國立臺灣大學生物資源暨農學院生物科技研究所

碩士論文

Institute of Biotechnology

College of Bioresources and Agriculture

National Taiwan University

Master Thesis

評估土壤根際潛力菌株搭配菊科植物添加物之生物防治功效

Evaluation of the biocontrol effects exerted by an elite rhizobacterial strain formulated with Asteraceae plant additive

劉彥妤

Yen-Yu Liu

指導教授：劉啓德 博士

Advisor: Chi-Te Liu, Ph.D.

中華民國 107 年 7 月

July 2018

doi:10.6342/NTU201804077

國立臺灣大學碩士學位論文
口試委員會審定書

中文題目: 評估土壤根際潛力菌株搭配菊科植物添加物之生物防治功效

英文題目: Evaluation of the biocontrol effects exerted by an elite rhizobacterial strain formulated with Asteraceae plant additive

本論文係 劉彥妤 君（R05642001）在國立臺灣大學生物科技所完成之碩士學位論文，於民國 107 年 07 月 24 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：

劉啓德 （指導教授簽名）

林詩舜 （簽名）

[illegible] （簽名）

李兒達 （簽名）

楊文欽 （簽名）

系主任、所長 [illegible]（簽名）

doi:10.6342/NTU201804077

誌謝

回想過去兩年在研究所的點點滴滴，騎著單車來回穿梭系館所在的長興街，男七自助餐和長興小七為我午餐及晚餐的好夥伴，當朋友問起你等一下要幹嗎？我常常回答「做實驗」，我的生活似乎充滿了實驗，但也因為鑽研科學而充實，當中遭遇不少挫折，卻也因從中解決問題而感到痛快，總因為家人、師長及朋友的陪伴和鼓勵下，最終完成期盼已久的碩士論文，論文完成之際，心中的激動實在無法言喻，唯有感謝再感謝。

首先感謝我的指導教授劉啟德博士，感謝老師的細心指導，以及撥空時間與我討論實驗進度，我們總是能在每星期的進度報告中，對於新的實驗結果賦予新的詮釋，並且督促實驗的進行。除此之外，更感謝老師提供參與研討會的機會，讓我能在台灣農化學會及日本微生物學會上發表我的碩論研究，讓我在僅僅兩年的研究生涯中能有所表現。不僅在學業上的給予指導，同時也給予生活上無微不至的關懷，在此致上誠摯的謝意與崇高敬意。

謝謝口試委員楊文欽老師、劉嘉睿老師、李昆達老師以及林詩舜老師撥冗指導，給予學生碩論的建議並指出缺失。本研究因為楊文欽老師所提供的實驗關鍵材料才得以進行後續的科學研究，中研院郭志鴻老師與蔡乙鳴助理提供全基因解序的協助，得以讓碩論變得更完整，在此由衷感謝。

在台大生技所 R412 的所有夥伴們，因為有你們在我生活上增添不少樂趣，讓我能度過無以計數的枯燥實驗，同時也給予實驗上不少建言，在我處於實驗不順的輪迴裡拉我一把，讓我能順利完成碩論。

最後感謝我的父母，在我研讀碩士期間，給予我最大的自由，也尊重及支持我的決定，讓我能夠盡全力專注於我的學業上，我愛你們！

doi:10.6342/NTU201804077

中文摘要

瓜類細菌性果斑病 (bacterial fruit blotch) 由細菌性果斑病菌 (*Acidovorax avenae* subsp. *citrulli*, Aac) 所引起。種子為其初級感染源，高溫、下雨等因素會造成病原菌快速蔓延，導致嚴重的經濟損失。傳統法會將種子浸泡在低濃度鹽酸或是次氯酸鈉等化學藥劑藉以抑制果斑病原菌，但衍生出對環境非友善或是食品安全疑慮等問題。本研究的目的為評估一株具有防治植物病害的潛力菌株搭配菊科植物添加物後對於瓜類細菌性果斑病的防治效果。根據對峙培養的實驗結果，潛力菌株與菊科植物添加物之處理組可成功抑制果斑病原菌的生長，然而除去菌體的上清液則無抑制效果，顯示其拮抗效果來自於活菌。我們進一步探討菊科植物添加物對於潛力菌株的微生物生理影響，結果顯示菊科植物添加物不僅可促進潛力菌株的生物膜生成能力，也提高菌體活性。在盆栽試驗發現，預先接種潛力菌株或是接種潛力菌株搭配菊科植物添加物的美濃瓜種子其病徵較為輕微，而且植株內的 Aac 的數量都較未接種組低。全基因解序結果顯示該潛力菌株基因體大小約為 4.00 Mb，經過序列比對與功能註解分析，潛力菌株含有約 3,900 條蛋白質編碼基因 (protein-coding gene)，包括數種抗生物質相關基因。此外，基因體中亦含有與生物膜形成相關的同源基因，這些基因與促進植物生長和生物防治有著密切關係。綜合上述結果，潛力菌株與菊科植物添加物的配方具有成為防治細菌性果斑病潛力的綜效型生物製劑。

關鍵字：細菌性果斑病、果斑病原菌、土壤根際潛力菌株、菊科植物添加物、生物防治

doi:10.6342/NTU201804077

Abstract

Bacterial fruit blotch (BFB) of curcubits is caused by *Acidovorax avenae* subsp. *citrulli* (Aac) which is a seed-borne pathogen and cause serious threat in cucurbit industry worldwide. Although seed treatments with hydrochloric acid or sodium hypochlorite can suppress Aac infection, they generally fail to eradicate the bacterium. The aim of this study was to evaluate the synergistic effects of an elite rhizobacterial strain and Asteraceae plant additive on inhibition of BFB. Under *in vitro* antagonistic test, both rhizobacterial strain and the combination of rhizobacterial strain with Asteraceae plant additive could inhibit Aac. We noted that the supernatant of rhizobacterial strain did not inhibit Aac, suggesting the biocontrol effectiveness derived from the living cells. We further investigated the effects of Asteraceae plant additive on physiological activities of rhizobacterial strain. We found the plant additive enhanced not only its biofilm formation, but also the cell vitality. In pot experiments, while pre-treating the melon seeds with either single-strain (rhizobacterial strain) inoculant or with the mixed treatment (rhizobacterial strain + plant additive) showed higher inhibitory effects against BFB than that without inoculation. The complete genome of rhizobacterial strain was analyzed by next generation sequencing. The results of whole genome sequencing indicated that rhizobacterial strain has one circular chromosome that is around 4.00Mb with 3,900 protein-coding genes, including several antibiotic-related genes. In addition, we also identified the homologous genes related to biofilm formation that is associated with the beneficial traits of growth promotion and biocontrol activities. Taken together, the rhizobacterial strain formulated with Asteraceae plant additive can act as a potential biocontrol agent against BFB.

