馬鈴薯瘡痂病菌、馬鈴薯瘡痂病

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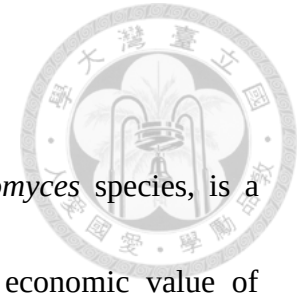

Abstract

Potato common scab, which is caused by soil-borne *Streptomyces* species, is a severe plant disease that results in significant reduction in the economic value of potatoes worldwide. Due to the lack of efficacious pesticides, crop rotations, and resistant potato cultivars against the disease, we investigated whether biological control can serve as an alternative approach. In this study, multiple *Bacillus* species were isolated from healthy potato tubers, and *Bacillus amyloliquefaciens* Ba01 was chosen for further analyses based on its potency against the potato common scab pathogen *Streptomyces scabies*. Ba01 inhibited the growth and sporulation of *S. scabies* and secreted secondary metabolites such as surfactin, iturin A, and fengycin with potential activity against *S. scabies* as determined by imaging mass spectrometry. In pot assays, the disease severity of potato common scab was decreased from 55.6 ± 11.1% (inoculated with *S. scabies* only) to 4.2 ± 1.4% (inoculated with *S. scabies* and Ba01). In the field trial, the disease severity of potato common scab was reduced from 14.4 ± 2.9% (naturally occurred) to 5.6 ± 1.1% after Ba01 treatment, representing the first documented instance that *Bacillus* species controls potato common scab in nature.

Keywords: *Bacillus amyloliquefaciens*, Ba01, biocontrol agent, *Streptomyces scabies*, potato common scab

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1. Introduction

Potato is one of the most important crops worldwide but is easily affected by serious diseases such as late blight, bacterial wilt, soft rot, and common scab. Potato common scab can be caused by at least four gram-positive bacteria from the *Streptomyces* genus, including *S. scabies*, *S. acidiscabies*, *S. turgidiscabies*, and *S. ipomoeae*. Of these, *S. scabies* is the best characterized pathogen (1, 2). The typical scab symptom on potato tubers is superficial, raised, deep-pitted corky lesions that affect tuber quality and marketability in fresh markets or processing operations. Scab symptoms are mainly caused by secreted toxins from *Streptomyces* species such as thaxtomins, concanamycin, borrelidin, or FD-891 (1, 3, 4). Among these, thaxtomin A, a cellulose biosynthesis inhibitor, is well characterized and considered the main virulence factor of most *Streptomyces* species (5).

Because of the limited understanding of the genetic diversity of *S. scabies* and the genetic differences in various potato cultivars, developing effective control strategies for potato common scab is challenging (6-10). Traditional control methods such as soil amendment/chemistry to lower soil pH, soil fumigation with chloropicrin, pre-sowing treatment of seed tubers with fluazinam or flusulfamide, and crop rotation are usually not efficacious and may harm the environment (6, 11, 12). Research in biological control as an alternative approach is emerging. Several studies have used biocontrol

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agents to combat potato common scab, including non-pathogenic *Streptomyces* spp. (13-15), *Pseudomonas* spp. (16-18), and *Bacillus* spp. (19, 20). Currently, only two studies have reported the effects of *Bacillus* spp. on potato common scab. Han *et al*. demonstrated that *Bacillus sp.* sunhua secreted iturin A and macrolactin A as potential antibacterial agents and inhibited the sporulation of *S. scabies* (19). Meng *et al*. showed that *Bacillus amyloliquefaciens* BAC03 secretes LCI protein as an antibacterial product and increases plant height and tuber weight, in addition to reducing the disease severity of potato common scab in pot assays (20). However, whether these two *Bacillus* species can control the scab pathogen *Streptomyces* species in agricultural fields is unclear.

Bacillus species, including *B. subtilis* and *B. amyloliquefaciens* produce endospores, resulting in a long shelf life (~2 years), a desirable characteristic for a biocontrol agent. Although *B. amyloliquefaciens* is a close relative of *B. subtilis*, the secondary metabolites produced by the two species are distinct. For example, *B. amyloliquefaciens* FZB42, a commercial strain, dedicates 8.5% of the genome (~340 kb) to synthesize secondary metabolites, which is two-fold higher (4.5% of its genome; ~350 kb) than that of the *B. subtilis* 168 isolate (21-24). Based on genomic analyses, FZB42 can secrete many secondary metabolites, including lipopeptides (surfactin, iturin, and fengycin), polyketides (macrolactin, bacillaene, and difficidin), and volatiles (acetoin/2,3-butandiol), which may directly suppress the growth of plant pathogens or

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elicit induced systemic resistance (ISR) of the plant host.

In this study, we isolated a biocontrol agent, *B. amyloliquefaciens* Ba01, from healthy potato tubers and showed its inhibitory effects on the growth and sporulation of the potato common scab pathogen *S. scabies*. The potential inhibitory mechanism of Ba01 against *S. scabies* is possibly through the secretion of lipopeptides such as surfactin, iturin A, and fengycin. Ba01 not only reduced the disease severity of potato common scab in pot assays, but also in scab naturally occurring in the field, representing the first documented example that *Bacillus* species reduce the disease severity of potato common scab in nature.

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2. Materials and Methods

2.1 Strains, growth media, chemicals, and phylogenetic analysis

Bacillus species and *S. scabies* strains are listed in Table 1. *B. amyloliquefaciens* Ba01, Ba02, Ba03, Ba04, Bs01, *S. scabies* PS01, PS02, PS07 and PS08 were provided by Dr. Chia-Hsin Tsai. *Bacillus sp.* was isolated from soil of potato field in Dounan, Taiwan. One gram of soil was serial diluted with ddH_2O and spread on LB medium. *S. scabies* YC1020, YC1028 were isolated from scabby potato tubers in Tanzi, Taiwan; while CL2, CL3, CL4 and CL5 were isolated from scabby potato tubers in Dounan, Taiwan. Scabby potato tubers were sliced and sterilized by 0.5% NaClO for 1 min and then put on SCAN agar medium. *Bacillus* species isolated from healthy potato tubers with nutrient agar medium were identified by sequencing 16S rRNA with primers fD1 and rP2 (25) (Table S1), and the *gyrase A* gene with primers p-*gyr*A-F and p-*gyr*A-R (26) (Table S1). *S. scabies* strains isolated from scabby potato tubers with nutrient agar or SCAN medium were identified by sequencing 16S rDNA with primers fD1 and rP2, and by PCR-RFLP with amplification of the partial *atpD* gene with primers atpDPF and atpDPR (27) (Table S1). The sequence results were compared in the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment and analysis of gene similarity were performed using the ClustalW program. The evolutionary history was inferred by using the Maximum Likelihood method based on

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the Tamura-Nei model (28). Phylogenetic trees were drawn using the MEGA7 program (29).

Media used in this study include nutrient agar medium (HIMEDIA, India), SCAN medium (0.001% $FeSO_4 7.H_2O$, 0.002% $CaCO_3$, 0.005% $MgSO_4.7H_2O$, 0.03% casein, 0.2% K_2HPO_4, 0.2% NaCl, 0.2% KNO_3, 1% starch, and 1.8% agar), YME medium (0.4% yeast extract, 1% malt extract, 0.4% dextrose, and 2% agar), MPYSC medium (0.1% $MgSO_4.7H_2O$, 0.1% casein, 0.1% yeast extract, 1% soluble starch, 0.05% K_2HPO_4, and 2% agar), LB medium (MDBio, Inc., Taiwan), and Mueller Hinton medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The following chemical agents were used in this study: iturin A (Sigma-SI-I1774, St Louis, MO, USA), surfactin (Sigma-SI-S3523), and fengycin (Sigma-SMB00292).

2.2 Disk diffusion assays

Disk diffusion assays were used to test anti-*S. scabies* activity of six *Bacillus* strains, including four *B. amyloliquefaciens* isolates, one *B. subtilis* isolate, and one *Bacillus sp.* isolate against the potato common scab pathogen. *Bacillus* species were grown overnight at 37℃ in LB liquid medium, and the cell concentrations were calculated by measuring OD_{600} (1 OD_{600} = ~ $2x10^7$ CFU/mL). *S. scabies* isolates were grown on solid MPYSC medium at 28℃ for 14 days. Spores were collected with cell

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scrapers and washed twice with ddH_2O before determining spore concentration by serial dilution. Then, 100 μL of 10^7 CFU/mL *S. scabies* cells were spread on YME solid medium, and two 6-mm disks were placed on the surface of each agar plate. Then, 3 μL of 1 OD_{600} *Bacillus* cells or ddH_2O were placed on the disk, and plates were cultured at 28℃ for five days before being photographed.

2.3 Determination of minimal inhibitory concentrations

Minimal inhibitory concentration (MIC) indices were determined following the CLSI-M07-A9 protocol. Freshly collected spores of *S. scabies* PS07 were diluted with Mueller Hinton medium, and 50 μL were added to each well to a final concentration at $5x10^5$ CFU/mL in a 96-well plate format. The compounds to be assessed were two-fold serially diluted with Mueller Hinton medium, and 50 μL of each dilution were added to the wells containing spores, yielding a total volume of 100 μL per well. Iturin A concentrations ranged from 0.125 to 64 μg/mL, while surfactin and fengycin concentrations ranged from 0.25 to 64 μg/mL. The plates were incubated at 28℃ for 48 h. The MIC was defined as the lowest concentration of a compound that completely inhibited the growth of *S. scabies* PS07 as detected by the unaided eye.

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2.4 Determination of fractional inhibitory concentrations

The fractional inhibitory concentration (FIC) of compounds was determined via checkerboard titration assays. Freshly collected spores of *S. scabies* PS07 were diluted with Mueller Hinton medium, and 50 µL were added to each well of a 96-well plate to a final concentration of $5x10^5$ CFU/mL. The two compounds to be assessed were two-fold serially diluted with Mueller Hinton medium, and 25 µL of each compound were added to the wells containing spores, yielding a total volume of 100 µL per well. Iturin A concentrations ranged from 1 to 64 µg/mL, while surfactin and fengycin concentrations ranged from 0.25 to 64 µg/mL. The plates were incubated at 28℃ for 48 h. The MIC or FIC of compounds, either alone or in combination, was defined as the lowest concentration of each compound that completely inhibited the growth of *S. scabies* PS07 as detected by the unaided eye. The FIC index was calculated by the following formula: FIC = (MIC of compound A combined) / (MIC of compound A alone) + (MIC of compound B combined) / (MIC of compound B alone). For calculation purposes, an MIC >64 µg/mL was assumed to be 128 µg/mL.

2.5 Scanning electron microscopy

The agar plates from the disk diffusion assay were cultured at 25℃ for five days, and the undifferentiating and non-inhibition zones were excised and fixed in 2%

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glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4℃ for 24 h. The samples were then rinsed three times with cold 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h each time, soaked in 2% osmium tetroxide in sodium cacodylate buffer at 4℃ for 5 h, and rinsed as described above. After fixation, the samples were dehydrated in a series of ethanol concentrations (50%, 70%, 80%, 90%, and 95%) at 4℃ for 10 min each time and in 100% acetone twice (first time at 4℃ for 15 min, second time at room temperature for 15 min). The samples were then critical point dried in liquid CO_2, mounted on metal stubs for gold coating, and observed under the scanning electron microscope JEOL JSM 6510 at 15 kV.

2.6 Imaging mass spectrometry

S. scabies PS07 was initially inoculated in a vertical line on 2% YME solid agar plates. After 12 h, *B. amyloliquefaciens* Ba01 was inoculated in a horizontal line on the same plate for an additional 24 h, and the region in which the two microbes interacted, as well as the individual regions with compounds secreted from each microbe, were excised and transferred to an indium tin oxide-coated glass target plate. Pure, serially diluted surfactin, iturin A, and fengycin were used as standards to determine the compound concentration secreted by Ba01. Surfactin and fengycin were two-fold serially diluted with methanol from 2,500 μg/mL to 39.1 μg/mL and from 312.5 μg/mL

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to 4.9 μg/mL, respectively, while iturin A was two-fold serially diluted with 50% ethanol from 10,000 μg/mL to 156.3 μg/mL, and 1 μL of each diluted compound was dropped onto the YME agar. The universal matrix powder (1:1 mixture of α-cyano-4-hydroxycinnamic acid and 2, 5-dihydroxybenzoic acid) was sprinkled on the top of samples. After covering with matrix, the samples were exposed to air overnight at 37℃ until dried completely.

Imaging mass spectrometry (IMS) data were collected on a Bruker Autoflex Speed MALDI TOF/TOF spectrometer at the Agricultural Biotechnology Research Center, Academia Sinica and analyzed by Bruker Compass Version 1.2 Software Suite (30). For the samples used in this study, linear positive ion mode was applied with 85% laser power and 333.3 Hz laser frequency. The 1,100 μm of raster interval in the X and Y dimensions were applied, and each raster summed up to 500 shots. The detection mass range was set from *m/z* 100 to 2125.

2.7 Pot assays

Pot assays were conducted to examine the biocontrol activity of Ba01 against *S. scabies* PS07. *S. scabies* was grown in solid MPYSC medium at 25℃ for 14 days, and the spores were collected by cell scrapers and washed twice with ddH_2O. Potato (cultivar: Kennebec) tuber pieces with a bud were air dried and planted in nursery pots

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filled with sterilized soil (1:1 peat moss:King Root plant substrate) at 25℃ for three weeks until seedlings emerged. Each potato seedling was transferred to a seven-inch pot in a greenhouse with the temperature maintained between 18℃ and 22℃. Five-week old potato plants were then inoculated with *S. scabies* PS07 by mixing 50 mL ($2x10^9$ CFU/mL) of inoculum with the soil. For *B. amyloliquefaciens* Ba01 treatment, Ba01 was grown overnight in LB liquid medium at 37℃ and rinsed twice with ddH_2O. Then, 50 mL containing 2.8 $x10^9$ Ba01 cells were applied to the treatments. Each of the five treatments described below included three potato plants, for a total of 15 plants: (a) mock control without PS07 or Ba01; (b) PS07 only; (c) Ba01 only; (d) PS07 and Ba01 inoculated on the same day; and (e) PS07 inoculated first for 14 days, followed by inoculation of Ba01. Potato plants were watered twice weekly, and fertilizer (HYPONeX 2) was applied weekly beginning the fifth week after planting. Potato tubers were harvested 12 weeks after planting, and the disease severity in each treatment was calculated as follows, originally described by Wanner *et al.* : Σ (percentage coverage by lesions x predominant lesion type x number of tubers with these scores) / (18 x total number of potato tubers evaluated) x 100. Lesion types were divided into four degrees: 0 = no symptoms, 1 = superficial, 2 = raised, and 3 = pitted. Percentage coverage by lesions for each tuber was classified in seven degrees: 0 = no scab, 1 = 0.1% to 2%, 2 = 2.1% to 5%, 3 = 5.1% to 10%, 4 = 10.1% to 25%, 5 = 25.1% to 50%,

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and 6 = ≥50%. Disease incidence was calculated by determining the proportion of tubers with >5% scab coverage from collected potato tubers.

2.8 Field trial

The 11-week trial was conducted in an agricultural field with naturally occurring potato common scab from December 2, 2015 to February 17, 2016 in Dounan, Taiwan (23.669919, 120.463699). The temperature in Dounan was between 15℃ and 21℃ during the experiment. The field was divided into 16 blocks (four blocks per treatment) with a randomized complete block design. Each block contained 20 potato plants, and 10 protective potato plants were located between blocks. Treatments were as follows: (A) $5x10^6$ CFU/mL Ba01, (B) $1x10^7$ CFU/mL Ba01, (C) $2x10^7$ CFU/mL Ba01, and (D) mock (no Ba01). Ba01 fermentation broth was provided by Dr. Chia-Hsin Tsai. The concentration of Ba01 fermentation broth was approximately 9 x 10^9 CFU/mL. Ba01 was applied at 0, 2, and 3 weeks after planting. The Ba01 cells contained in 200 mL were poured directly onto the soil that covered the tuber buds at week 0, and 400 mL of Ba01 were applied 2 and 3 weeks after planting. We randomly collected 25 potato tubers from each block to evaluate the disease severity and incidence of each block, and four blocks of the same treatment were used to determine the mean ± standard error and compared to other treatments based on Tukey's test.