doi:10.6342/NTU201804077

Keywords: bacterial fruit blotch (BFB), *Acidovorax avenae* subsp. *citrulli*, rhizobacterium, Asteraceae plant, biocontrol

doi:10.6342/NTU201804077

Contents

doi:10.6342/NTU201804077

doi:10.6342/NTU201804077

List of Figures

doi:10.6342/NTU201804077

List of Tables

doi:10.6342/NTU201804077

Introduction

Bacterial fruit blotch

Bacterial fruit blotch (BFB), which is caused by a gram-negative bacterium *Acidovorax avenae* subsp. *citrulli* (Aac), is the most economically considerable bacterial disease of cucurbits (such as watermelon, melon, squash, cucumber, etc.) in the world (Langston Jr et al. 1999; Martin et al. 1999; Somodi et al. 1991). BFB outbreaks occurred in commercial watermelon fields in Florida, USA in 1989, and the disease has become devastating threat (Somodi et al. 1991). After the outbreak, the disease began to spread and extend the host range in worldwide (Isakeit et al. 1997; Langston Jr et al. 1999; Martin et al. 1999). The annual yield loss of watermelon suffered from BFB in USA reached up to 50% (Hopkins 1995; Somodi et al. 1991), resulting in marketable watermelon fruit losses of over $100,000 to individual growers (data provided by Crop Protection Compendium. Wallingford, UK, www.cabi.org/cpc). Curcubits are one of the most important crop in Taiwan. The cultivation region is about 25,000 hectares per year. The cultivation region of watermelon and melon are about 12,448 and 4,394 hectares, respectively (趙 2007). The watermelon seeds of export trade accounts for approximately 23% to total seeds import market value (關 et al. 2015). The import value of watermelon and melon seeds have been up to three million dollars and five million U.S. dollars in Taiwan in recent ten years (data provided by National Statistics, Taiwan R.O.C; 胡 2017). The outbreak of bacterial fruit blotch was observed in the south of Taiwan during 1992 to 1993 (唐 1997). Up to 80% of marketable fruit were suffered from BFB around Taiwan in 1997 (Cheng et al. 2000). The pathogen detection and controlling BFB are considered to as important strategies to make sure the seed health before products launch (關 et al. 2015).

doi:10.6342/NTU201804077

Symptoms of BFB on susceptible fruits begin with small water-soaked lesion and later extend into larger lesion and crack (Burdman and Walcott 2012). The primary source of inoculum of BFB is mainly infested seeds. The pathogen is easily spreaded between plants by rain, high humidity, use of overhead irrigation (Bahar and Burdman 2010). Growers often assume that the vegetable seed they buy meets minimum health standards. The ability to treat seed with suitable disinfectants would mean a huge saving in money for seed companies due to current losses of contaminated seed and also provide extra protection for the plant to against disease. The young seedlings and fruits are highly susceptible to Aac, and usually suffer from cotyledon infection and seedling blight. When weak infection occurs, symptoms might not be detected, and the seedlings are transplanted into the field. Since mature plants are relatively tolerant to the pathogen, the infected plants may show weak or no symptom (Burdman and Walcott 2012; Hopkins 1995). These will cause the seed suppliers mis-judge the contaminated seeds as health.

Since Aac was a serious seed-borne pathogen, exclusion of the bacterium from the field and the use of pathogen-free seeds were the most effective control strategy (Latin 1996). The control option for eradicating the pathogen was seed fermentation, multiple applications of a copper-containing bactericide and acid treatments (peroxyacetic acid, hydrochloric acid and sodium hypochlorite, etc.) (Guzman et al. 1991; Hopkins et al. 1996; Hopkins et al. 2003). Copper-containing bactericide had effectiveness on controlling the disease development. However, slight phytotoxicity was observed with the application of copper-containing bactericide (Guzman et al. 1991). Although seed treatments like fermentation, hydrochloric acid, and sodium hypochlorite can suppress Aac inoculum, they generally fail to eradicate the bacterium (Hopkins et al. 2003; Rane and Latin 1992).

doi:10.6342/NTU201804077

Bacillus spp. are one of the most common biocontrol agents which is an excellent option to fight against plant pathogens

For good agronomic and cultural practices, growers rely on chemical pesticides application (Agrios 2005; Baker 1987). Although chemical pesticides can effectively control plant pathogens, they generally bring the negative impacts on environment and even on human health. The issue has been the growing concern of environment friendly methods and healthy food. However, scientists have been interested in biological control strategies with microorganisms for their environmental safety and viable method to control the plant diseases (Cook 1993). The organisms are able to suppress plant pests and pathogen are referred as biological control agents (BCAs). Several bacteria and fungi have been reported as BCAs against plant pathogens (Broadbent et al. 1971; Chowdhury et al. 2015; Harman et al. 1991; Merriman et al. 1974; Tjamos et al. 2004; Weller 1988).

Numerous biocontrol mechanisms for antagonizing plant pathogens have been studied widely, including the production of inhibitory compounds (Islam et al. 2005; Keel et al. 1990; Lanteigne et al. 2012; Yoshihisa et al. 1989), induction of resistance in plant tissue (Cook 1993) and lytic enzyme production for hydrolyzing variety of polymeric compounds (Bull et al. 2002; Palumbo et al. 2005). For example, pseudomonads are known for the antibiotic 2, 4-diacetylphloroglucinol (DAPG) production with antifungal, antibacterial activity (Keel et al. 1990; Lanteigne et al. 2012). *Bacillus cereus* strain UW85 can produce multiple antibiotics, such as zwittermicin (Milner et al. 1996; Silo-Suh et al. 1994) and kanosamine (Milner et al. 1996) to suppress the plant disease development. The determinants and pathways of induced resistance stimulated by biological control agents have been characterized. A number of microbial products have been identified as elicitors of systemic acquired

doi:10.6342/NTU201804077

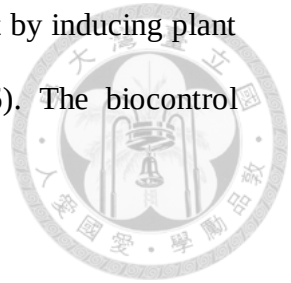
resistance (SAR) and induced systemic resistance (ISR), including chitinase, β-1, 3-glucanase, siderophore, lipopolysaccharides, and 2, 3-butanediol, and other volatile substances (Bargabus et al. 2002; Bargabus et al. 2004; Doornbos et al. 2012; Leeman et al. 1995; Pieterse et al. 2014; Ryu et al. 2004; Wu et al. 2015).

Among the bacterial antagonists, *Bacillus* spp. are one of the extensively-used bacteria in plant disease control. They are known for multiple advantages for plant disease due to antagonistic activity and endospore formation which are highly resistance to extreme environment to prolong the longevity (Borriss 2015; Elliott et al. 2001; Romero et al. 2007; Sansinenea and Ortiz 2011). They have been used for protecting plant species like tomato, maize, strawberry, etc. (Bacon and Hinton 2002; Choudhary and Johri 2009; Essghaier et al. 2009; Shafi et al. 2017). In the members of *Bacillus* spp., *B. amyloliquefaciens* and *B. subtilis* are the most efficient biological control agents so far. *B. amyloliquefaciens* was separated from *Bacillus subtilis* as a new species (Priest et al. 1987) and has been reported as BCAs controlling bacterial fruit blotch (Jiang et al. 2015). *B. amyloliquefaciens* produces a variety of antibacterial and antifungal antibiotics, and cyclic lipopeptides (LPs) such as surfactin, iturin and fengycin (Ongena and Jacques 2008). Both iturin and fengycin were shown to play major roles in biocontrol ability toward *Podosphaera fusca* infecting melon leaves (Romero et al. 2007). Moreover, surfactin produced by *Bacillus* spp. was reported to mediate antibacterial activity, colonization and suppress BFB development (Fan et al. 2017).

In this study, the bacterium *B. amyloliquefaciens* strain WF02 originally isolated from the soil collected at Wufeng Mountain, Taiwan, was used to as the potential biocontrol agent against bacterial fruit blotch. WF02 was demonstrated to secrete siderophore proteins to restrict the proliferation and root colonization by phytopathogen.

doi:10.6342/NTU201804077

It is also reported to protect tomato suffering from the bacterial wilt by inducing plant systemic immunity, such as SAR and ISR (Huang et al. 2016). The biocontrol effectiveness of WF02 on BFB was evaluated in this study.



The bioactivities of *Bidens pilosa* (BP)

Natural products, such as plant extracts, contain various antimicrobial compounds to provide unlimited opportunities for controlling microbial growth. *Bidens pilosa* L. is a species which belongs to plant family Asteraceae, commonly used as an ingredient in foods and medicines for human and animals (Bartolome et al. 2013; Pozharitskaya et al. 2010). It is an easy–to–grow, edible and safe plant so that the United Nations Food and Agriculture Organization (FAO) promoted the cultivation of *B. pilosa* in Africa in the 1970s (Bartolome et al. 2013). It contains various secondary compounds. Around 200 compounds comprising aliphatics, flavonoids, terpenoids, phenylpropanoids, aromatics, porphyrin and other compounds have been identified from this plant (Lima Silva et al. 2011).

The extracts of *B. pilosa* showed highly effectiveness on antibacterial and antifungal activity. One study indicated the compound polyyne, (R)-1,2 dihydroxytrideca-3, 5, 7, 9, 11-pentayne, from this plant effectively suppressed the growth of several drug-resistant bacteria *Staphylococcus aureus* N315 (MRSA) and *Enterococcus faecalis* NCTC12201 (VRE) (Tobinaga et al. 2009). Different extraction solvents, procedures and plant sources might cause different antimicrobial activities. For example, methanol extract of *B. pilosa* roots showed antagonistic activity against *Candida albicans* while there was no inhibitory effects of the extract obtained from Papua New Guinea (Khan et al. 2001). The hot water extracts of *B. pilosa* displayed more effective antibacterial activities against the plant fungal pathogens, such as

doi:10.6342/NTU201804077

Corticium rolfsii, *Fusarium solani* and *Fusarium oxysporu*, than those extracted by essential oils (Deba et al. 2007). However, none of the active compounds against fungi have been confirmed so far (Bartolome et al. 2013).

Previous studies have shown that the antioxidant compounds, such as quercetin 3-O-rutinoside, chlorogenic acid, 3, 5-Di-O-caffeoylquinic acid and so on, were found in the crude extracts of *B. pilosa*, indicating the antioxidant activity potential of *B. pilosa* extracts (Chiang et al. 2004). The antioxidant activities of various plant extracts were demonstrated to contribute to food storage or effect on viabilty of microbes (Michael et al. 2010; Xue et al. 2014). Plant extracts from oleoresin mixtures were considered as antioxidants that could improve the viability of *Lactobacillus delbrueckii* subsp. *bulgaricus* in nonfat yogurt (Michael et al. 2010). Xue et al. research group examined the shelf life of a biocontrol fungal strain *Esteya vermicola* and found out that the shelf life of this inoculant could be improved by additing the herbal extracts containing eleven kinds of herbs, such as *Fructus Trichosanthis*, *Radix Angclica*, *Polygala Tenuifolia*, etc. (Xue et al. 2014). Due to the multiple bioactivities of the extracts, one of the aims of this study is to apply the extract of *B. pilosa* as an additive in the potential biocontrol agents and evaluate its effects.

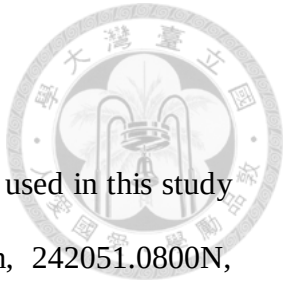
doi:10.6342/NTU201804077

Objectives of the study

The aim of the study was to develop an effective biocontrol agent to reduce the seed-to-seedling transmission of bacterial fruit blotch (BFB) of melon. The elite strain *B. amyloliquefaciens* strain WF02 was formulated with different concentrations of the phytogenic additive *B. pilosa*. We evaluated their biocontrol potential by *in vitro* antagonism assays as well as by pot experiments. To elucidate the effects of *B. pilosa* on bacterial physiology, we examined the growth curve, biofilm formation ability and the cell vitality of WF02. For further functional analysis of the essential genes in WF02, we performed next-generation sequence analysis.

doi:10.6342/NTU201804077

Materials and Methods



Bacterial strains, media and growth condition

Bacillus amyloliquefaciens strain WF02 (designated as WF02) used in this study was isolated from Wufeng mountain (Taichung county, Taiwan, 242051.0800N, 12042048.5700E) (Huang et al. 2016). *Bacillus subtilis* strain Y1336 (designated as Y1336) was derived from a commercial biocontrol product BIOBAC® WP (BION TECH INC., Taiwan), which was reported to be able to suppress bacterial wilt on tomato plants, *Fusarium oxysporum*, *Phytophthora palmivora*, etc. (Yang et al. 2012; 石 2008), was used for control treatment. *B. amyloliquefaciens* strain BPD1 (designed as BPD1) was provided by Dr. Feng-Chia Hsieh (Product Development Division, Taiwan Agrculture Chemicals and Toxic Substances Research Institute). BPD1 were reported to be able inhibit the fungal pathogen growth, such as *Rhizoctonia solani*, *Botrytis cinera*, etc. (謝 et al. 2003). For preparation of respective above inoculant, a single bacterial colony was selected, inoculated into 3 mL of LB broth, and incubated for 24 h at 37°C (200 rpm). Then, 10 µl of above broths was sub-cultured to another 3 mL of LB broth at 37°C (200 rpm). After 24 h incubation, 0.1 ml of the above broths were inoculated into 250-mL Erlenmeyer flasks containing 50 mL of fresh medium for 8 h at 37°C (200 rpm).