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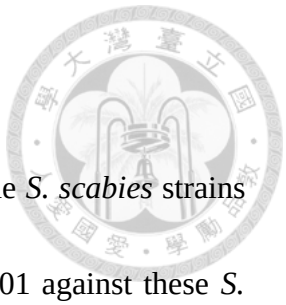

2.9 Tuber slice assays

Tuber slice assays were used to test the pathogenicity of multiple *S. scabies* strains isolated from the field trial, as well as the biocontrol activity of Ba01 against these *S. scabies* strains. The procedures have been described previously by Loria *et al*. (31), with minor modifications. In brief, the surface of potato tuber was sterilized with 1% NaOCl and cores (1.2 cm) of pith tissue were removed from the tubers. The cores were then sliced into pieces (0.25 cm thick) and placed on moist filter paper in glass petri dishes. Three tuber pieces were used for each treatment. Test strains were grown on solid MPYSC medium for 14 days at 28℃, and agar disks with the sporulating colony were inverted onto the tuber pieces. Tuber pieces were incubated in moist glass petri dishes at 28℃ for six days in the dark and photographed.

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3. Results

3.1 *B. amyloliquefaciens* inhibited the growth and sporulation of *S. scabies*

Bacillus strains, including four *B. amyloliquefaciens* isolates (Ba01, Ba02, Ba03 and Ba04), a *B. subtilis* (Bs01) isolate, and a *Bacillus sp.* isolate with species unidentified were used to test anti-*S. scabies* activity *in vitro*. For phylogenetic assignment, 16S rRNA and *gyrA* gene sequences of *Bacillus* strains were determined. Ba01 clustered closely with Ba02 and Ba03 clustered closely with Ba04, while Bs01 and *Bacillus sp*. strains were distinct from Ba01~04 (Fig. 1A). In the disk diffusion assays, we found that Ba01, Ba02, Ba03, and Ba04 isolates effectively inhibited the growth of *S. scabies* PS07, while *B. subtilis* Bs01 and *Bacillus* sp. demonstrated subtle inhibition (Fig. 1B). Due to the similar activity of the four *B. amyloliquefaciens* isolates, we chose Ba01 to conduct further experiments. We first tested antibacterial activity of Ba01 against multiple *S. scabies* isolates (Fig. 1C) and found that clear and undifferentiating inhibition zones were formed when Ba01 inhibited the growth of *S. scabies* (Fig. 2A). Furthermore, based on observations from scanning electron microscopy, the hyphae morphology of *S. scabies* PS07 in the non-inhibition zone was spiral and hyphae formed sporulation septa with constrictions (Fig. 2C), while hyphae in the undifferentiating zone displayed vegetative septa without constrictions (Fig. 2D).

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3.2 Identification of secondary metabolites secreted from Ba01 that potentially inhibited *S. scabies* growth

IMS is a powerful technique used to visualize the spatial distribution of various chemical compounds based on their molecular masses (eg, *m/z* ratio) (30). We used this technique to identify potential secondary metabolites secreted from Ba01 while it inhibited the growth of *S. scabies* PS07 on YME solid medium (Fig. 3A). Three major peaks representing surfactin (*m/z* 1046.38), iturin A (m/z 1095.76), and fengycin (m/z 1516.19) were detected in the region in which Ba01 inhibited the growth of *S. scabies*. Of these, surfactin was secreted largely to neighboring regions at a maximum amount of 2.5 μg, as evidenced by density gradient and IMS spectra. Iturin A was secreted at the strongest intensity with a maximum amount of 10 μg, while fengycin was secreted at a maximum amount of 0.32 μg (Figs. 3A and B). In order to confirm our findings, pure iturin A, surfactin, and fengycin were used to test the anti-*S. scabies* activity. Each of the three compounds demonstrated anti-*S. scabies* activity as evidenced by undifferentiating inhibition zones, but none of the compounds completely inhibited the growth of *S. scabies* (Fig. 4A). Surfactin (2.5 μg) exhibited better inhibitory effects on the growth of *S. scabies* than iturin A (10 μg) or fengycin (0.32 μg) based on the disk diffusion assays (Fig. 4A). Meanwhile, *S. scabies* treated with surfactin, iturin A, or fengycin exhibited defects in the formation of spiral hyphae, while *S. scabies* treated

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with iturin A demonstrated additional morphological defects such as clumped hyphae (Fig. 4B). These results suggested that these three compounds identified by IMS inhibit the growth and differentiation of *S. scabies*. However, each compound was determined to have a MIC value >64 μg/mL against *S. scabies* (Table 2). The contrasting results between the disk diffusion assays (i.e., partial inhibition [undifferentiating] zone on solid medium) and the high MIC values of surfactin, iturin A, and fengycin in liquid medium against *S. scabies* might be because the definition of MIC endpoint requires the complete inhibition of growth, whereas some growth persisted even with extensive inhibition. Further experiments were performed to test if these three compounds demonstrated synergistic activity against *S. scabies*. However, synergistic activity was not detected based on determined FICs of 2, representing no interaction between compounds (Table 2).

3.3 Ba01 reduced the disease severity of potato common scab in pot assays

Pot assays were performed to test whether Ba01 can reduce the disease severity of potato common scab. Fifteen potato plants were divided into five treatments: (a) mock control without PS07 or Ba01; (b) PS07 only; (c) Ba01 only; (d) PS07 and Ba01 inoculated on the same day; and (e) inoculation of PS07 first, then Ba01 inoculation after 14 days. Potato tubers were harvested 12 weeks after planting, and the disease

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severity was calculated based on an index of percentage coverage by lesions multiplied by the index of predominant lesion type, divided by 18 (13). Treatment with Ba01 reduced the disease severity of potato common scab from 55.6 ± 11.1% (inoculated with *S. scabies* only) to 4.2 ± 1.4% (inoculated with *S. scabies* and Ba01 on the same day) (Figs. 5A and 5B) ($P < 0.01$, Tukey's test). However, when Ba01 was applied two weeks after *S. scabies* inoculation, it did not reduce the disease severity and disease incidence of potato common scab (Figs. 5A and 5B), indicating that Ba01 demonstrated preventive rather than therapeutic activity.

3.4 Ba01 reduced the disease severity of potato common scab in the field

A field trial was conducted in an agricultural field with naturally occurring potato common scab (Fig. 6A). Three treatments of Ba01 at $5x10^6$, $1x10^7$, and $2x10^7$ CFU/mL, in addition to a mock control of 0 CFU/mL, in a randomized complete block design were used to test inhibitory activity against potato common scab disease (Fig. 6A). Ba01 treatment at $2x10^7$ CFU/mL significantly reduced disease severity from 14.4 ± 1.9% (no Ba01 treatment) to 5.6 ± 1.2% ($P < 0.05$; Tukey's test) and decreased disease incidence from 21% to 5% for the naturally occurring potato common scab. While Ba01 treatment at $5x10^6$ and $1x10^7$ CFU/mL did not reduce disease severity, disease incidence decreased from 21% to 7% and 8%, respectively (Figs. 6B, C, and D). Ba01 treatment

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did not stimulate plant growth or increase potato tuber yield (Figs. 6E and F).

We isolated four *Streptomyces* isolates (CL2 to CL5) from scabby potato tubers in different experimental blocks and identified these strains as pathogenic *S. scabies* based on potato tuber slice assays (Fig. 7A). Ba01 exhibited inhibitory effects toward four *S. scabies* isolates (CL2 to CL5), but these isolates were slightly less susceptible to Ba01 than PS07 based on disk diffusion assays. Interestingly, our data showed similar inhibitory activity of surfactin, iturin A, and fengycin against four *S. scabies* strains (CL2 to CL5) isolated from the field and *S. scabies* PS07 (Figure 7B), indicating that differential tolerance of *S. scabies* strains to Ba01 might be due to specific characteristics of *S. scabies* and not simply attributed to the secretion of surfactin, iturin A, or fengycin.

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4. Discussion

Biological control agents have been extensively studied to combat plant pathogens in order to reduce environmental pollution, ecological disturbance due to pesticides used in fumigation, and pre-sowing tuber/seed treatments. In this study, we tested six *Bacillus* isolates and found that *B. amyloliquefaciens* Ba01 exhibited stronger inhibitory effects than *B. subtilis* or *Bacillus* sp. against the potato common scab pathogen. Additionally, *S. scabies* hyphae in the undifferentiated zone were still undifferentiated after 20 days, indicating that the hyphal growth of *S. scabies* was inhibited and was not simply due to growth delay. To our knowledge, only two studies have investigated the use of *Bacillus* isolates (*Bacillus* sp. sunhua and *B. amyloliquefaciens* BAC03) to control this plant pathogen (19, 20). Our findings that surfactin, iturin A, and fengycin acted as antibiotics against *S. scabies* are partially supported by studies in which Han and colleagues showed that iturin A was responsible for combating *Streptomyces* species. In addition, these three compounds, controlled by *srf*, *bmy* and *fen* genes, respectively, have been shown to be secreted from *B. amyloliquefaciens* FZB42 as demonstrated by cassette mutagenesis (32). Although surfactin and fengycin were not identified by Han *et al*. to be secreted by *Bacillus* sp. sunhua, these compounds have been detected in studies against other pathogens (33). In this study, we provide evidence that Ba01 secretes surfactin, iturin A, and fengycin based on IMS analysis, while the other peaks

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seen on the spectra are derivatives of surfactin, iturin, or fengycin. Ba01 itself produces better inhibitory effects than a single pure compound (surfactin, iturin A, or fengycin), which indicates that compounds secreted from Ba01 might synergistically enhance the killing effects on *S. scabies*. Further experiments may include disrupting a single gene (*srfA*D, *ituD*, or *fenA*), two genes (*srfA*D *ituD*, *srfA*D *fenA*, or *ituD fenA*), or three genes (*srfA*D *ituD fenA*) from the Ba01 isolate and testing the ability of these mutants to inhibit *S. scabies* growth and sporulation or their biocontrol efficacy against potato common scab in pot assays and field trials. Such experiments will provide additional evidence to show that synthesis of these compounds is required to inhibit the growth of *S. scabies* and reduce scab symptoms. However, several attempts to obtain these mutants via homologous recombination or in-frame deletion strategies were unsuccessful (Supplementary Materials), possibly due to the 'wild' nature of Ba01, with a low level of genetic competence and transformation amenability.

We tested if surfactin, iturin A, and fengycin exhibited synergistic effects by determining the FIC, but did not observe synergistic activity between any two compounds (Table 2). Because the endpoint of FIC requires complete inhibition, the combination of any two compounds might partially inhibit growth but does not reach complete inhibition. Meanwhile, these results may also indicate that other compounds or proteins secreted from Ba01 may enhance the effects of surfactin, iturin A, or

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fengycin on growth suppression of *S. scabies*. Previous studies showed that *Bacillus* species secrete compounds such as macrolactin A, bacillaene, and difficidin in the presence of plant pathogens (33-35). Therefore, additional studies to identify other secondary metabolites or proteins secreted from *B. amyloliquefaciens* are warranted. Interestingly, several studies have demonstrated that *B. amyloliquefaciens* not only can suppress diseases but also promote plant growth (20, 36). Nevertheless, in this study Ba01 only suppressed scab symptoms and did not promote potato plant growth or increase tuber weight.

In addition to secreting antibacterial compounds against *S. scabies*, it is possible that Ba01 elicits ISR of the potato plant. Previous studies have shown that *Bacillus* isolates can elicit ISR of various plant hosts, including tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco, cucumber, and loblolly pine, to combat pathogens (37-39). For example, Chowdhury and colleagues found that cyclic lipopeptides and volatiles produced by *B. amyloliquefaciens* FZB42 can trigger ISR pathways and protect plants against pathogens (40). Future studies involving pot experiments and field trials can address whether Ba01 triggers ISR signaling.

The less potent inhibitory effects of Ba01 against potato common scab in the field trial than those in the pot assays may be due to the complicated microbial community in the soil of the field trial. We isolated several *S. scabies* isolates from scabby potato

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tubers in the field and found that these isolates were pathogenic in potato tuber slice assays. However, these *S. scabies* isolates were less susceptible to Ba01 than *S. scabies* PS07, which was used in the pot assays, suggesting that *S. scabies* isolates in the field were relatively tolerant to Ba01 and that Ba01 demonstrates differential inhibitory effects against various *S. scabies* isolates. In the future, we may consider combining two or more *B. amyloliquefaciens* isolates in order to control multiple *Streptomyces* isolates in the field. Meanwhile, additional field tests with diverse moisture, temperature, soil pH, and environmental conditions can be conducted in order to test the efficacy of Ba01 in various situations.

In summary, we present the first report that *B. amyloliquefaciens* reduces symptoms of naturally occurring potato common scab. The potential mechanisms by which Ba01 inhibits the growth of *S. scabies* at least in part is through the secretion of surfactin, iturin A, or fengycin. The evidence that Ba01 inhibits the growth and sporulation of *S. scabies* and reduces scab symptoms in pot assays and field trials suggests that Ba01 is a potential biocontrol agent for controlling potato common scab.

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5. Tables

Table 1. Strains used in this study.

Strain	Species	Source
Ba01	*Bacillus amyloliquefaciens*	Houli, Taiwan
Ba02	*Bacillus amyloliquefaciens*	Houli, Taiwan
Ba03	*Bacillus amyloliquefaciens*	Houli, Taiwan
Ba04	*Bacillus amyloliquefaciens*	Houli, Taiwan
Bs01	*Bacillus subtilis*	Douliu, Taiwan
B. sp.	*Bacillus sp.*	Dounan, Taiwan
PS01	*Streptomyces scabies*	(41)
PS02	*Streptomyces scabies*	(41)
PS07	*Streptomyces scabies*	(41)
PS08	*Streptomyces scabies*	(41)
YC1020	*Streptomyces scabies*	(41)
YC1028	*Streptomyces scabies*	(41)
A3(2)	*Streptomyces coelicolor*	(42)
CL2	*Streptomyces scabies*	Dounan, Taiwan
CL3	*Streptomyces scabies*	Dounan, Taiwan
CL4	*Streptomyces scabies*	Dounan, Taiwan
CL5	*Streptomyces scabies*	Dounan, Taiwan

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Table 2. Minimum inhibitory and fractional inhibitory concentrations of compounds against *Streptomyces scabies* PS07.

Strain	MIC alone (μg/mL)			MIC combined (μg/mL)			*FIC index		
	Iturin A	Surfactin	Fengycin	Iturin A, Surfactin	Iturin A, Fengycin	Surfactin, Fengycin	Iturin A + Surfactin	Iturin A + Fengycin	Surfactin + Fengycin
PS07	>64	>64	>64	>64, >64	>64, >64	>64, >64	2	2	2

*FIC ≤0.5 (synergy); FIC >0.5 but ≤4 (no interaction); FIC >4 (antagonism)

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6. Figure legends

Figure 1. *B. amyloliquefaciens* Ba01 showed antibacterial activity against *S. scabies* causing potato common scab. A. Phylogenetic trees based on either the (a) 16S rRNA sequence or (b) *gyrA* gene sequence showed the evolutionary relationships between *Bacillus* isolates. The numbers at the nodes represent bootstrap values. The scale bars indicating the numbers of substitutions per nucleotide position were (a) 0.005 and (b) 0.02. **B.** Multiple *Bacillus* isolates exhibited a diverse degree of antibacterial activity against *S. scabies* PS07. Disk diffusion assays were used to test the antibacterial activity of *Bacillus* species against *S. scabies*. In this experiment, 10^6 *S. scabies* spores in 100 μL were spread on solid YME medium, and 3 μL of 1 OD_{600} (~$6x10^4$ cells) of *Bacillus* isolates were loaded on the right disk, while 3 μL of ddH_2O were loaded on the left disk as a control. **C.** Ba01 was selected to test its antibacterial activity against multiple *S. scabies* isolates. *S. scabies* isolates were spread on solid YME medium, and 3 μL of 1 OD_{600} of Ba01 were added to the right disk and ddH_2O to the left disk. All plates were incubated at 28℃ for five days and photographed.

Figure 2. *B. amyloliquefaciens* Ba01 inhibited the growth and sporulation of *S. scabies* PS07. A. A disk diffusion assay was used to observe the antibacterial effects of Ba01 against *S. scabies* PS07 on solid YME medium. Clear (C) and undifferentiating (U)

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zones were observed around the disk loaded with the Ba01 isolate. **B.** The morphology of Ba01 from panel A was observed under a scanning electron microscope. **C.** *S. scabies* PS07 without Ba01 treatment (ddH_2O treated) produced spiral hyphae and sporulation septa with constrictions. **D.** *S. scabies* PS07 in the undifferentiating zone exhibited vegetative/smooth hyphae and septa without constrictions. Resolution is 10,000X. The scale bar represents 1 μm.

Figure 3. Imaging mass spectrometry of Ba01 against *S. scabies* PS07. A. (a) *S. scabies* PS07 was initially inoculated in a vertical line on a 2% YME solid agar plate. After 12 h, *B. amyloliquefaciens* Ba01 was inoculated in a horizontal line on the same plate for another 24 h. (b) The IMS image of an ion with *m/z* 1046.38 represents surfactin. (c) The image represents two-fold serial diluted surfactin as a standard control ranging from 2.5 to 0.04 μg. (d) The IMS image of an ion with *m/z* 1095.76 represents iturin A. (e) The image represents two-fold serial diluted iturin A as a standard control ranging from 10 to 0.16 μg. (f) The IMS image of an ion with *m/z* 1516.19 represents fengycin. (g) The image represents two-fold serial diluted fengycin as a standard control ranging from 0.32 to 0.005 μg/mL. Intensity gradients for surfactin, iturin A, and fengycin are normalized and illustrated by color histogram (maximum, white; minimum, black). **B.** The mass spectra of IMS regions include three major peaks: *m/z* 1046.38

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(surfactin), 1095.76 (iturin A), and 1516.19 (fengycin).

Figure 4. Surfactin, iturin A, and fengycin inhibited the growth and formation of spiral hyphae of *S. scabies* PS07. A. Disk diffusion assays were used to test the anti-*S. scabies* activity of surfactin, iturin A, and fengycin. Approximately 10^6 *S. scabies* spores were spread on YME solid agar, and a 6-mm disk containing iturin A (dissolved in ethanol), surfactin (dissolved in methanol), or fengycin (dissolved in methanol) were pressed on the surface of an agar plate and incubated at 28℃ for five days. **B.** The morphology of *S. scabies* PS07 in the undifferentiating zone of panel A was observed under a scanning electron microscope. (a) The magnification is 5,000X, and the scale bar represents 10 μm. (b) The magnification is 15,000X, and the scale bar represents 2.5 μm.

Figure 5. Ba01 reduced the disease severity of potato common scab in pot assays. The growth conditions of *S. scabies* PS07, Ba01, and potato plants were described in the materials and methods. **A.** Three five-week-old potato plants were used for each treatment: (a) mock control without inoculation of *S. scabies* PS07 or Ba01; (b) inoculation of *S. scabies* PS07 only; (c) inoculation of Ba01 only; (d) inoculation of PS07 and Ba01 on the same day; and (e) inoculation of PS07 first, and then Ba01

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inoculation after 14 days. The bar represents 1 cm. **B.** The disease severity and disease incidence of the potato common scab were reduced when potato plants were inoculated with PS07 and Ba01 on the same day. Data were expressed as the average of tubers collected from three potato plants ± standard error of the mean. *P* values were calculated using Tukey's test. Asterisks (**) indicate $P < 0.01$ as compared to the treatment of PS07 only.

Figure 6. Ba01 reduced the severity of naturally occurring potato common scab in the field. A. (a) An 11-week potato field trial was completed in Dounan, Taiwan. Bars = 100 cm. (b) Four treatments were treated with the Ba01 isolate at the concentrations indicated. (c) Each treatment had four blocks assigned by a randomized complete block design. **B.** Potato tubers were harvested from each Ba01 treatment: (1) Ba01 at $5x10^6$ CFU/mL; (b) $1x10^7$ CFU/mL; (c) $2x10^7$ CFU/mL; and (d) water. Bars = 5 cm. **C.** The percentage of disease severity was calculated from 100 randomly selected tubers of each treatment. **D.** Disease incidence was calculated by determining the proportion of tubers with >5% scab coverage from 100 randomly selected tubers in each treatment. **E.** Potato plant height (left panel) and tuber weight (right panel) were not affected by Ba01 application. We randomly chose 25 potato tubers from each block to evaluate the disease severity and incidence of each block, and four blocks of the same treatment

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were used to determine the mean ± standard error and compare to other treatments. *P* values were calculated using Tukey's test. Asterisks *, **, and *** represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Figure 7. Ba01 inhibited the growth of multiple *S. scabies* strains isolated from the field trial. A. Potato slide assays were used to test the pathogenicity of *S. scabies* isolates. Agar disks with or without *S. scabies* spores were inverted onto potato tuber slices and incubated at 28℃ for six days in the dark and photographed. Nonpathogenic *Streptomyces coelicolor* was used as a negative control, and a MPYSC agar disc was used as a mock control. **B.** Disc diffusion assays were used to test the anti-bacterial activity of Ba01 and three pure compounds against multiple *S. scabies* strains.