The BFB pathogen of melon, *Acidovorax avenae* subsp. *citrulli* (designated as Aac), was isolated from melon and provided by KNOWN-YOU SEED CO., LTD. For preparation of Aac inoculant, a single bacterial colony was selected, inoculated into 3 mL of LB broth, and incubated for 24 h at 37°C (200 rpm). Then, 10 µl of above broths was sub-cultured to another 3 mL of LB broth at 37°C (200 rpm). After 24 h incubation, 0.1 ml of the above broths were inoculated into 250-mL Erlenmeyer flasks containing 50 mL of fresh medium for 8 h at 37°C (200 rpm).

doi:10.6342/NTU201804077

Preparation of plant materials

The oriental melon (*Cucumis melo* L. var. *makuwa* cv. "Silver Light") is one of the most economically cultivated cucurbits widely grown in the tropical and subtropical climatic countries (Choi et al. 2012). Seeds of "Silver Light" were obtained from KNOWN-YOU SEED CO., LTD (Kaohsiung, Taiwan). The seeds were surface-sterilized with 1% HCl for 20 min, and rinsed three times with sterile distilled water. The sterilized seeds were then inoculated with 20 ml Aac bacterial suspension (OD_{600} = 0.3) for 5 min, and later treated with WF02 and various dose of BP extracts under a continuous vacuum (73 cmHg) in a desiccator. Each pots with the surface diameter 4.5 cm and a depth of 4.5 cm, were filled in soil containing 80% Fine Peat and 20% Perlite Mix (BVB Substrates, the Netherlands). Pots were spaced on the greenhouse benches under conditions of 25~27°C and 70~80% of relative humidity (RH). Plants were harvested after ten days (1 to 2 leaves stage). The crude extracts of *B. pilosa* were provided by Dr. Wen-Chin Yang (Agricultural Biotechnology Research Center, Academia Sinica, Taiwan). Crude extracts were stored at -20℃ and dissolved in LB medium or sterile ddH_2O which only for cell vitality assay.

doi:10.6342/NTU201804077

In vitro antagonistic ability of biocontrol agents

Antagonists were confirmed using the filter paper method described by Sinclair and Dhingra (1995). *Bacillus* spp. strain WF02, Y1336 and BPD1 were used to evaluate the antagonistic abilities to inhibit Aac. In addition, extract of *B. pilosa* was supplemented with WF02 at final concentrations of 300 and 600 ppm, which was according to the effective dose in preliminary assays. 81.3% KASUGAMYCIN+COPPER OXYCHLORIDE (1000X Ksm+oxc) (Great victory chemical Industry CO., LTD) and 100 ppm nalidixic acid were used as positive controls for inhibiting Aac (Cheng et al. 2000; 陳 2014). The pathogen was cultivated as mentioned above. OD_{600} of the Aac suspension was adjusted to 0.3 (approximately ~10^8 cfu/ml), and 100 μl of it was evenly spread over the LB agar plate. The bacterial strains for testing were incubated as mentioned above. OD_{600} of respective bacterium was adjusted to 1.0 (approximately ~10^8 cfu/ml) (Huang et al. 2016), and 20 μl of the individual suspension was added to the sterile filter paper disk (8 mm) placed on the LB plate with Aac lawn. Twenty microliter of the extract of *B. pilosa* as well as the commercial pestcide Ksm+oxc at 1:1000 dilution were also spotted on the sterile paper filter disk, respectively. The testing plates were incubated at 37°C for 48 h. The inhibition zone was determined by measuring the diameter of inhibition zone as described by Lightbown (1954).

doi:10.6342/NTU201804077

Effects of BP on growth of strain WF02 cultivated on LB agar

Strain WF02 was incubated as mention above. OD_{600} of the bacterial suspension was adjusted to 1.0 (approximately ~10^8 cfu/ml) and 100 μl of it was evenly spread over the LB agar plate. Crude extracts of BP was dissolved in LB broth with following final concentrations: (i) high concentration: 12,500, 25,000, 50,000, 100,000 and 200,000 ppm; (ii) low concentration: 300, 600, 1,000, and 2,000 ppm. Twenty microliter of respective dose of BP, fresh LB broth as well as 100,000 ppm ampicillin was added to the sterile filter paper disk (8 mm) placed on the LB plate with WF02 lawn. Ampicillin and LB were used as positive and negative control, respectively. The testing plates were incubated at 37 °C for 48 h. The inhibition zone was determined by measuring the diameter of inhibition zone as described by Lightbown (1954).

Effects of BP on growth of strain WF02 cultivated in LB broth

Strain WF02 was incubated as mention above. Using 100 μL of the prepared culture was inoculated to 50 mL of LB broth containing 600, 5,000, 25,000, 200,000 ppm BP (250-ml flask), respectively. Place the flask of the remaining WF02 in a 37 °C shaking incubator. Remove 1ml of bacterial suspension for cell counting at 0, 2, 4, 6, 8, 12, 16 and 24 h incubation. Subsequently, serial dilution was conducted to count the cell number. Take 100 μl of the diluted bacterial suspension, spread with glass beads on LB agar plates and incubated at 37°C. The number of CFUs per milliliter was calculated using the following formula described by Miles et al. (1938): Average CFU per milliliters = Average number of colonies for a dilution × 100 × Dilution factor.

doi:10.6342/NTU201804077

Biofilm assay

Biofilm formation assay was performed according to a protocol proposed by Merritt et al. with some modifications (Merritt et al. 2011). A single bacterial colony of WF02 was selected, inoculated into tube containing 3 mL of LB broth, and then incubated at 37°C and shaken (200 rpm) overnight. For sub-culturing, the above culture broth (3 ml) was taken, and inoculated into 250-mL Erlenmeyer flasks containing 50 mL of LB for 8 h incubation. OD_{600} of the suspension was adjusted to 1.0 (approximately ~10^8 cfu/ml), and 10 μl of the suspension was added to plastic tube containing 3 ml LB with or without BP extract (high concentration: 12,500, 25,000, , 50,000, 100,000 and 200,000 ppm; low concentration: 50, 100, 200, 300, 600, 1,000 and 2,000 ppm, respectively). Bacteria were incubated at 37°C in darkness. For biofilm quantification, the broth in the tube was slowly emptied and then rinsed with sterile distilled, deionized water (DDW) to remove the incomplete biofilm. The biofilm formed on the surface of tube was stained with 3.5 ml of 0.1% crystal violet for 15 min. The crystal violet solution was removed, and the tube was washed with DDW three times prior to observation. Subsequently, 3.5 ml of absolute ethanol was added into each tube and sonificated for 20 min to release the dye from the biofilms. Following, the absorbance at 570 nm was determined by using Ultraspec 2100 *pro* UV/visible spectrophotometer (Amersham Biosciences). The cell number of each tube was also calculated by colony counting on serial dilution plate.