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7. Figures

Figure 1

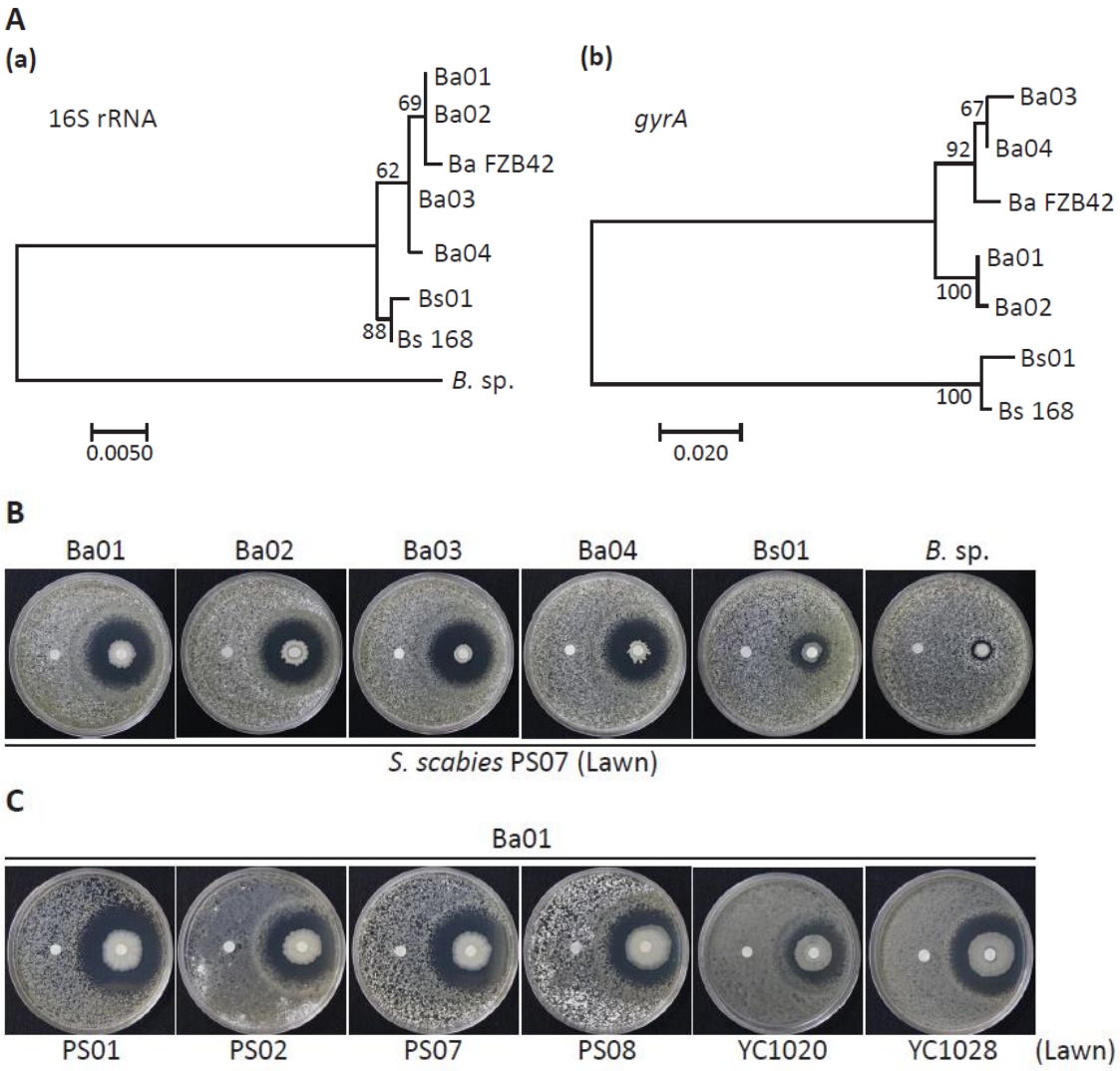

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Figure 2

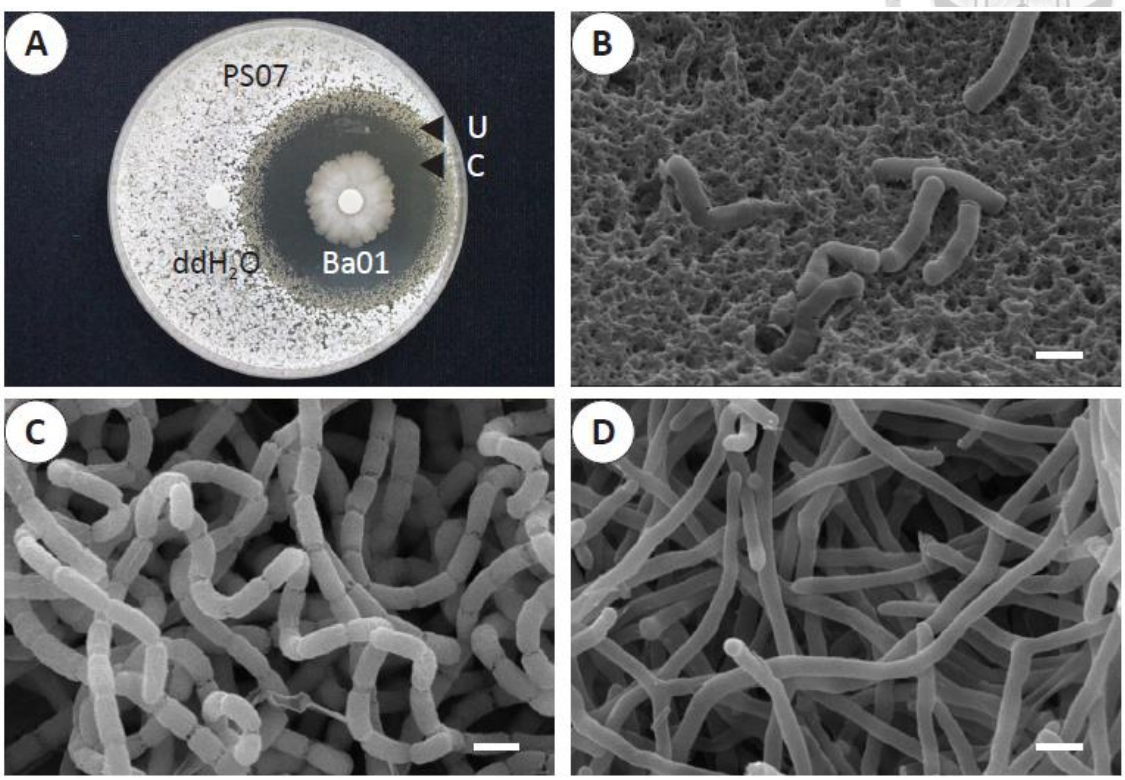

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Figure 3

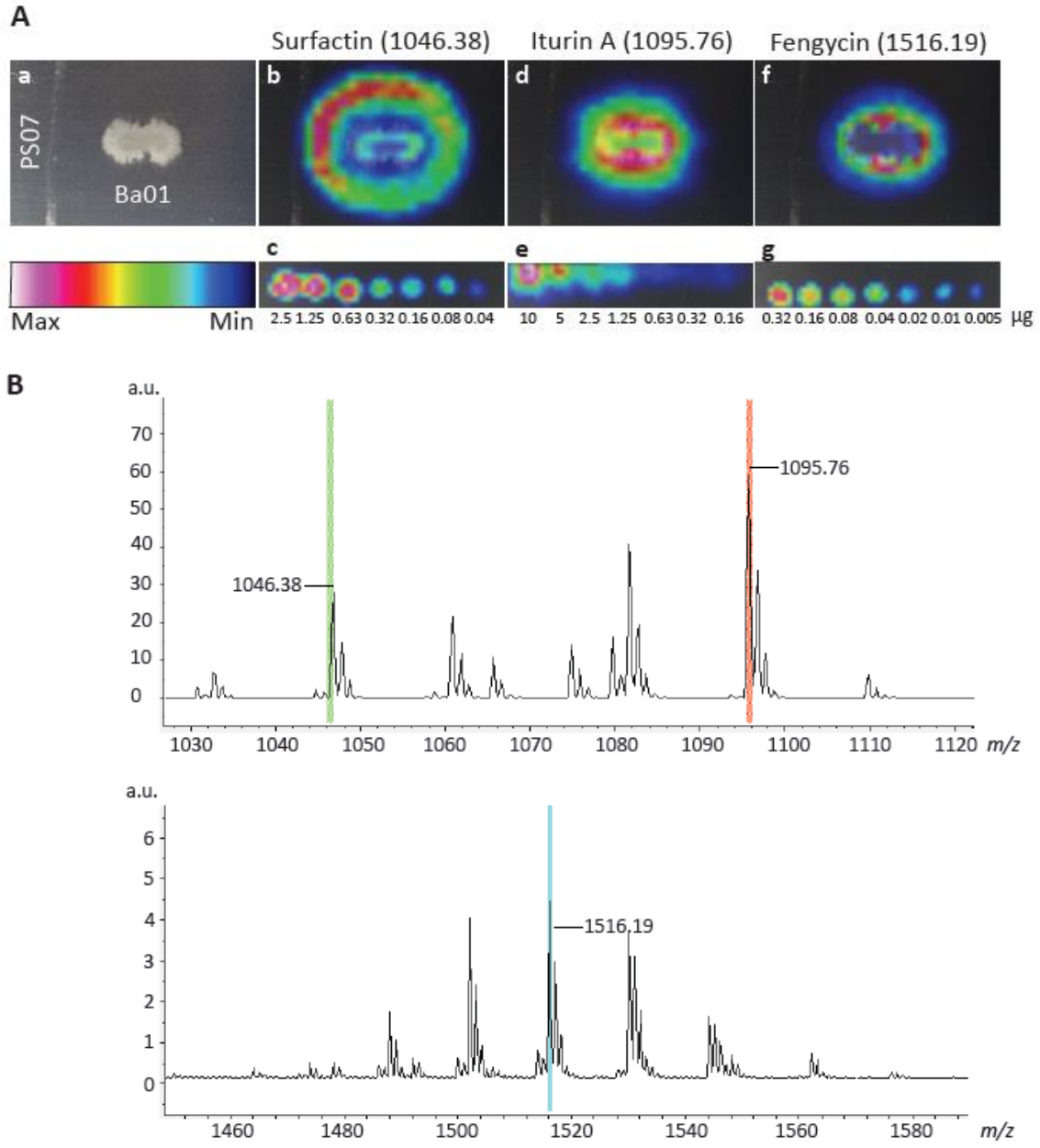

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Figure 4

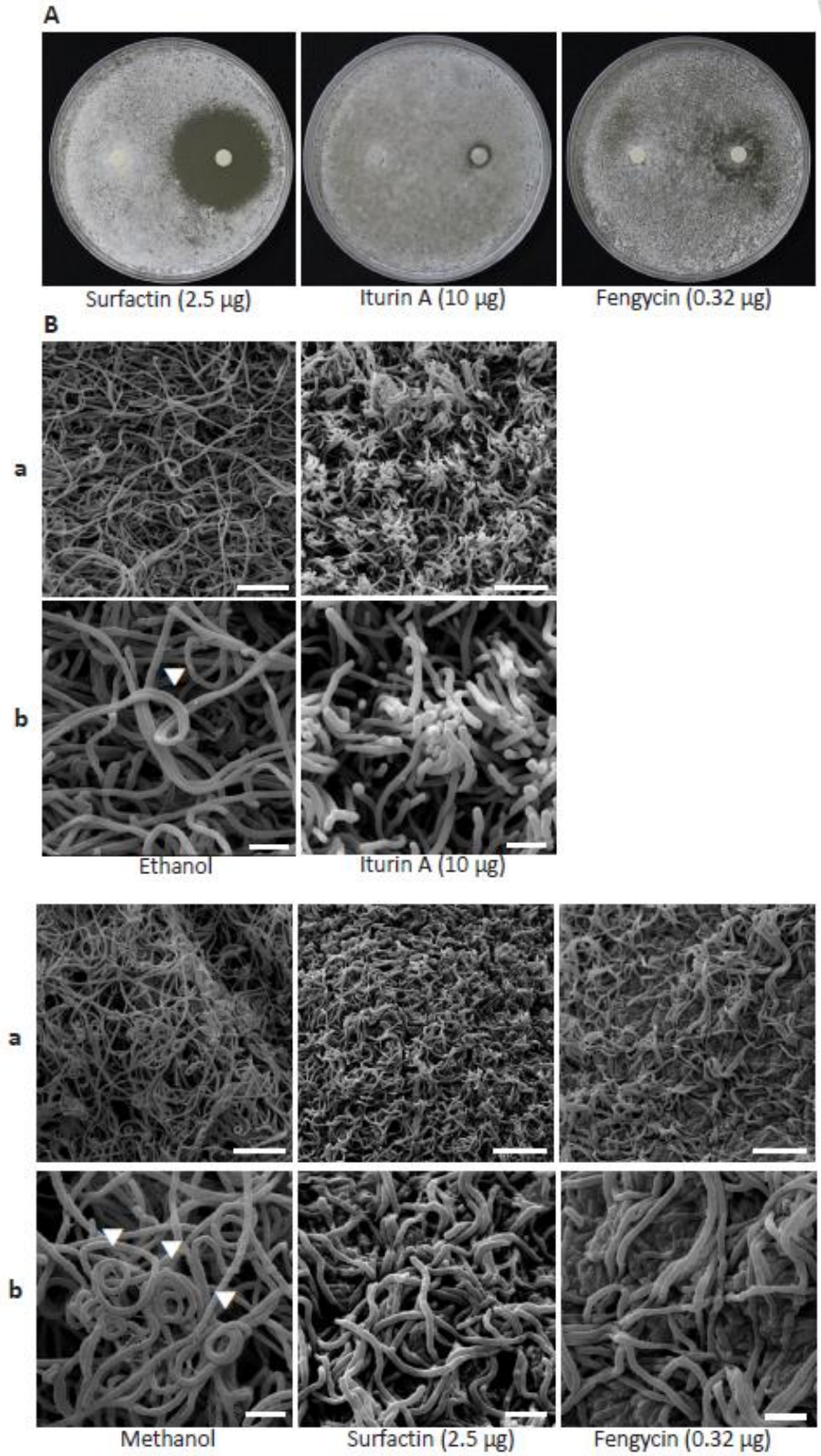

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Figure 5

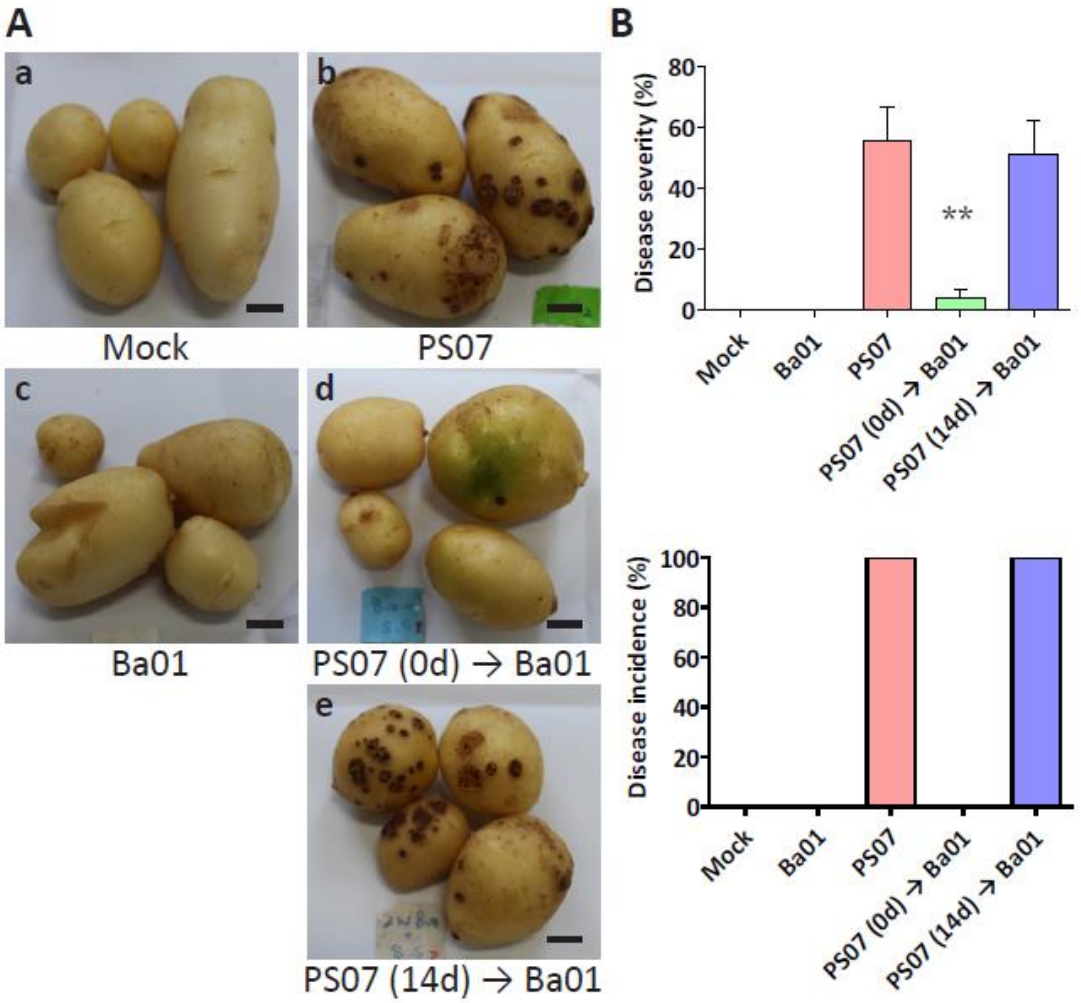

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Figure 6

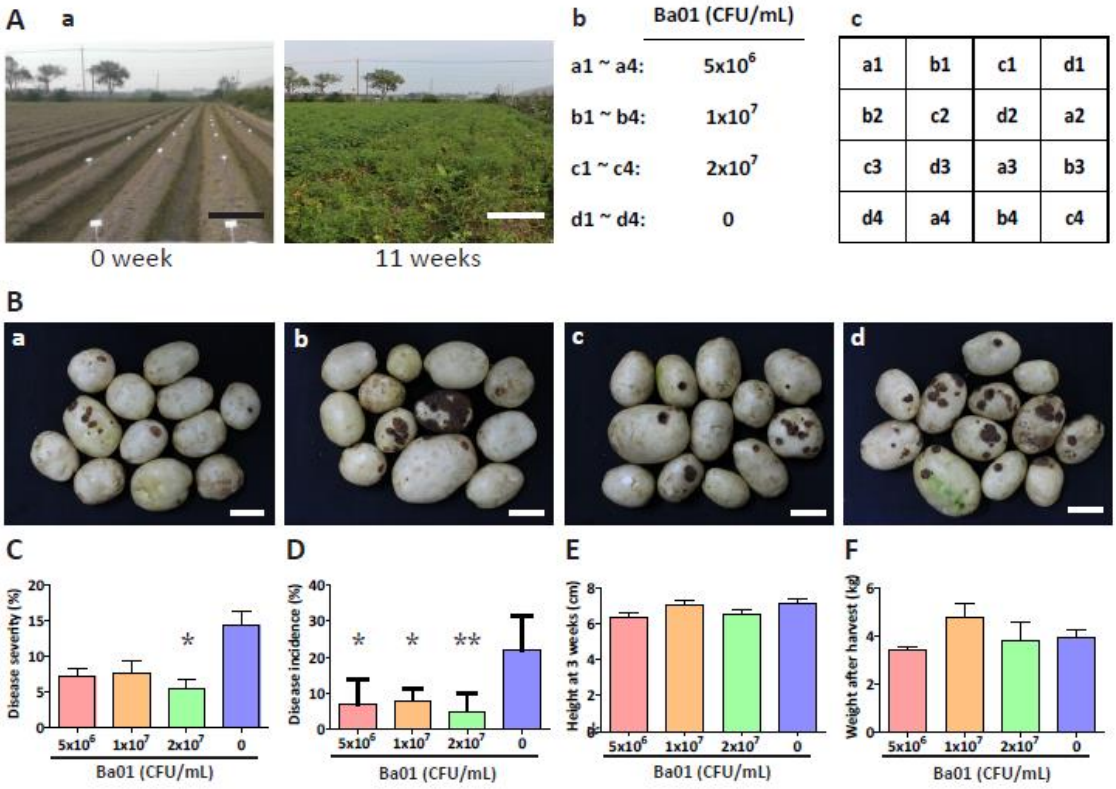

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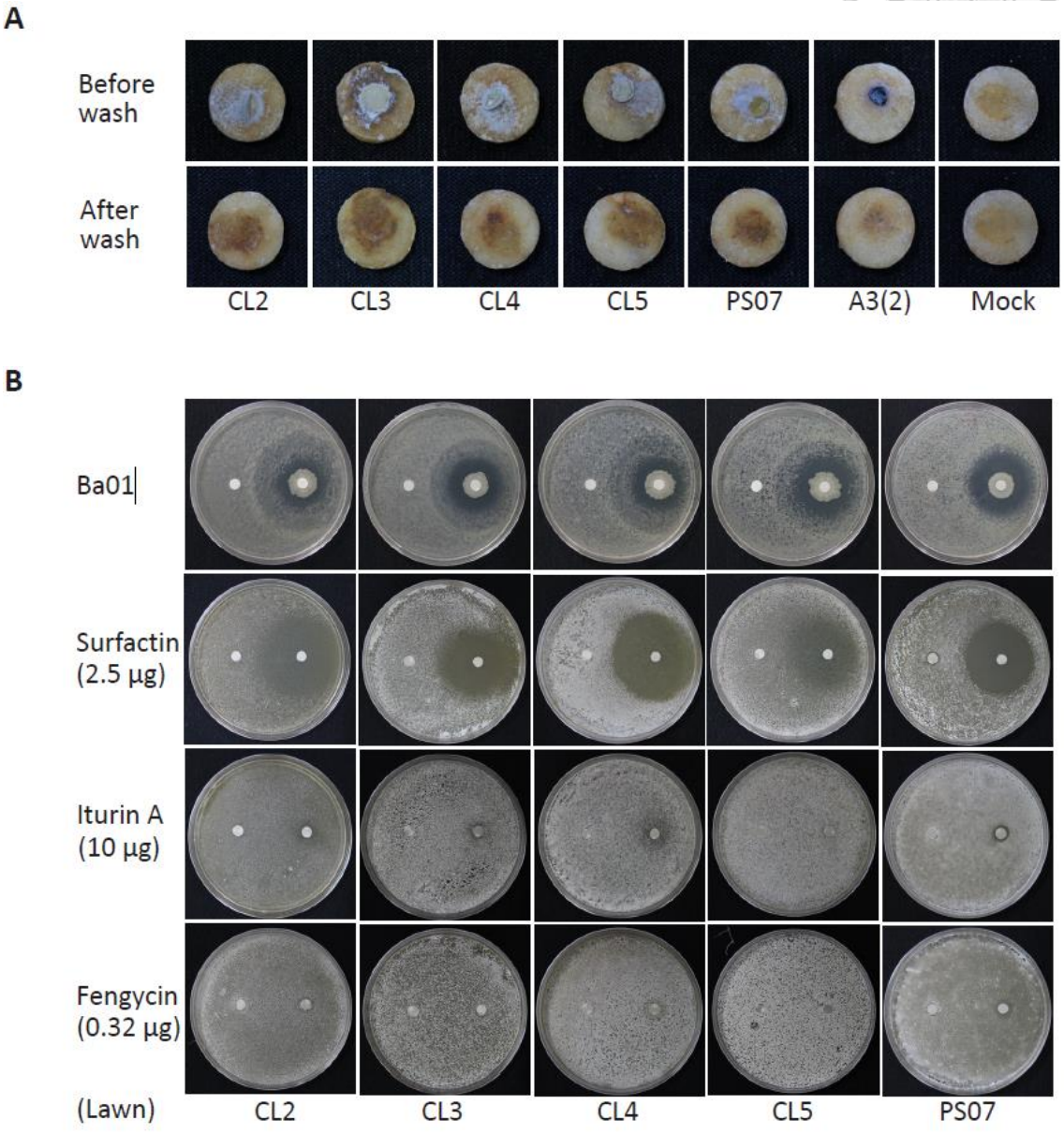
Figure 7



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9. Appendix

9.1 Supplementary Materials and Methods

Attempts to obtain mutants via homologous recombination

1. Construction of *srfA*D, *ituD,* and *fenA* disruption cassettes

We attempted to generate all *Bacillus amyloliquefaciens* Ba01 deletion mutants by homologous recombination. To disrupt the surfactin synthesis gene *srfA*D, approximately 1 kb of the 5' and 3' noncoding regions (NCRs) of the *srfA*D ORF were PCR amplified with primers JC1548/JC1549 (for the 5' NCR) or JC1551/JC1552 (for the 3' NCR) from Ba01 genomic DNA. The 5' and 3' NCR PCR products of the *srfA*D gene were digested with the restriction enzymes BspEI and BamHI/HindIII, respectively. The BamHI/HindIII-digested PCR products of the 3' *srfA*D^{NCR} were purified using the Gel/PCR DNA Isolation System kit (Viogene, Taiwan) and then cloned into plasmid pMiniMAD (which carries the erythromycin resistance gene *Erm*^{R}; Table S2), resulting in plasmid pCL13 (Table S2). The BspEI-digested 5' *srfA*D^{NCR} PCR product was purified and cloned into pCL13 to create the *srfA*D disruption plasmid pCL21 (Table S2). The pCL21 plasmid was PCR amplified with primers JC1548/JC1552 to obtain the 5' *srfA*D^{NCR}-*Erm*^{R}-3' *srfA*D^{NCR} disruption cassette to transform into Ba01.

A similar approach was used to disrupt the iturin A synthesis gene *ituD*. To disrupt

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ituD, approximately 1 kb of the 5’ and 3’ NCRs of the *ituD* ORF were PCR amplified with primers JC1553/JC1554 (for the 5’ NCR) or JC1555/JC1556 (for the 3’ NCR) from Ba01 genomic DNA. The 5’ and 3’ NCR PCR products of the *ituD* gene were digested with the restriction enzymes BspEI and BamHI/HindIII, respectively. The BamHI/HindIII-digested PCR product of 3’ *ituD*^{NCR} was purified using the Gel/PCR DNA Isolation System kit and then cloned into the plasmid pMiniMAD, resulting in plasmid pCL15 (Table S2). The BspEI-digested 5’ *ituD*^{NCR} PCR product was purified and cloned into pCL15 to create the *ituD* disruption plasmid pCL19 (Table S2). The pCL19 plasmids were PCR amplified with primers JC1550/JC1556 to obtain the 5’ *ituD*^{NCR}-*Erm*^{R}-3’ *ituD*^{NCR} disruption cassette to transform into Ba01.

To disrupt the fengycin synthesis gene *fenA*, approximately 1 kb of the 5’ and 3’ NCRs of the *fenA* ORF were PCR amplified with primers JC1557/JC1558 (for the 5’ NCR) and JC1559/JC1560 (for the 3’ NCR) from Ba01 genomic DNA. The 5’ and 3’ NCR PCR product of the *fenA* gene was digested with the restriction enzymes BspEI and EcoRI/HindIII, respectively. The EcoRI/HindIII-digested PCR product of 3’ *fenA*^{NCR} was purified using the Gel/PCR DNA Isolation System kit and then cloned into the plasmid pMiniMAD, resulting in plasmid pCL18 (Table S2). The BspEI-digested 5’ *fenA*^{NCR} PCR product was purified and cloned into pCL18 to create the *fenA* disruption plasmid pCL23 (Table S2). The pCL23 plasmid was PCR amplified with primers

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JC1557/JC1560 to obtain the 5' *fenA*^{NCR}-*Erm*^{R}-3' *fenA*^{NCR} disruption cassette to transform into Ba01.