doi:10.6342/NTU201804077

Bacterial cell vitality test

A simple resazurin-based assay was performed as described by Lee et al. (2016) to evaluate the bacterial vitality of WF02 in the presence of BP extract. Strain WF02 was incubated in 250-ml flask containing 50 ml LB broth at 37°C under shaking (200 rpm) in darkness for 8 h. OD_{600} of the suspension was adjusted to 1.0 (approximately ~10^8 cfu/ml), and 7.5 µl of the bacterial suspension was added to individual 96-well filled with 135 µl reagent. This reagent contained both carbon and nitrogen sources, including complete L2 medium (Suzuki et al. 2007) and 7.6 mM ammonium sulfate with 0.01% (w/v) resazurin dye (Sigma-Aldrich, St. Louis, MO, USA). Fifteen microliter (15 µl) of the BP extract (50, 100, 200, 300, 600, 1,000 and 2,000 ppm respectively) dissolved in ddH_2O was added to the reagent. The reactions were conducted under light-shielded conditions and were incubated at 37°C for 15 h. Cell vitality (i.e., metabolic activity) was confirmed through the changes in the color of resazurin. These color changes were from blue (oxidized, resazurin) to purple/pink (reduced, resorufin) and then to colorless (hydroresorufin). The cell vitality of strain WF02 was getting higher as the color getting lighter. Subsequently, the cell number of each tube was also calculated by colony counting on serial dilution plate.

doi:10.6342/NTU201804077

Evaluation of the seed treatments with chemistry or biology for controlling BFB

WF02 and Aac were pre-cultured in LB broth at 37°C overnight, respectively. Cultures were sub-cultured in 50 ml flask at 37°C for 8 h (WF02: OD_{600} = 1.0, containing ~10^8 cfu/ml; Aac: OD_{600} = 0.3, containing ~10^8 cfu/ml). Melon seeds were surface sterilized with 1% HCl for 20 min and washed with sterile water for 20 min. After air-drying overnight, the seeds were incubated with 20 ml of Aac suspension under a continuous vacuum for 20 min to cause the pathogen can be localized under the seed coat (Fessehaie and Walcott 2005). Aac treated seeds (n = 40 seeds/treatment) were air-dried overnight at room temperature, and subsequently vacuum infiltrated with 10ml biocontrol agents for 20 min (eight treatments: healthy seedlings, Aac inoculated seedlings, WF02, 300 ppm BP, 600 ppm BP, WF02+300 ppm BP, WF02+600 ppm BP, 81.3% KASUGAMYCIN+COPPER OXYCHLORIDE). In the healthy seedlings treatment, seeds were merely soaked with 10 ml of LB broth. The treated seeds were sowed in pots, which were placed on the greenhouse benches under 25-27°C with approximately 70-80% relative humidity, and 16 h of light daily. After 10 days culturing, bacterial fruit blotch symptoms (necrosis and water-soaking) were appeared on the melon leaves. Severity of disease for cotyledons of individual plant was determined by the descriptive scale (Cheng et al. 2000), with scores ranging from 0 to 4 (0 = no symptoms; 1 = small necrotic lesions on less than 1/3 of the cotyledon; 2 = necrotic lesions 1/3 to 2/3 of the cotyledon; 3 = large spreading lesions, greater than 2/3 of cotyledon; 4 = dead plant, as shown in Fig. M-1). The disease index, disease incidence and biocontrol efficacy were obtained by the following formula:

doi:10.6342/NTU201804077

Biocontrol Efficacy (%) = [(disease index of control plants – disease index of antagonist treated plants) /disease index of control] ×100%

Disease Incidence (%) = (number of diseased plants / total number of plants in each treatment) ×100%.

Disease index = Σ (disease severity × number of corresponding disease severity melon cotyledons)/(the highest disease severity × total number of melon cotyledons) × 100%

Figure M-1. The category of disease severity on the melon leaves.

The disease severity was determined by the descriptive scale from range 0 to 4 according to the percentage of lesion area.

doi:10.6342/NTU201804077

Quantifying the population of Aac in leaves

To extract Aac from the infected leaves, cotyledons of 10 plants were collected and weighted after 10 days harvesting. The modified method for pathogen extraction was performed according to the protocol proposed by Jiang et al. (2015). The mixed leaf tissue was suspended in 40 ml PBS solution containing 8 g/L NaCl, 0.2 g/L KCL, 1.42 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4 (Dulbecco and Vogt 1954) with shaking (200 rpm) for 30 min at room temperature. To count the cell number of Aac, 100 µl of the diluted bacterial suspension was spreaded on the AacG semi-selective agar plates using glass beads, and incubated at 37°C for two days. The contents of AacG medium was as follow: [containing, per liter: 0.5 g KH_2PO_4, 2 g $Na_2HPO_4 \cdot 12H_2O$, 2 g $(NH_4)_2SO_4$, 5 g L-glutamic acid, 12.5 mg bromothymol blue, 15 g agar, 20 mg ampicillin, 25 ppm cycloheximide] (陳 2014). L-glutamic acid is the main source of nitrogen nutrient in the selective medium. The amino acid will be utilized to produce alkali compounds by *A. avena*e subsp. *citrulli*, thus the medium containing bromothymol blue would turn green into blue color. The number of CFUs/ml per gram of cotyledon weight was calculated using the following formula described by Miles et al. (1938) : Average CFU per milliliters = Average number of colonies for a dilution × 100 × Dilution factor. Subsequently, the average CFU/per milliliters are divided into total gram of leaves in each treatments. A range of 30 to 300 colonies/plate was suggested for suitable number for counting on plates for counting (Breed and Dotterrer 1916).

doi:10.6342/NTU201804077

Genomic DNA extraction

Cultivation of strain WF02 was as mention above. After 8 h incubation at 37°C, 100 µl of the bacterial suspension was removed to a 2 ml microtube and centrifuged at 9,000 g for 5 min. After removing the supernatant, a sterile stainless bead (4 mm diameter) was added to the tube. The tube containing bacterial pellet was frozen by liquid nitrogen, then homogenized by SH-100 tissue grinder (Kurabo industries LTD.) immediately with shaking vigorously (700~800 rpm) for several times until the pellet turned into powder. Bacterial DNA was extracted and purified using the Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's instructions. The quantity and quality of the extracted DNA was assessed by UV spectrophotometry (Nanodrop, ND-1000, J&H technology Co., Ltd). High quality DNA (concentration > 600 ng/µl; A260/230 > 2.0; A260/280 = 1.8~2.0) was prepared for sequencing of MiSeq (Illumia) system and PacBio RS Ⅱ (Pacific Biosciences) platforms. Agarose gel electrophoresis (0.7%) was performed to analyze the completeness of genomic DNA.

doi:10.6342/NTU201804077

Whole genome sequencing and assembly

Genomic DNA of WF02 was sequenced by both MiSeq (Illumia) system and PacBio RSII (Pacific Biosciences) platforms at Genomics BioSci & Tech. Co., Ltd (Taipei, Taiwan). In total, 2,062,995 reads were produced from Illumia system with an average read length of 301 bp. The average read length of the PacBio with maximum read length of about 57,544 bases. The raw Illumia reads were trimmed at first position from 5'end and 3'end that has a quality score lower than 20 with average length of 270 bp. These trimmed Illumia reads and PacBio reads were used as the input for SPAdes Genome assembler version 3.11.1 with the default parameter (Bankevich et al. 2012). The reads were assembled into one contig with a total size of 4,026,648 bp.