We attempted to generate *Bacillus amyloliquefaciens* FZB42 deletion mutants by homologous recombination. To disrupt the surfactin synthesis gene *srfA*D, approximately 1 kb of the 5' and 3' noncoding regions (NCRs) of the *srfA*D ORF were PCR amplified with primers JC1548/JC1549 (for the 5' NCR) or JC1551/JC1552 (for the 3' NCR) from Ba01 genomic DNA. The 5' and 3' NCR PCR products of the *srfA*D gene were digested with the restriction enzymes BspEI and BamHI/HindIII, respectively. The BamHI/HindIII-digested PCR products of the 3' *srfA*D^{NCR} were purified using the Gel/PCR DNA Isolation System kit (Viogene, Taiwan) and then cloned into plasmid pMiniMAD (which carries the erythromycin resistance gene *Erm*^{R}; Table S2), resulting in plasmid pCL25 (Table S2). The BspEI-digested 5' *srfA*D^{NCR} PCR product was purified and cloned into pCL25 to create the *srfA*D disruption plasmid pCL28 (Table S2). The pCL28 plasmid was PCR amplified with primers JC1548/JC1552 to obtain the 5' *srfA*D^{NCR}-*Erm*^{R}-3' *srfA*D^{NCR} disruption cassette to transform into Ba01.

2. Transformation of *B. amyloliquefaciens* Ba01

For the competence assay, Ba01 was grown in 2 mL of 1X modified competence

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(MC) medium supplemented with 20 μL of 300 mM $MgSO_4$ for 4.5 h at 37℃. MC medium (1X) was made with a solution containing 100 mM K_2HPO_4, 100 mM KH_2PO_4, 2% glucose, 22 mg/L ferric ammonium citrate, 3 mM trisodium citrate, 0.1% casein hydrolysate, and 0.2% potassium glutamate. Then, 3 μg of the disruption cassette were added into 400 μL of the culture in an eppendorf tube. The culture was grown an additional 1.5 h at 37°C and plated onto LB containing 1 μg/mL erythromycin and 25 μg/mL lincomycin and grown for three days at 28℃. Colony PCR was used to analyze *srfA*D, *ituD*, and *fenA* mutants with primers JC1468/JC1469, JC1466/JC1467, or JC1470/JC1471, respectively (1).

Attempts to obtain mutants via an in-frame deletion strategy

1. Construction of *srfA*D, *ituD*, and *fenA* disruption cassettes

To disrupt the *srfA*D gene, approximately 1 kb of the 5' and 3' NCRs of the *srfA*D ORF was PCR amplified with primers JC1458/JC1459 (for the 5' NCR) or JC1460/JC1461 (for the 3' NCR) from Ba01 genomic DNA. The 5' and 3' NCR PCR products of the *srfA*D gene were digested with two restriction enzymes (BamHI/XhoI and XhoI/KpnI, respectively). The two fragments were simultaneously ligated with the BamHI and KpnI sites of pMiniMAD to generate pCL7.

A similar approach was used to disrupt the *ituD* gene. To disrupt the *ituD* gene,

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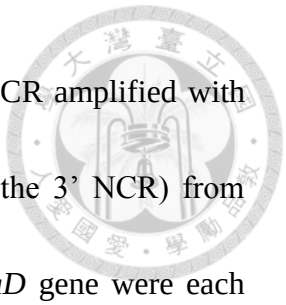

approximately 1 kb of the 5’ and 3’ NCRs of the *ituD* ORF was PCR amplified with primers JC1454/JC1455 (for the 5’ NCR) or JC1456/JC1457 (for the 3’ NCR) from Ba01 genomic DNA. The 5’ and 3’ NCR PCR products of the *ituD* gene were each digested with two restriction enzymes (HindIII/XhoI and XhoI/KpnI, respectively). The two fragments were simultaneously ligated with the HindIII and KpnI sites of pMiniMAD to generate pCL9.

A similar approach was used to disrupt the *fenA* gene. To disrupt the *fenA* gene, approximately 1 kb of the 5’ and 3’ NCR of the *fenA* ORF was PCR amplified with primers JC1462/JC1463 (for the 5’ NCR) or JC1464/JC1465 (for the 3’ NCR) from Ba01 genomic DNA. The 5’ and 3’ NCR PCR products of the *fenA* gene were digested with two restriction enzymes (BamHI/XhoI and XhoI/HindIII, respectively). The two fragments were simultaneously ligated with the BamHI and HindIII sites of pMiniMAD to generate pCL11.

To disrupt the *srfA*D gene, approximately 1 kb of the 5’ and 3’ NCRs of the *srfA*D ORF was PCR amplified with primers JC1458/JC1459 (for the 5’ NCR) or JC1460/JC1461 (for the 3’ NCR) from FZB42 genomic DNA. The 5’ and 3’ NCR PCR products of the *srfA*D gene were digested with two restriction enzymes (BamHI/XhoI and XhoI/KpnI, respectively). The two fragments were simultaneously ligated with the BamHI and KpnI sites of pMiniMAD to generate pCL26.

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2. Transformation of *B. amyloliquefaciens* Ba01

We introduced 3 μg of plasmid pCL7 into Ba01 by transformation as described above, and two colonies were chosen to grow in 3 mL LB liquid medium containing 1 μg/mL erythromycin and 25 μg/mL lincomycin overnight at 28℃ for plasmid replication. The cultures were serially diluted and plated onto LB medium containing erythromycin and lincomycin overnight at 37℃ (restrictive temperature for plasmid replication). To evict the integrated plasmid, three colonies were incubated in 3 mL LB broth for 12 h at 25℃ and subcultured 1:100 in fresh LB broth. The cultures were incubated at 25℃ for an additional 12 h. Subcultures were repeated two more times, and the final serially diluted solution was plated onto LB medium at 37℃ overnight. Colonies were patched onto LB plates and LB containing erythromycin and lincomycin, and drug-sensitive colonies representing potential mutants were chosen to conduct colony PCR with primers JC1468/JC1469, JC1466/JC1467, or JC1470/JC1471 for *srfA*D, *ituD*, or *fenA* ORFs, respectively.

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9.2 Supplementary Tables

Table S1. PCR primers used in this study.

Primer	Use	Sequence (5'-> 3')
fD1	16s rRNA	AGAGTTTGATCCTGGCTCAG
rP2	16s rRNA	ACGGCTACCTTGTTACGACTT
p-*gyr*A-F	*gyrA*	CAGTCAGGAAATGCGTACGTCCTT
p-*gyr*A-R	*gyrA*	CAAGGTAATGCTCCAGGCATTGCT
atpDPF	*atpD*	GTCGGCGACTTCACCAAGGGCAAGGTG TTCAACACC
atpDPR	*atpD*	GTGAACTGCTTGGCGACGTGGGTGTTCT GGGACAGGAA
JC1458	5' NCR of *srfAD* (BamHI)	aggagggatccCGGCGAAAGAATGGATCGGG
JC1459	5' NCR of *srfAD* (XhoI)	aggagctcgagCTGGACCATTGGCGGGCTTC
JC1460	3' NCR of *srfAD* (XhoI)	aggagctcgagTTGTTATAGGATATGACAGA CAGC
JC1461	3' NCR of *srfAD* (KpnI)	aggagggtaccCGCGTAATTTTCCTTCGTC
JC1454	5' NCR of *ituD* (HindIII)	aggagaagcttCGTAAACATTCAAAATGGCG GA
JC1455	5' NCR of *ituD* (XhoI)	aggagctcgagTTAAAATAAAGCGCCCAGGA
JC1456	3' NCR of *ituD* (XhoI)	aggagctcgagATTGTTCATGAGATTCCTCC
JC1457	3' NCR of *ituD* (KpnI)	aggagggtaccTCAACGGACTGATCGGTTTT
JC1462	5' NCR of *fenA* (BamHI)	aggagggatccCCGTCTGAACGTCCTAGCCA
JC1463	5' NCR of *fenA* (XhoI)	aggagctcgagGAAAGCATGGTCGGCGTGCT
JC1464	3' NCR of *fenA* (XhoI)	aggagctcgagGTTCTTCAATGGAATCCCTCC
JC1465	3' NCR of *fenA* (HindIII)	aggagaagctt CGGACATCCATGCCTCTTTC
JC1468	*srfAD* ORF	aaaCCGCCGTTGAGGATTTTGAA
JC1469	*srfAD* ORF	aaaCATGTGGCCGTCCGAAAACT
JC1466	*ituD* ORF	aaaAGTGTATGCCGCACCCTTTT
JC1467	*ituD* ORF	aaaGAGCGATGCGATCTCCTTGG
JC1470	*fenA* ORF	aaaGCGAGAGGCTGGTATTGCAT
JC1471	*fenA* ORF	aaaGAACACCTTTCACTGGCGGA
JC1548	5' NCR of *srfAD* (BspEI)	aaatccggaGTTAACGACAAACGGGAAGG
JC1549	5' NCR of *srfAD* (BspEI)	aaatccggaCTGGACCATTGGCGGGCTTC
JC1550	Check direction of 5' NCR	AGACAATCTCCCGTCCTCTGTT
JC1551	3' NCR of *srfAD* (BamHI)	aaaggatccTTGTTATAGGATATGACAGACA GC
JC1552	3' NCR of *srfAD* (HindIII)	aaaaagcttCGCGTAATTTTCCTTCGTC

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JC1553	5’ NCR of *ituD* (BspEI)	aaatccggaCGTAAACATTCAAAATGGCGGA
JC1554	5’ NCR of *ituD* (BspEI)	aaatccggaTTAAAATAAAGCGCCCAGGA
JC1555	3’ NCR of *ituD* (BamHI)	aaaggatccATTGTTCATGAGATTCCTCC
JC1556	3’ NCR of *ituD* (HindIII)	aaaaagcttTCAACGGACTGATCGGTTTT
JC1557	5’ NCR of *fenA* (BspEI)	aaatccggaCCGGGCGAAGATGTCTTGTA
JC1558	5’ NCR of *fenA* (BspEI)	aaatccggaTGCAAGGGCAGTTTCCGTTA
JC1559	3’ NCR of *fenA* (EcoRI)	aaagaattcGTTCTTCAATGGAATCCCTCC
JC1560	3’ NCR of *fenA* (HindIII)	aaaaagcttAGAAAAGTGGTACCCGGCTT

*Lowercase letters represent restriction enzyme cutting sites, including adenine nucleotides to protect primers.

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Table S2. Plasmids used in this study.

Plasmid	Relevant insert	Parent	Strategy	Strain
pMiniMad		(2)		
pCL13	3'NCR of *srfA*D	pMiniMad	Homologous recombination	Ba01
pCL21	5'NCR of *srfA*D	pCL13	Homologous recombination	Ba01
pCL15	3'NCR of *ituD*	pMiniMad	Homologous recombination	Ba01
pCL19	5'NCR of *ituD*	pCL15	Homologous recombination	Ba01
pCL18	3'NCR of *fenA*	pMiniMad	Homologous recombination	Ba01
pCL23	5'NCR of *fenA*	pCL18	Homologous recombination	Ba01
pCL9	3'NCR and 5'NCR of *srfA*D	pMiniMad	In-frame deletion	Ba01
pCL7	3'NCR and 5'NCR of *ituD*	pMiniMad	In-frame deletion	Ba01
pCL11	3'NCR and 5'NCR of *fenA*	pMiniMad	In-frame deletion	Ba01
pCL25	3'NCR of *srfA*D	pMiniMad	Homologous recombination	FZB42
pCL28	5'NCR of *srfA*D	pCL25	Homologous recombination	FZB42
pCL26	3'NCR and 5'NCR of *srfA*D	pMiniMad	In-frame deletion	FZB42

9.3 References

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9.4 Supplementary Figures

Figure S1

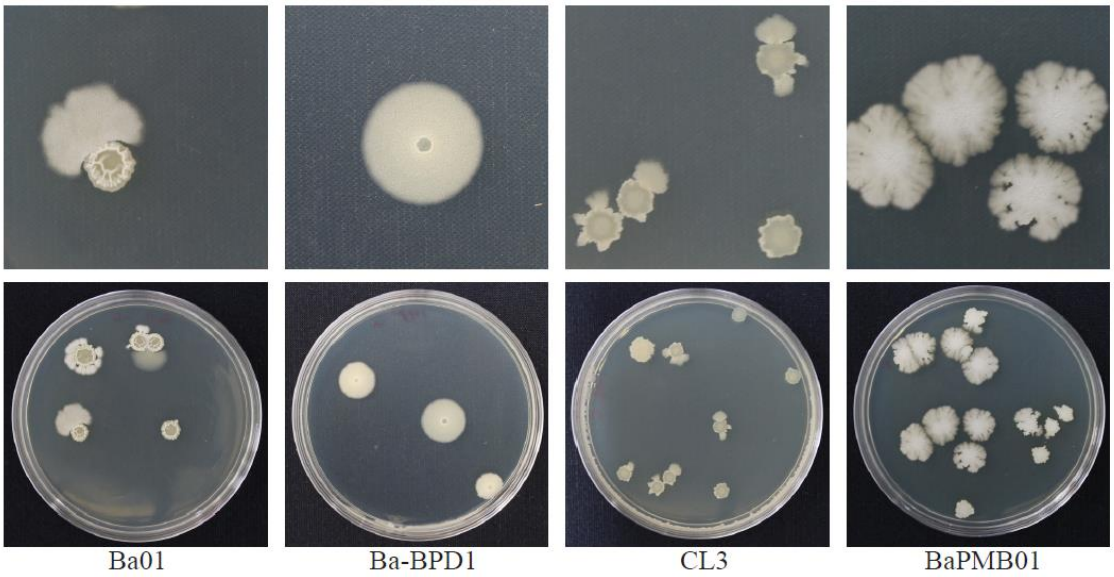

Figure S1. The colony morphology of Ba01 on LB is distinct from those of commercial *B. amyloliquefaciens* strains. *B. amyloliquefaciens* isolates were serially diluted and spread on LB medium. The plates were incubated at 37℃ for 2 days.

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Figure S2

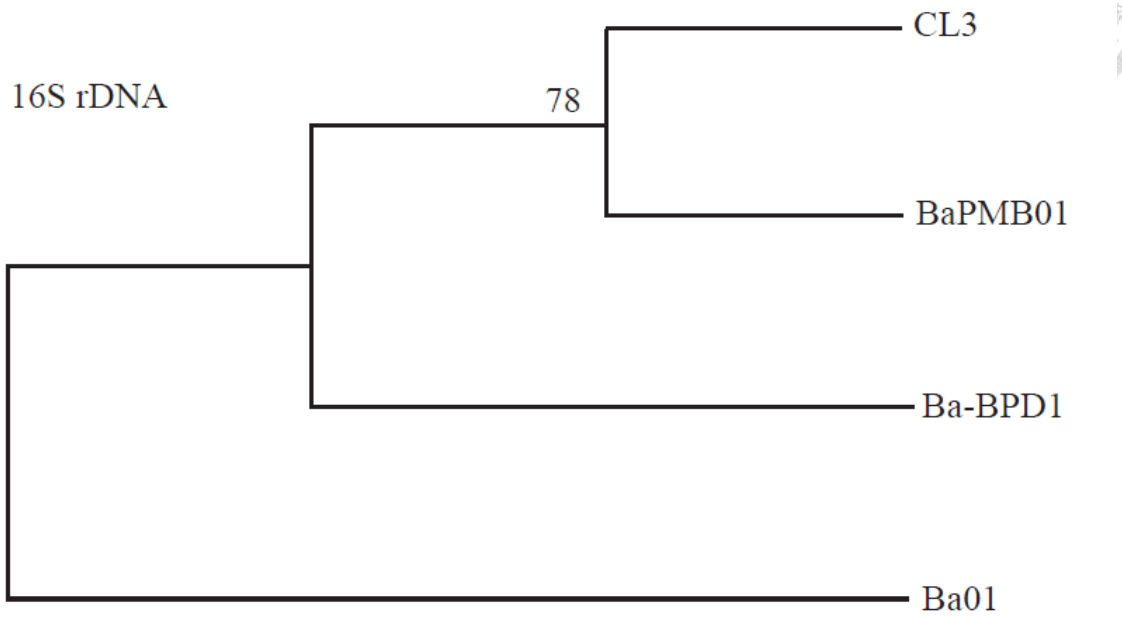

Figure S2. Ba01 is distinct from those of commercial *B. amyloliquefaciens* strains based on phylogenetic tree analysis. A. Phylogenetic trees based on the 16S rDNA sequence showed the evolutionary relationships between *B. amyloliquefaciens* isolates. Numbers at the nodes represent the bootstrap values.