Genome annotation and average nucleotide identity (ANI)

The circular chromosome was rotated and used *dnaA* as the first gene according to *Bacillus amyloliquefaciens* SQR9 that is known for plant growth promoting and biocontrol characteristic (Zhang et al. 2015). The programs RNAmmer 1.2 (Lagesen et al. 2007), tRNAscan-SE-1.3.1 (Lowe and Eddy 1997) and PRODIGAL (Hyatt et al. 2010) were used for gene prediction. Functional annotation of protein coding genes was based on BlastKOALA/KEGG (Kanehisa et al. 2016), homologous genes in *B. amyloliquefaciens* SQR9 (Zhang et al. 2015) as identified by OrthoMCL (Li et al. 2003) with a BLASTP (Camacho et al. 2009) e-value cutoff of $1e^{-15}$ and an inflation value of 1.5, BLASTP searches against NCBI non-redundant (nr) database (Benson et al. 2015). For average nucleotide identity (ANI), the multiple sequence alignment was based on MUSCLE (Edgar 2004) and the sequence identities were calculated using PHYLIP (Felsenstein 1989). CIRCOS (Krzywinski et al. 2009) was used to plot the final annotated chromosomes and show the GC-skew and GC content.

doi:10.6342/NTU201804077

Results

In vitro antagonistic assay against Aac by strain WF02

To understand the biocontrol potential of WF02, *in vitro* antagonistic assay against Aac was examined. Two commercial strains (*B. subtilis* strain Y1336 and *B. amyloliquefaciens* strain BPD1) were introduced for comparison. In addition, 81.3% KASUGAMYCIN+COPPER OXYCHLORIDE (1000X) which was reported to successfully inhibit Aac growth was used as a positive control (Cheng et al. 2000). The diameter of the clear area surrounding the individual colony (inhibition zone) was determined, and the antagonistic result was shown in Fig. 1 and Table 1. WF02 revealed antagonistic activity against Aac, and the expected antagonistic effect of this strain was larger than that of BPD1, and smaller than that of Y1336.

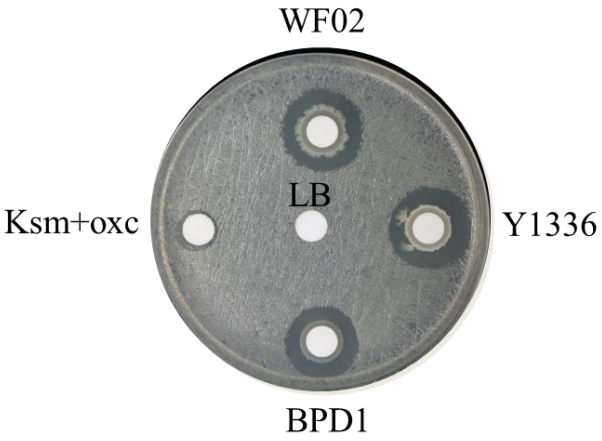


Figure 1. *In vitro* antagonistic activity against Aac by three strains of *Bacillus* species.

Zones of growth inhibition for *Acidovorax avenae* subsp. *citrulli* on LB medium. LB : negative control treatment. Ksm+oxc : commercial pesticide at 1:1000 dilution. WF02 and BPD1 are *Bacillus amyloliquefaciens* strains. Y1336 is a commercial strain of *Bacillus subtilis*.

doi:10.6342/NTU201804077

Table 1. *In vitro* inhibitory activity of three strain of *Bacillus* species against Aac.

	Diameter of Inhibition Zone (mm)
WF02	21.28 ±0.07 [b]
Y1336	22.85 ±0.84 [a]
BPD1	19.77 ±0.20 [c]

Values are mean inhibition zone (mm) ± S.D of three replicates. Means within a column followed by different letters are statistically significant by Tukey's test at $P < 0.05$.

In vitro antagonistic assay against Aac by the BP extraction

We evaluated the effects of BP extraction on the growth of WF02 by *in vitro* antagonistic assay. Since ampicillin has been demonstrated to have antibacterial effect on *Bacillus amyloliquefaciens* (Kadaikunnan et al. 2015), 100,000 ppm of ampicillin was used as a positive control. As shown in Fig. 2, ampicillin showed 18.4 ±0.54 mm inhibition zone. No antagonistic activity was observed while treating with either high concentration (12,500~200,000 ppm) or low concentration (300~2,000 ppm) of BP. We deduced that BP extraction does not influence the growth of WF02.

doi:10.6342/NTU201804077

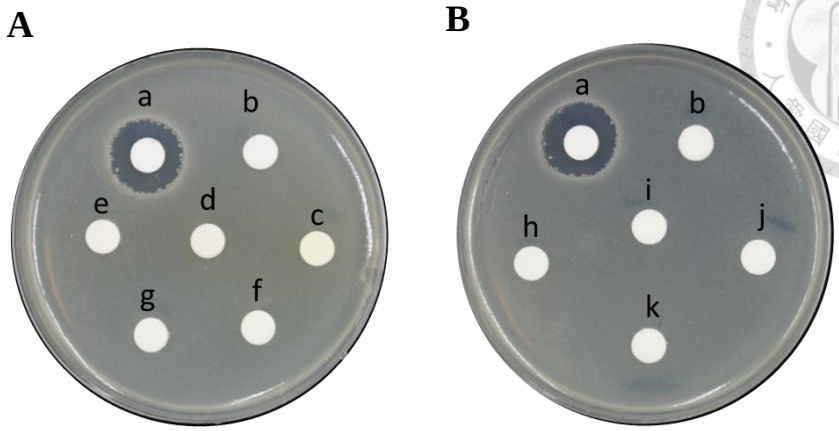


Figure 2. Antagonistic test of BP extraction against strain WF02.

WF02 suspension (~10^8 cfu/ml) was spreaded on the LB agar plate. Sterile filter paper disks (8mm) were placed on agar. (A) Disks contained high concentrations of BP extraction (12,500~200,000 ppm). a: 100,000 ppm ampicillin (positive control); b: LB (negative control); c: 200,000 ppm BP; d: 100,000 ppm BP; e: 50,000 ppm BP; f: 25,000 ppm BP; g: 12,500 ppm BP (B) Disks contained low concentrations of BP extraction; h: 2,000 ppm; i: 1,000 ppm; j: 600 ppm; k: 300 ppm.