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Figure S3

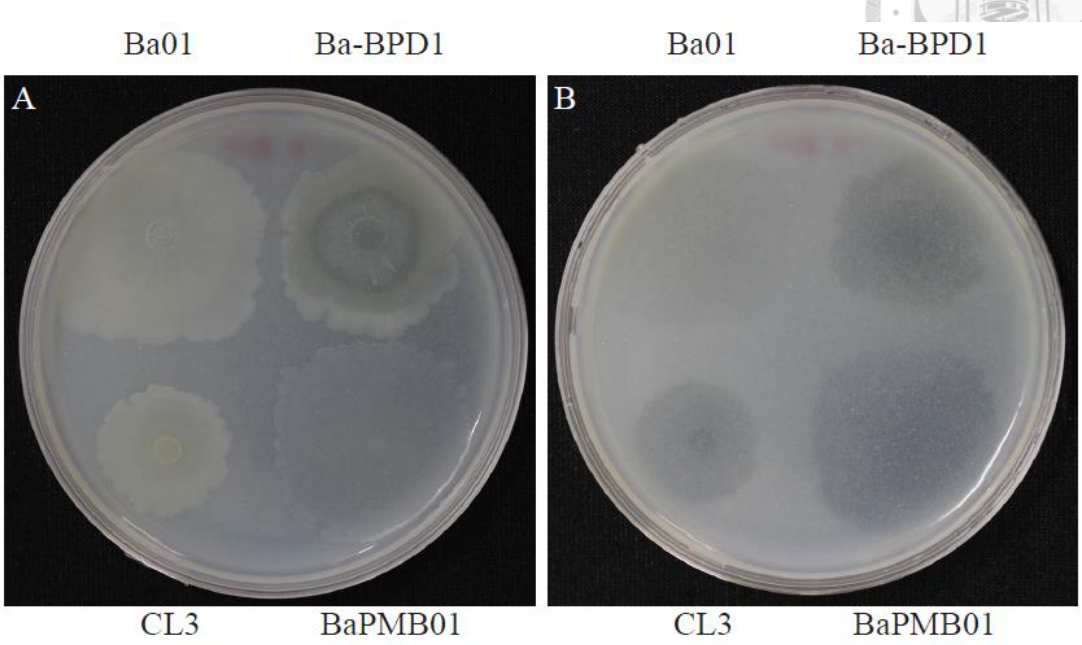

Figure S3. Ba01 is distinct from those of commercial *B. amyloliquefaciens* strains based on phosphate-solubilizing activity analysis. *B. amyloliquefaciens* isolates were grown overnight at 37℃ in LB liquid medium, and the cell concentrations were calculated by measuring OD_{600}. The 5 μL OD_{600} = 1 of *B. amyloliquefaciens* isolates were dropped on Pikovskayas agar medium. The plates were incubated at 37℃ for 5 days. Pikovskayas agar medium (0.00001% $MnSO_4$, 0.00001% $FeSO_4$, 0.01% $MgSO_4$, 0.02% KCL, 0.05% $(NH_4)_2SO_4$, 0.05% yeast extract, 0.5% $Ca_3(PO_4)_2$, 1% dextrose and 1.5% agar; pH = 7±0.1). **A.** Before and **B.** after *B. amyloliquefaciens* isolates were washed.

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Figure S4

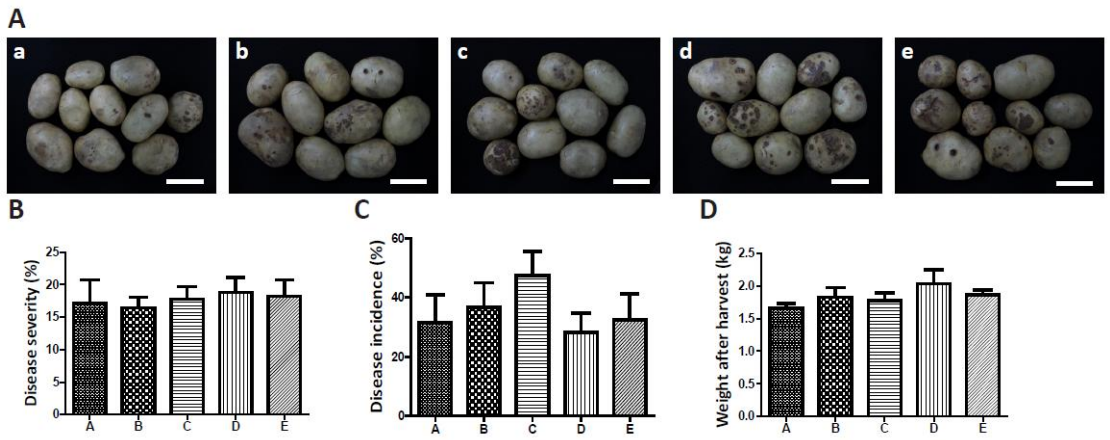

Figure S4. Ba01 and other commercial products can not reduce the severity of potato common scab in low concentration in the field. A. (a) An 11-week potato field trial was completed in Dounan, Taiwan. Five treatments were treated with the Ba01 isolate at the concentrations indicated. Each treatment had eight blocks assigned by a randomized complete block design. **A.** Potato tubers were harvested from each Ba01 treatment: (1) Ba01 at $5x10^6$ CFU/mL; (b) Ba-BPD1 at $5x10^6$ CFU/mL; (c) CL3 at $5x10^6$ CFU/mL; (d) BaPMB01 at $5x10^6$; and (e) water. Bars = 5 cm. **B.** and **C.** Twenty five potato tubers from each block to evaluate the disease severity and incidence of each block, and eight blocks of the same treatment were used to determine mean ± standard error and compared to other treatments based on Tukey's test. **D.** Potato tuber weight were not affected after application of Ba01. *P* values were calculated using Tukey's test. Asterisks *, **, and *** represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

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9.5 殺真菌劑得克利能抑制馬鈴薯瘡痂病原細菌

林芝[1]、馮如瑩[1]、蔡佳欣[2]、陳穎練[1,3]

[1]國立台灣大學　植物病理與微生物學系

[2]行政院農業委員會農業試驗所 植物病理組

[3]聯絡作者，E-mail：ychen28@ntu.edu.tw

簡題：得克利能抑制馬鈴薯瘡痂病菌

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The fungicide tebuconazole inhibits potato common scab caused by *Streptomyces scabies*

Chih Lin[1], Ru-Ying Feng[1], Chia-Hsin Tsai[2], Ying-Lien Chen[1,3]

[1]Department of Plant Pathology and Microbiology, National Taiwan University, Taipei, Taiwan

[2]Department of Plant Pathology, Taiwan Agricultural Research Institute, Taichung, Taiwan

[3]Correspondence, Email: ychen28@ntu.edu.tw

Running title: Tebuconazole inhibits *Streptomyces scabies*

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摘要

馬鈴薯瘡痂病主要由植物病原細菌馬鈴薯瘡痂病菌 *Streptomyces scabies* 引起，其可在馬鈴薯薯塊表面造成褐色凸起或凹陷的瘡痂，造成市場價格及數量下降。然而現今尚未研發出有效的防治方法。本研究利用最低藥劑濃度測試平台快篩一系列農藥，發現殺真菌劑得克利可有效抑制馬鈴薯瘡痂病菌的生長，其藥物最低抑制濃度值為 64 μg/mL，為全球首次發現。濾紙片擴散實驗發現得克利亦可抑制番茄青枯病菌 *Ralstonia solanacearum* 及人體病原菌金黃色葡萄球菌 *Staphylococcus aureus* 的生長。另發現滅達樂或蓋普丹與得克利共同施用時，對馬鈴薯瘡痂病菌的生長有加乘的抑制作用。於盆栽試驗，先接種 *S. scabies* PS07，一天後再澆灌得克利的組別，可有效降低罹病嚴重度從 54.9 ± 7% (只接種 PS07) 到 11.4 ± 4.5% ($P < 0.001$)，顯示得克利有潛力成為抑制馬鈴薯瘡痂病的防治藥劑。

關鍵詞：馬鈴薯瘡痂病、馬鈴薯瘡痂病菌、得克利、化學防治

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緒言

馬鈴薯瘡痂病是台灣種植馬鈴薯期間重要之病害，此病害由土傳性放線菌病原 *Streptomyces* 屬造成，包含 *S. scabies, S. acidiscabies, S. turgidiscabies, S. europaeiscabiei* 等[1]，當中以 *S. scabies* 最為常見，也是目前發現造成台灣馬鈴薯瘡痂病的主要病原。*S. scabies* 已知可分泌多種植物毒素，包含 thaxtomins, concanamycins, FD-891 及 borrelidin[1, 2]。Thaxtomin A 能抑制植物細胞纖維素的生合成，造成細胞壁形成受阻[3]，使得薯塊表面產生凸起或凹陷的木栓化病斑[4]，嚴重影響收成之馬鈴薯的商業價值。目前管理方法主要利用健康種薯、耐病品種與水稻田輪作、土壤酸鹼度的調整及土壤燻蒸[5-7]，但效果有限。

得克利，為 G1 類抑制麥角固醇 (ergosterol) 生合成的三唑類殺真菌劑，作用目標為真菌之 Cyp51：lanosterol-14α-demethylase (在 *Candida albicans* 裡為 *ERG11* 基因產物)。當三唑類藥物與 lanosterol-14α-demethylase 結合後，會使 lanosterol 無法去甲基化，進而影響麥角固醇的合成，使真菌細胞膜失去正常功能而細胞死亡[8]。在台灣得克利主要用來防治蔬果、花卉及果樹之炭疽病，以及瓜類白粉病、落花生銹病、葡萄晚腐病及茶赤葉枯病等。

在台灣田間，農民主要利用種植水稻田與馬鈴薯輪作來降低瘡痂病的發生，但防治效果有限且目前並無核准使用於此病害防治之藥劑，無法利用化學藥劑來防治，故找尋具潛力之藥劑為此實驗目的。本研究在最低藥劑濃度測試及濾紙片擴散實驗發現得克利可有效抑制 *S. scabies* 的生長，在盆栽試驗也有良好防治效果，說明得克利為有潛力防治馬鈴薯瘡痂病之藥劑。

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材料與方法

試驗菌株、培養基及藥品

表一為本研究使用到的菌株。*S. scabies* 菌株從病薯塊上分離並培養於 NA 固態培養基 (0.3% beef extract, 0.5% peptone 及 1.5% agar) 或 SCAN 固態培養基 (0.001% $FeSO_47.H_2O$, 0.002% $CaCO_3$, 0.005% $MgSO_4.7H_2O$, 0.03% casein, 0.2% K_2HPO_4, 0.2% NaCl, 0.2% KNO_3, 1% starch 及 1.8% agar) ，並用引子 fD1 (AGAGTTTGATCCTGGCTCAG) 和 rP2 (ACGGCTACCTTGTTACGACTT)[9] 定序其 16S rDNA ，並利用 Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) 進行比對。使用白色念珠菌 *Candida albicans* SC5314 的 Cyp51 (Erg11) 胺基酸序列在 tblastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) 上找尋不同菌株的 Cyp51；而 Cyp51 胺基酸序列對列調整(alignment) 則使用 Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/)。

S. scabies 培養在 YME 固態培養基 (0.4% yeast extract, 1% malt extract, 0.4% dextrose, 及 2% agar)，使之大量產孢時另用 MPYSC 固態培養基 (0.05% K_2HPO_4, 0.1% $MgSO_4.7H_2O$, 0.1% casein, 0.1% yeast extract, 1% soluble starch 及 2% agar)。*S. aureus*, *Escherichia. coli* 及 *Pseudomonas aeruginosa* 培養於 LB 液態培養基 (0.5% yeast extract, 1% tryptone 及 1% NaCl) / 固態 (1.5% agar)；*R. solanacearum* 培養於 523 液態培養基 (0.00358% $MgSO_4.7H_2O$, 0.2% KH_2PO_4 , 0.4% yeast extract, 0.8% casamino acid , 及 1% sucrose) 及 CPG 固態培養基 (0.1% casamino acid, 0.5% glucose , 1% peptone 及 1.7% agar)；*Candida albicans* 培養於 YPD 液態培養基 (1% yeast extract, 2% peptone 及 2% glucose) / 固態 (2% agar)。細菌的藥物最低抑制濃度實驗使用的培養基為 Mueller Hinton 液態培養基 (0.15% starch, 0.2% beef extract 及 1.75% acid hydrolysate of casein)[10]；而真菌的藥物最低抑制濃度實驗使用 RPMI1640 液態培養基 (0.84% RPMI1640, 2% glucose 及 0.165M MOPS)。本篇研究

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使用藥品為 tebuconazole (Sigma-Aldrich)、fluconazole (Selleckchem, Houston, TX)、voriconazole (Sigma-Aldrich)、posaconazole (Merck)、市售 25.9%得克利水基乳劑 (獲達多®，台灣拜耳)、35%滅達樂可濕性粉劑 (露多滅®，生力) 及 50%蓋普丹可濕性粉劑 (好速剎®，好速)。

濾紙片擴散實驗

濾紙片擴散實驗用以測試不同菌株對藥品的感受性。首先 *S. aureus*, *E. coli*, *P. aeruginosa* 及 *C. albicans* 液態培養於 37 ℃ 生長箱一個晚上，*R. solanacearum* 則液態培養於 28 ℃ 生長箱 24 小時。接著將 100 µL OD_{600}= 1 的菌液以玻璃珠均勻抹於固態培養基；*S. scabies* 則塗抹 100 µL 的 $1x10^7$ CFU/mL 孢子懸浮液，再將直徑 6 mm 的濾紙片放置固態培養基上，滴上適量藥品 (體積控制為 6~7.5 µL)。塗有 *R. solanacearum* 和 *S. scabies* 的培養基培養在 28 ℃ 生長箱 3 天，其餘菌株的培養基則放在 37 ℃ 生長箱 24 小時。

藥物最低抑制濃度測定

細菌的藥物最低抑制濃度 (minimum inhibitory concentration; MIC) 測定是遵守 CLSI M07-A9 流程進行[11]。用 Mueller Hinton 培養基稀釋新鮮的 *S. scabies* PS07 孢子，96 孔盤每格加 50 µL 的 $1x10^6$ CFU/mL 孢子液。待測試藥物用 Mueller Hinton 培養液兩倍序列稀釋，並加 50 µL 的液體至每格，使最終每格有 100 µL 的液體。得克利濃度範圍從 0.5 到 256 µg/mL。96 孔盤置於 28℃ 生長箱 48 小時。 最低抑制濃度值定義為藥物可完全抑制 *S. scabies* PS07 生長的最低濃度。

真菌的藥物最低抑制濃度測定是遵守 CLSI M27-A3 流程進行[12]。先將養好的菌以無菌水清洗兩次，將菌液濃度調整至 OD_{600}= 1，再將 10 µL OD_{600}= 1 的菌液加入 990 µL 的 RPMI 稀釋。稀釋好的菌液取 500 µL 出來，再加入 9.5 mL 的 RPMI 稀釋，使最終菌液的 OD_{600}=0.0005。待測試藥物以兩倍序列稀釋。96 孔盤每格加

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98 µL 的菌液且加 2 µL 的藥物。得克利濃度範圍從 0.5 到 256 µg/mL。96 孔盤置於 35℃ 生長箱 24 小時。