Effects of BP extraction on the growth of strain WF02 in broth

To evaluate the effects of BP extraction on WF02 growth, LB broth supplemented with high (25,000 and 200,000 ppm, respectively) and low (600 and 5,000 ppm, respectively) concentrations of BP extraction were used to determine the growth curve of strain WF02. The initial inoculating concentration of strain WF02 was approximately 10^6~10^7 CFU/ml. As shown in Fig. 3, rapid exponential growths were exhibited in the treatments of WF02 and WF02+5,000 ppm BP, and achieved stationary state (~10^8 to 10^9 CFU/ml) at 6 h after inoculation. On the other hand, the growth of the WF02+600 ppm BP treatment remained static in the initial 2 h, and then the growth increased

doi:10.6342/NTU201804077

rapidly and achieved stationary state (~10^8 to 10^9 CFU/ml) at 8 h after inoculation. We noted the growth of WF02+25,000 ppm BP was also retarded in the initial 2 hours, and the growth rate was slower than the other treatments. It reached the steady state at 10 h after inoculation. We also found that the growth of WF02+200,000 ppm BP treatment was completely inhibited. According to the result, less than 5,000 ppm of BP did not influence the growth of strain WF02 during 24 h of incubation. By contrast, that would be significantly inhibited while treating with more than 25,000 ppm of BP extraction.

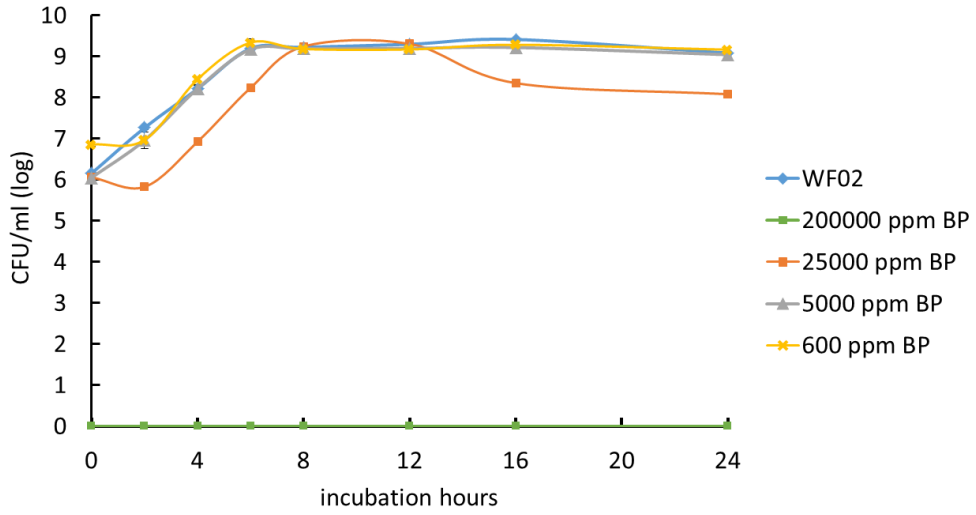


Figure 3. Effects of BP (600 ppm~200,000 ppm) on bacterial growth of strain WF02.

Four doses of BP extraction (625, 5000, 25,000, 200,000ppm) were used to determine the growth curve of strain WF02 during 24 h. Values are mean cell number ± S.D of three replicates.

doi:10.6342/NTU201804077

Effects of BP on biofilm formation of strain WF02

Colonization of plant surfaces by bacterial inoculants plays an important role in plant disease control. Biofilms are highly structured microbial communities on surfaces. The microbes inside are engulfed by a self-produced extracellular matrix consisting of proteins, polysaccharides and/or extracellular DNA (Costerton 1995; Das et al. 2013). The presence of biofilm improves the root colonization of beneficial bacteria and biocontrol efficacy (Bais et al. 2004; Kearns et al. 2005). To understand the effects of BP on the biofilm formation of strain WF02, we conducted a modified biofilm assay (Merritt et al. 2011). As shown in Fig. 4A, the bacterial biofilm formation of WF02 was enhanced by the addition of BP. Moreover, we determined the cell number of WF02 after biofilm formation (Fig. 4B). Intriguingly, there was no significant difference among the treatments (Tukey's test at $P > 0.05$). This result suggest that while treating WF02 with BP at the concentrations of 300 ppm to 2,000 ppm does not influence the bacterial growth, furthermore, can improve the bacterial biofilm formation.

doi:10.6342/NTU201804077

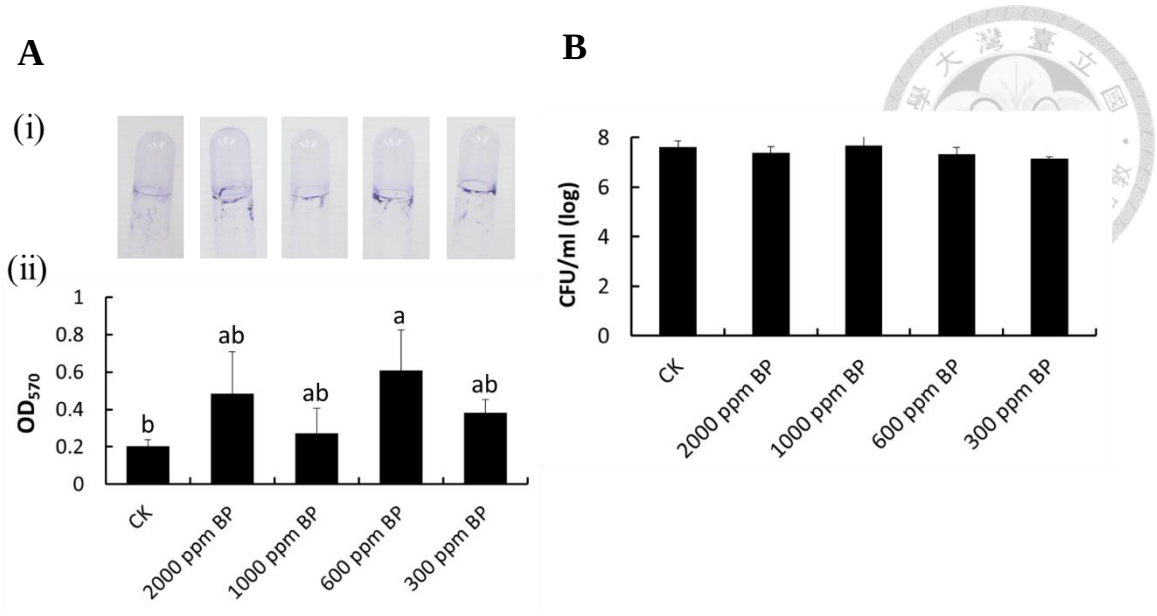


Figure 4. Effects of BP on biofilm formation of strain WF02.