盆栽試驗

S. scabies PS07先培養在MPYSC固態培養基28℃生長箱14天，接著用牙籤蒐集孢子並用無菌水清洗兩次，存在-20℃冰箱備用。馬鈴薯使用G4克尼伯品種，先放在室外催芽，等芽眼長出後將之切塊，一小塊留兩芽眼，風乾後備種。土壤使用泥炭土 (KEKKILA OY®，芬蘭) 與三號根基旺 (南海®) 以1：1比例混合，每盆栽放一馬鈴薯薯塊，植株一周澆水兩次，第2周開始澆花寶二號 (HYPONEX®，美國) 一周一次直到澆灌得克利之前。本實驗使用六周大的馬鈴薯植株進行馬鈴薯瘡痂病的接種，使用混土的方式，每盆盆栽含有$3.8x10^8$ CFU的*S. scabies* PS07。市售25.9%得克利水基乳劑以及35%滅達樂可濕性粉劑稀釋2000倍後，每盆盆栽澆灌150 mL，每隔七天澆灌一次，共澆灌三次。一處理組為五盆盆栽，試驗設計之八處理組分別為：(A) 以下八處理組每組有五株六周大之馬鈴薯植株：(a) 只澆灌水； (b) 只澆灌得克利； (c) 只澆灌滅達樂； (d) 只接種*S. scabies* PS07；(e) 先澆灌得克利，六天後再接種*S. scabies* PS07； (f) 先接種*S. scabies* PS07，一天後再澆灌得克利；(g) 先接種*S. scabies* PS07，六天後再澆灌得克利；(h) 先接種*S. scabies* PS07，一天後再澆灌得克利及滅達樂。植株在第10周收成，罹病度 (disease severity) 以下列公式計算[13]： Σ (病斑覆蓋比例 x 病斑型態) / 18 x 100% 。病斑覆蓋比例分成七級： 0 = 沒有病斑，1 = 0.1% – 2%，2 = 2.1% – 5%，3 = 5.1% – 10%，4 = 10.1% – 25%，5 = 25.1% – 50%，以及6 = ≥50%。病斑型態分成四級： 0 = 沒有病徵；1 = 病徵平滑， 2 =病徵突起，以及3 =病徵凹陷。

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結果

得克利可有效抑制 *S. scabies* 的生長

為了尋找有效抑制 *S. scabies* 的藥劑，首先使用實驗室已有的 30 種農藥進行藥物最低抑制濃度測定的快速篩選，在其中發現得克利能抑制 *S. scabies* 的生長，進而使用得克利做後續的實驗。得克利為三唑類農藥，其結構式如圖一 A 所示。三唑類為殺真菌藥物，由圖一 B 可發現使用西藥之三唑類藥物：fluconazole、voriconazole、posaconazole 對細菌都沒有抑菌效果，只能抑制真菌 *C. albicans* 的生長。特別的是使用三唑類農藥得克利時，除可抑制 *S. scabies* 之外，另可抑制番茄青枯病 (*R. solanacearum*)、人類金黃色葡萄球菌 (*S. aureus*) 及白色念珠菌 *C. albicans* 的生長。除此之外，我們進一步進行得克利的最低抑制濃度測定，結果顯示得克利對 *S. scabies* 及 *C. albicans* 都是 64 μg/mL，對 *R. solanacearum* 為 128 μg/mL，對 *S. aureus* 則大於 256 μg/mL (表二)。

得克利可有效抑制不同來源的 *S. scabies*

為了得知得克利是否可廣效的抑制不同來源的 *S. scabies*，使用從嘉義田間馬鈴薯薯塊上分離到的 *S. scabies* PS01 及 *S. scabies*PS02，台中后里馬鈴薯薯塊上分離到的 *S. scabies* PS07 及 *S. scabies*PS08 以及台中潭子馬鈴薯薯塊上分離到的 *S. scabies* YC1020 及 *S. scabies*YC1028 等菌株做測試。由濾紙片擴散實驗可知除了對 *S. scabies* YC1028 的抑制生長效果比較不明顯外，得克利純藥物對其餘 *S. scabies* 菌株都有良好的抑制效果 (圖二)。

得克利與滅達樂或蓋普丹具加乘作用

合併農藥使用以減少病原菌抗藥性及農藥用量為目前主流策略之一。為了找到與得克利具加乘作用的農藥，我們利用濾紙片擴散實驗篩選了不同的藥劑。結果發現滅達樂或蓋普丹兩種藥劑，與得克利共同施用時較單一藥物的抑制圈更澄

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清，表示能更有效的抑制 *S. scabies* 的生長 (圖三)。但因為蓋普丹在高劑量使用下具小鼠腫瘤性，目前為限制登記使用藥，又滅達樂已登記為馬鈴薯晚疫病用藥，所以選擇滅達樂做後續的盆栽試驗。

得克利於盆栽試驗可降低馬鈴薯瘡痂病罹病嚴重度

盆栽實驗為期十周，總共有八個處理組，每處理組含五株馬鈴薯植株，各處理組詳見材料與方法。實驗結果顯示先澆灌得克利六天再接種 *S. scabies* 或接種 *S. scabies* 後一天再澆灌得克利的組別，均有效降低罹病嚴重度，分別從 54.9 ± 7% (只接種 *S. scabies*) 降低至 14.1 ± 5% ($P < 0.001$；Tukey's test) 以及 11.4 ± 4.5% ($P < 0.001$；Tukey's test) (圖四 A 與 B)。但先接種 *S. scabies* 六天後再澆灌得克利就無法降低罹病嚴重度，說明得克利較佳使用時機為病原菌入侵馬鈴薯薯塊前使用。另外先接種 *S. scabies* PS07，一天後再澆灌得克利及滅達樂的組別亦可以有效降低罹病嚴重度從 54.9 ± 7% (只接種 *S. scabies*) 到 27 ± 7% ($P < 0.01$；Tukey's test)。

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討論

殺真菌劑得克利能對馬鈴薯瘡痂病菌產生拮抗作用及降低馬鈴薯瘡痂病之病害嚴重度為全球首次之發現。得克利能抑制多種植物病原真菌的生長，也是田間病害 (如炭疽病、白粉病及銹病) 防治常用的農藥之一。為了探討得克利的作用機制，故使用不同的細菌一同做測試，意外發現其能抑制*S. scabies*、*R. solanacearum*與*S. aureus* 之生長，暗示這些菌可能存在得克利之藥物標靶。得克利屬於G1三唑類之農藥，其於真菌之作用標靶為sterol 14α-demethylase (Cyp51)[8]，因此推測細菌體內可能也有相似於此酵素之標靶存在。根據前人研究，發現在*Mycobacterium tuberculosis*有Cyp51的同源基因-*Rv0764c*產物[14]，而在另一研究指出*M. tuberculosis*的Cyp121的同源基因-*Rv2276*產物[15]對三唑類藥物的親和性較*Rv0764c*產物好[16]；該研究亦指出模式放線菌*Streptomyces coelicolor* A3(2)的*CYP51*及*CYP105D5*突變株跟野生株相比，對三唑類藥物最低濃度抑制值稍微變高[16]，表示*CYP51*及*CYP105D5*可能為三唑類藥物的標靶。另用*C. albicans* SC5314的Cyp51/Erg11 (CaO19.922)胺基酸序列於NCBI以tblastn比對，發現*M. tuberculosis* RGTB423、*S. scabies* 87.22、*S. coelicolor* A3(2)及*R. solanacearum* CMR15有同源蛋白存在，分別為：MRGA423_04780 (identity 28%)、SCAB_9321 (identity 23%)、SCO5223 (identity 22%) 及CMR15_20043 (identity 24%) (圖五)，因此推測得克利拮抗*S. scabies*之機制有可能透過抑制此酵素而達到。*S. aureus*、*P. aeruginosa* 及*E. coli* 在比對下沒有發現Cyp51的同源蛋白，其中已知*E. coli*沒有P450[17]，*S. aureus*雖然沒有發現Cyp51的同源蛋白，卻依然有抑制圈 (圖一B)，推測可能有其他的P450為得克利作用標靶。同屬於三唑類藥物的fluconazole、voriconazole及posaconazole雖然可抑制真菌*C. albicans*的生長卻無法抑制*S. scabies*、*S. aureus*及*R. solanacearum*的生長 (圖一B)，推測是因為結構式不同，造成與Cyp51的同源蛋白結合的親和力不同。如欲探討得克利抑制馬鈴薯瘡痂病菌之機制，可將*S. scabies*合成此酵素之基因 (*SCAB_9321*) 敲除而獲得突變株。如此突變株對得克利具耐受性，則可推論此酵素基因可能為

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得克利之藥物標靶。如此突變株仍與野生株一樣，展現對得克利具敏感性，則推論得克利可能針對異於sterol 14α-demethylase之基因產物進行抑制，以達到拮抗*S. scabies* 之目的。在濾紙片擴散實驗中發現，當使用市售25.9%水基乳劑得克利其抑制圈比使用得克利純藥物還小 (圖一與二)，推測可市售的得克利藥物有效物質跟純藥物相比之下較不純，或其他溶劑抑制得克利的效用。

由於得克利為系統性農藥[18]，於植物體內具向上移行性，而馬鈴薯瘡痂病為土棲性病原菌，因此盆栽試驗設計以澆灌方式取代傳統之地上部噴灑方式行之。確實，得克利以澆灌方式可於盆栽試驗獲得對馬鈴薯瘡痂病的良好防治效果 (圖四)，當中又以接種 *S. scabies* 後一天再澆灌得克利的組別防治效果最好，罹病嚴重度由 54.9 ± 7%降低至 11.4 ± 4.5%；而先澆灌得克利六天後再接種 *S. scabies*，其罹病嚴重度也從 54.9 ± 7%降低至 14.1 ± 5%，顯示實際應用上可在薯塊開始形成前使用，若本身土壤就帶有病原菌，亦可嘗試提早開始施用，以達到防治的最佳效果。滅達樂為系統性移行藥劑，其作用機制為抑制真菌的核糖核酸聚合酶 I (RNA polymerase I)，進而抑制真菌合成蛋白質[19]；而蓋普丹為非專一性且多作用點抑制劑[20, 21]。先接種 *S. scabies* PS07，一天後再澆灌得克利及滅達樂的組別亦可有效降低罹病嚴重度從 54.9 ± 7%到 27 ± 7%，只是不如接種 *S. scabies* 後一天再澆灌得克利的組別好，顯示得克利與滅達樂的加乘作用僅存在於濾紙片擴散實驗。未來如欲評估得克利於實際田間應用之可能性，則需以多場田間試驗佐證其對馬鈴薯瘡痂病具防治效果。

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表格

表一、本篇研究使用菌株

Table 1. Strains used in this study.

Strain	Species	Source
PS01	*Streptomyces scabies*	Chiayi, Taiwan
PS02	*Streptomyces scabies*	Chiayi, Taiwan
PS07	*Streptomyces scabies*	Houli, Taichung, Taiwan
PS08	*Streptomyces scabies*	Houli, Taichung, Taiwan
YC1020	*Streptomyces scabies*	Tanzi, Taichung, Taiwan
YC1028	*Streptomyces scabies*	Tanzi, Taichung, Taiwan
S01-10-0202	*Staphylococcus aureus*	Taiwan
DH5α	*Escherichia coli*	Bethesda Research Laboratories[22]
S07-10-0059	*Pseudomonas aeruginosa*	Taiwan
KRS03	*Ralstonia solanacearum*	Taiwan
SC5314	*Candida albicans*	Gillum *et al.*[23]

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表二、得克利對不同菌株之最低抑制濃度與最低殺細菌/真菌濃度

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of tebuconazole against multiple microbial strains

Strains	MIC (μg/mL)	MBC/MFC (μg/mL)
Streptomyces scabies (PS07)	64	>256
Staphylococcus aureus (S01-10-0202)	>256	—*
Escherichia coli (DH5α)	>256	—
Pseudomonas aeruginosa (S07-10-0059)	>256	—
Ralstonia solanacearum (KRS03)	128	256
Candida albicans (SC5314)	64	128

*Due to MIC >256 μg/mL, MBC was not determined.

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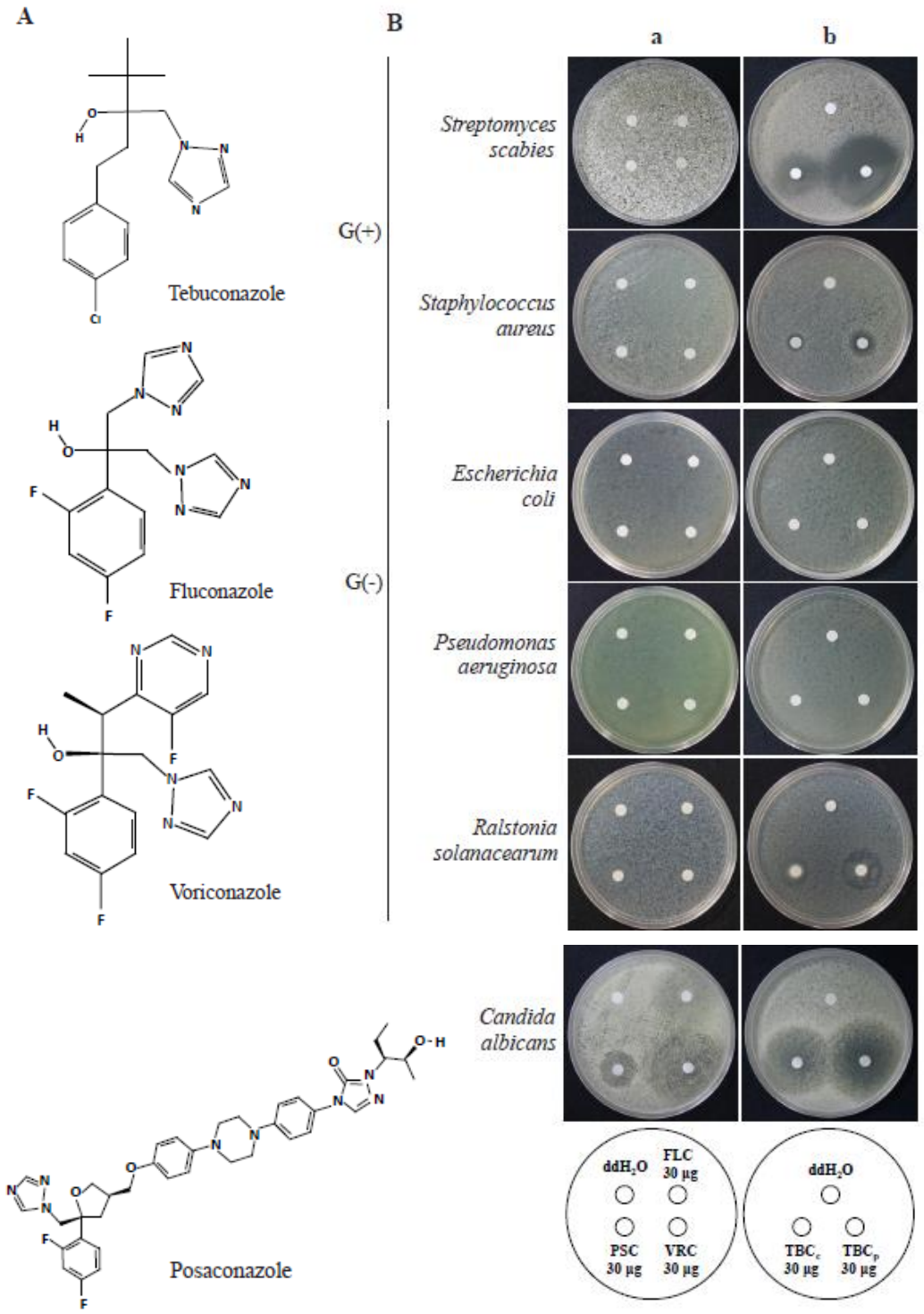
Figure 1



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圖一、殺真菌劑得克利能抑制*S. scabies*。(A) 三唑類藥物之化學結構式。(B) 以濾紙片擴散實驗測試三唑類藥物對細菌及真菌菌株的感受性。 (a) 放在培養基上的濾紙片含有30 μg的fluconazole (FLC)、voriconazole (VOC) 和posaconazole (PSC)。(b) 放在培養基上的濾紙片含有30 μg的純得克利藥物 (TEBp) 以及市售農藥得克利 (TEBc)。6 μL的ddH_2O作為負控制組。

Fig. 1. The fungicide tebuconazole exhibits antibacterial activity against *S. scabies*. (A) Chemical structure of azoles used in this study. (B) Disc diffusion assays were used to determine azoles susceptibility of bacterial and fungal strains. (a) Discs containing 30 μg of fluconazole (FLC), voriconazole (VOC) or posaconazole (PSC) were placed on the surface of the medium. (b) Discs containing 30 μg of tebuconazole pure compound (TEBp) or tebuconazole commercial product (TEBc) were placed on the surface of the medium. 6 μL ddH_2O was used as negative control.

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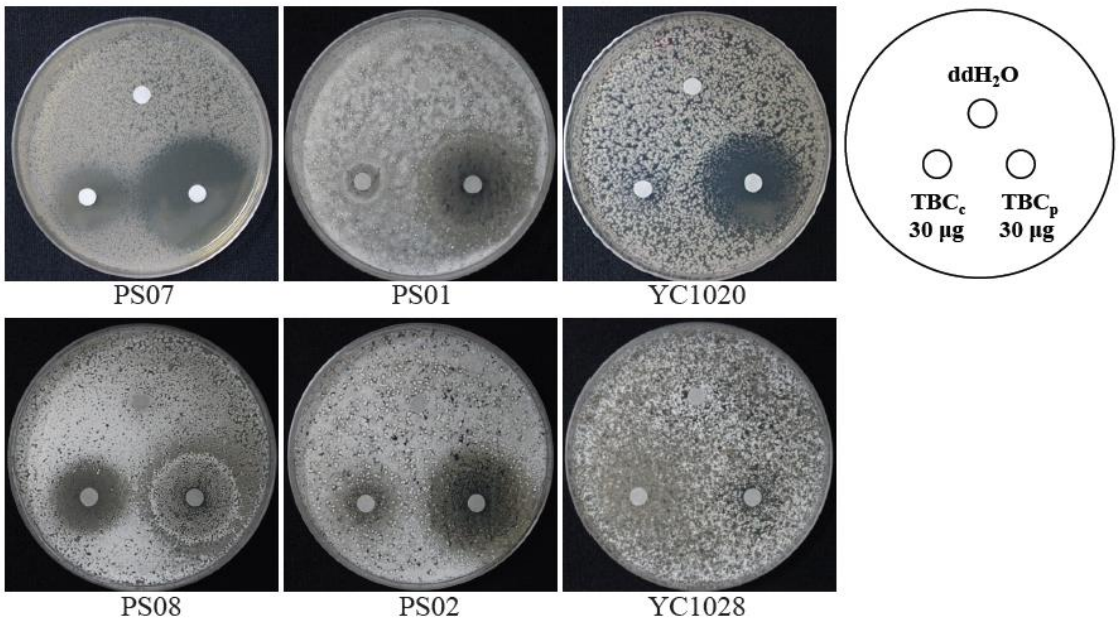
Figure 2



圖二、得克利可抑制不同來源的*S. scabies*。10^6 CFU的*S. scabies*孢子懸浮液塗抹在YME固態培養基上。放在培養基上的濾紙片含有30 µg的純得克利藥品 (TEBp) 以及有效成分30 µg的市售農藥得克利 (TEBc)。6 µL的ddH_2O作為負控制組。

Fig. 2. Tebuconazole is effective against multiple *S. scabies* isolates. Discs containing 30 µg of tebuconazole pure compound (TEBp) or 30 µg active gradient of tebuconazole commercial product (TEBc) were placed on the surface of the medium. 6 µL ddH_2O was used as negative control.

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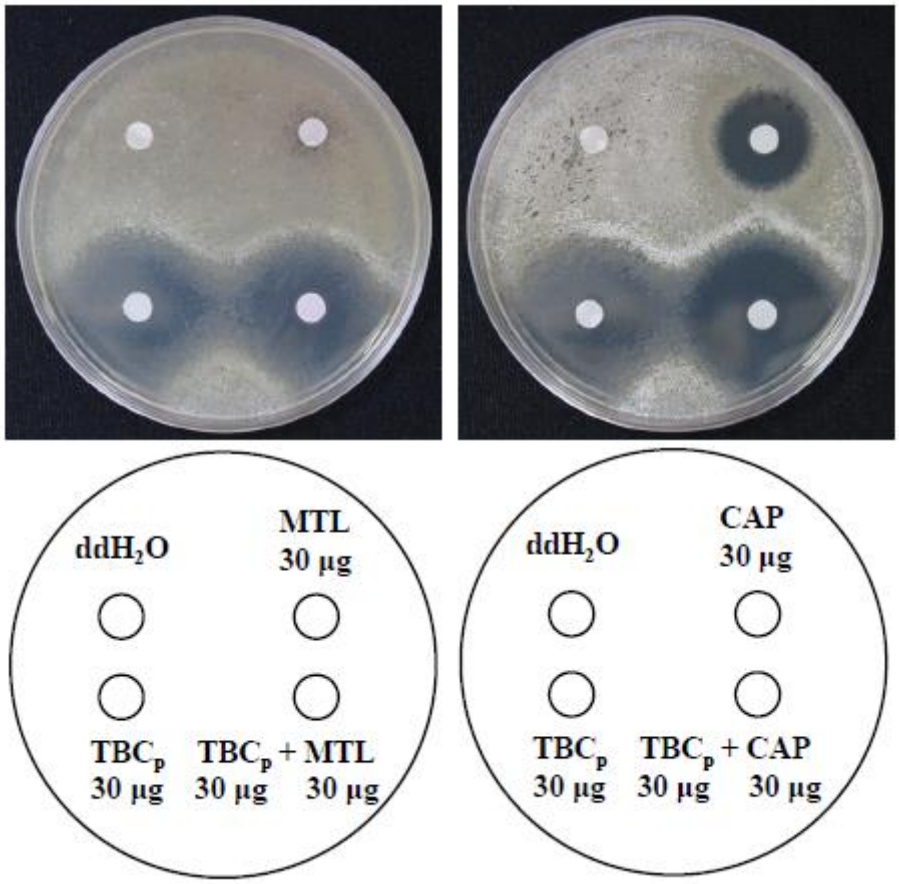
Figure 3



圖三、得克利與滅達樂或蓋普丹對拮抗*S. scabies*具加乘作用。10^6 CFU的*S. scabies*孢子懸浮液塗抹在YME固態培養基上。放在培養基上的濾紙片含有30 µg的純得克利藥品 (TEBp)、市售農藥滅達樂或市售農藥蓋普丹。6 µL的ddH_2O作為負控制組。

Fig. 3. Tebuconazole has combination effect with metalaxyl or captan against *S. scabies*. The 10^6 CFU spores of *S. scabies* were spread on YME agar plates Discs containing 30 µg of tebuconazole (TEBp; pure compound), metalaxyl (MTL; commercial product) or captan (CAP; commercial product) were placed on the surface of the medium. 6 µL ddH_2O was used as negative control.

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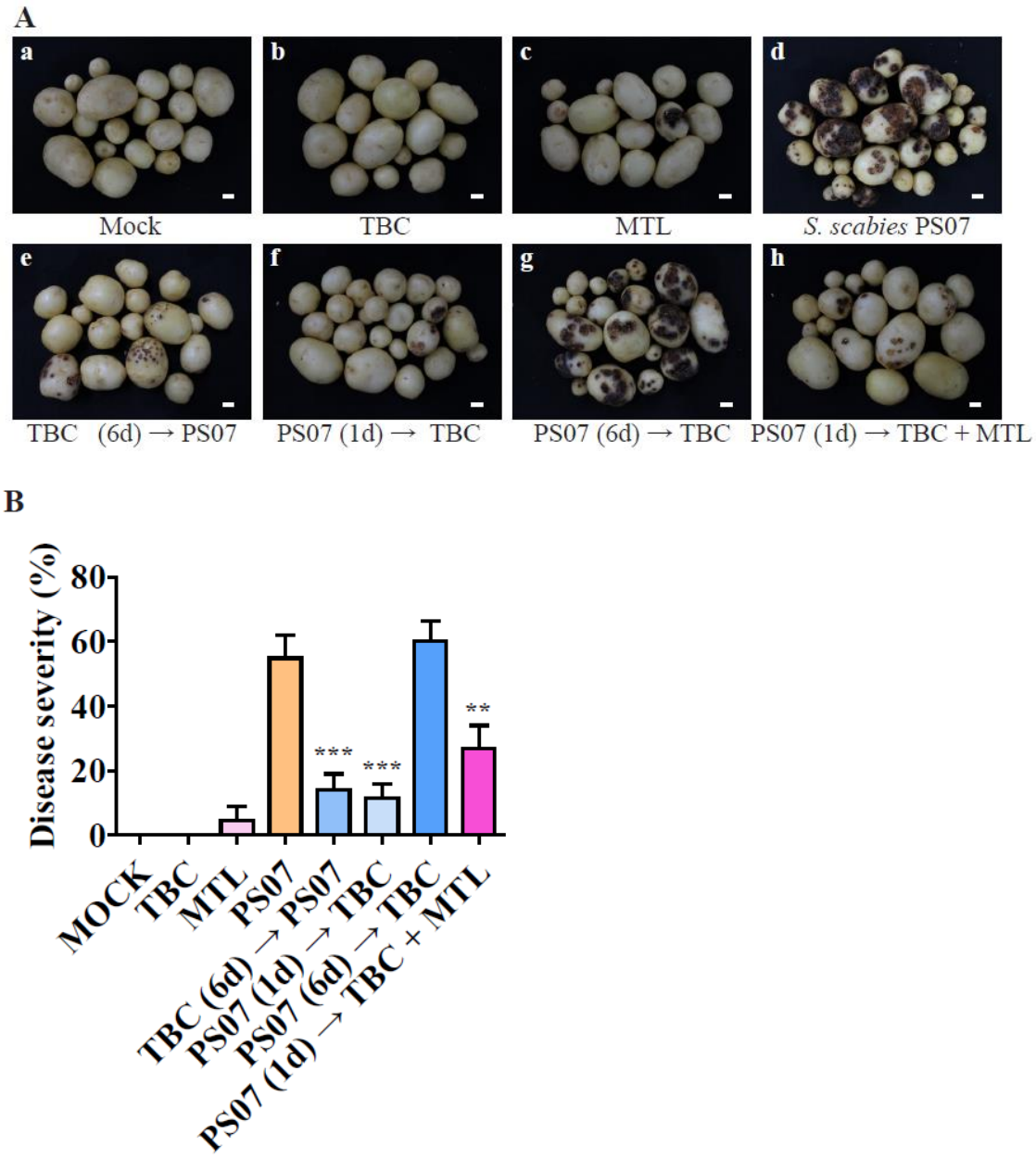
Figure 4



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圖四、得克利可有效降低馬鈴薯瘡痂病之罹病嚴重度。 (A) 以下八處理組每組有五株六周大之馬鈴薯植株：(a) 只澆灌水。 (b) 只澆灌得克利。 (c) 只澆灌滅達樂。 (d) 只接種*S. scabies* PS07。(e) 先澆灌得克利，六天後再接種*S. scabies* PS07。 (f) 先接種*S. scabies* PS07，一天後再澆灌得克利。 (g) 先接種*S. scabies* PS07，六天後再澆灌得克利。 (h) 先接種*S. scabies* PS07，一天後再澆灌得克利及滅達樂。市售25.9%水基乳劑得克利以及35%可濕性粉劑滅達樂稀釋2000倍後，每盆盆栽澆灌150 mL，每隔七天澆灌一次，共澆灌三次。比例尺代表一公分。(B) 馬鈴薯瘡痂病罹病嚴重度。每組數據呈現為五株馬鈴薯植株的平均 ± 平均標準差。統計使用Tukey's test並和處理組 (d) 比較; **代表顯著性 < 0.01，***代表顯著性 < 0.001。

Fig. 4. Tebuconazole reduced the disease severity of potato common scab in pot assays. (A) Five potato plants with six-week old were used for each treatment below. (a) mock control without application of pesticides and PS07; (b) application of tebuconazole (TBC) only; (c) application of metalaxyl (MTL) only; (d) inoculation of PS07 only; (e) application of tebuconazole first for 6 days, then inoculation of PS07; (f) inoculation of PS07 first for 1 day, then application of tebuconazole; (g) inoculation of PS07 first for 6 days, then application of tebuconazole; (h) inoculation of PS07 first for 1 day, then application of tebuconazole and metalaxyl. Commercial tebuconazole or metalaxy were diluted 2000 folds and then 150 mL of tebuconazole or metalaxyl were applied to each pot once a week and total three times. The bar represents 1 cm. (B) The disease severity of the potato common scab. Data were expressed as the average of tubers collected from

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five potato plants ± standard error of mean. *P* values were calculated using Tukey's test; '**' and '***' represent $P < 0.01$ and $P < 0.001$, respectively, as compared to the treatment of PS07 only.

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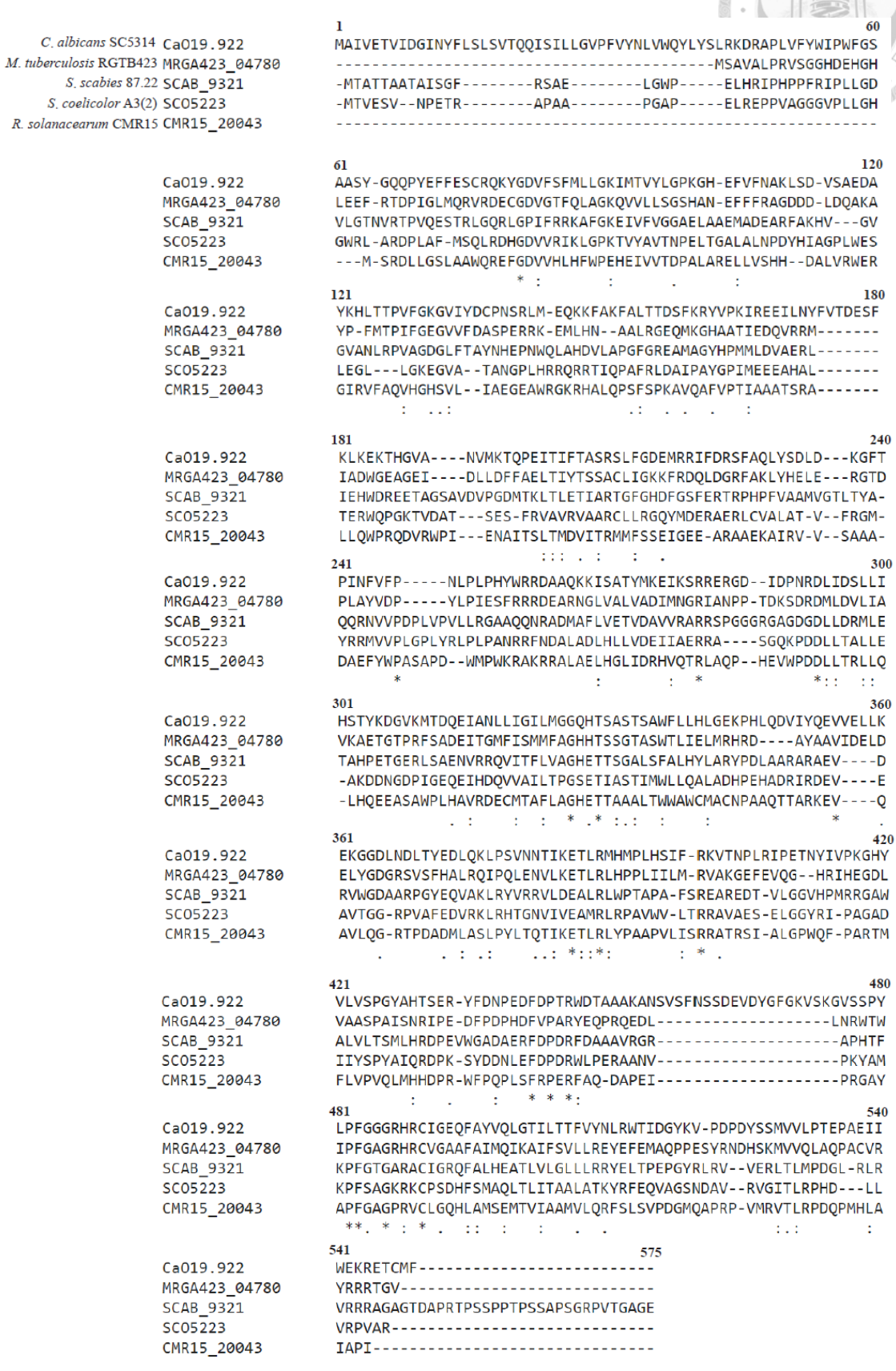
Figure 5



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圖五、Cyp51胺基酸序列比對。‘*’代表所有的胺基酸完全相同；‘：’代表在Gonnet PAM 250 matrix分數> 0.5，胺基酸間具極高的相似性；‘.’代表在Gonnet PAM 250 matrix分數≦ 0.5或> 0，胺基酸間相似性較弱。

Fig. 5. Multiple sequence alignment of Cyp51 in bacterial and fungal strains. Asterisk ‘*’ indicates positions which are conserved. Colon ‘：’ indicates conservation between groups with strongly similar properties as below - roughly equivalent to scoring > 0.5 in the Gonnet PAM 250 matrix. Period ‘.’ indicates conservation between groups of weakly similar properties as below - roughly equivalent to scoring≦ 0.5 and > 0 in the Gonnet PAM 250 matrix.

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