(A) The biofilm formation was measured at OD_{570} in treatment with 300~2,000 ppm BP. Values are mean OD_{570} ± S.D of three replicates. Different letters are statistically significant by Tukey's test at $P < 0.05$. (B) The cell number of strain WF02 in each treatment. Values are mean cell number ± S.D of three replicates. No significance was observed between five treatments (Tukey's test at $P > 0.05$).

doi:10.6342/NTU201804077

Effects of BP on cell vitality of strain WF02

To evaluate the cell vitality of individual treatment, we performed a resazurin-based assay. Strain WF02 was cultured in the L2 broth with nutrient deficiencies, and the BP at different concentrations (50~2,000 ppm) acted as nutrition additives. As shown in Fig. 5A, the sample solution turned dark blue to light purple or pink after 15 h of incubation. Strong intensity of reactions (values around 3 to 4) were occurred at relatively high concentrations of BP (600 ppm~2,000 ppm). We found that the living cell number was significantly decreased (from 7.0 log CFU/ml to 2.5 log CFU/ml, approximately equivalent to 10^7 to 10^3 CFU/ml) after incubation while no BP was added (WF02 only) (Fig. 5B). On the other hand, the living cell numbers were maintained approximately at 10^6 to 10^7 CFU/ml after 15 h incubation while treating WF02 with concentration of BP at 1,000 ppm and 2,000 ppm. On the other hand, while treating WF02 with BP at the concentrations lower than 300 ppm, the living cell numbers were significantly reduced after 15 h incubation in comparison with those in variant dose of BP. Taken together, These results indicated that proper dose of BP could be the nutrient source for strain WF02 growth during incubation in the nutrient-deficient environments.

doi:10.6342/NTU201804077

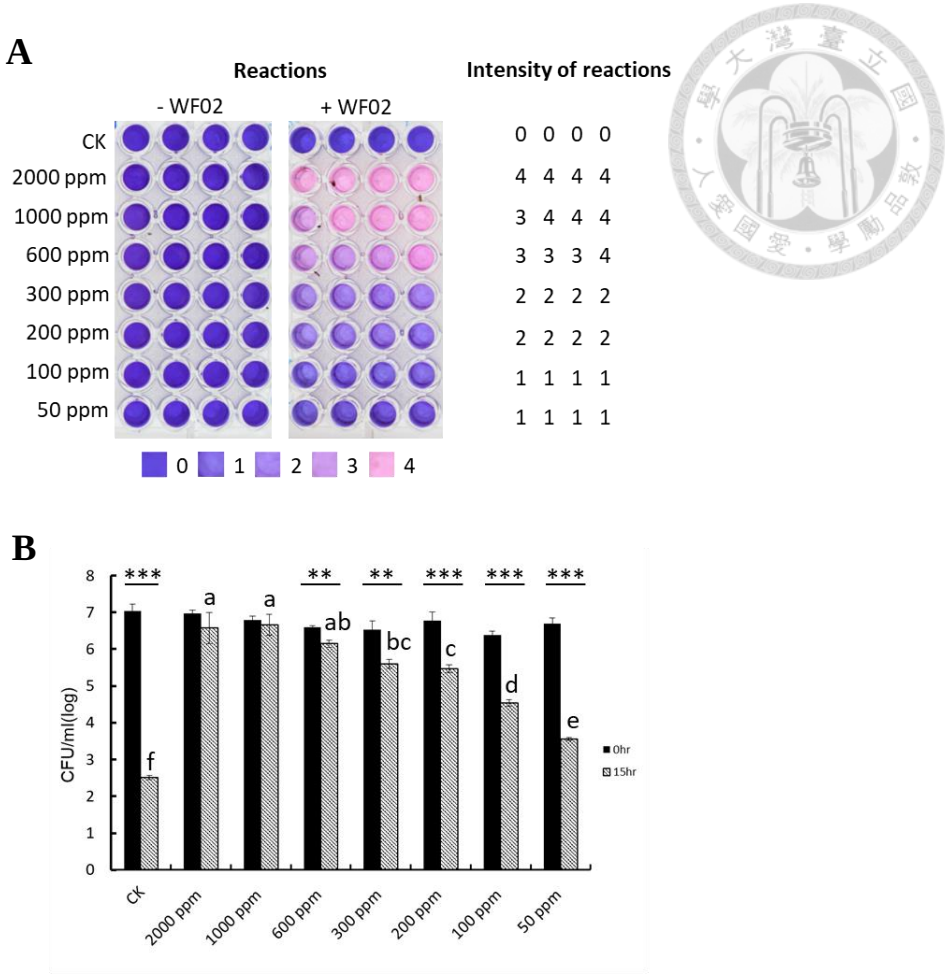


Figure 5. Effects of BP (50~2,000 ppm) on the cell vitality of strain WF02.

(A) Resazurin-based assay. The color changes were from blue (oxidized form, resazurin) to purple/pink (reduced form, resorufin) and then to colorless (hydroresorufin). For numerical interpretation in all cases, "0" designated negative reactions, and "1-4" designated positive reactions with different intensities. "L2" indicates that L2 broth without BP. (B) The cell number of strain WF02 in each reaction. Values are mean cell number ± S.D of three replicates. Means with the different letters are statistically significant by Tukey's test at $P < 0.05$. The asterisks means statistically significant compared with each reaction at 0 h by student *t* test (**$P < 0.01$, ***$P < 0.001$).

doi:10.6342/NTU201804077

***In vitro* antagonistic ability of the supernatant of strain WF02**

We collected the cell free supernatant from the broth of WF02 at 8 h, 24 h and 48 h after incubation. As shown in Fig. 6, none of the supernatant could inhibit the pathogen growth, indicating only living bacterium had antagonistic activity. To develop a more effective microbial inoculant, we referred to the above results and applied BP additive with 300 ppm and 600 ppm, respectively, to the broth of WF02. Since nalidixic acid and the chemical pesticide KASUGAMYCIN+COPPER OXYCHLORIDE (1000X 81.3% Ksm+oxc) were reported to inhibit the growth of *A. avenae* subsp. *citrulli* (Cheng et al. 2000; 陳 2014), we used them as positive controls in this study. However, we didn't observe inhibitory effect on Aac by the chemical pesticide (Ksm+oxc) treatment, which was contrary to the result shown in Fig 1. This contradict result might due to the impurity of the pesticide. The impurities of pesticide may affect to the toxicity of the pesticide (Ambrus et al. 2003; Armenta et al. 2005). As shown in Fig. 6B, the individual treatment with WF02 and BP showed antagonistic activities against Aac. However, the diameters of the inhibition zones were not influenced by the concentrations of BP, indicating that the antagonistic effects were not dose related. In addition, we didn't observe any antagonistic activity by the BP additive (300, 600 ppm) itself (Fig. 6B-g, h). It indicates that adding BP to WF02 neither improving the antagonistic activity toward WF02 on the plate, nor affecting the inhibition activity of WF02.

doi:10.6342/NTU201804077

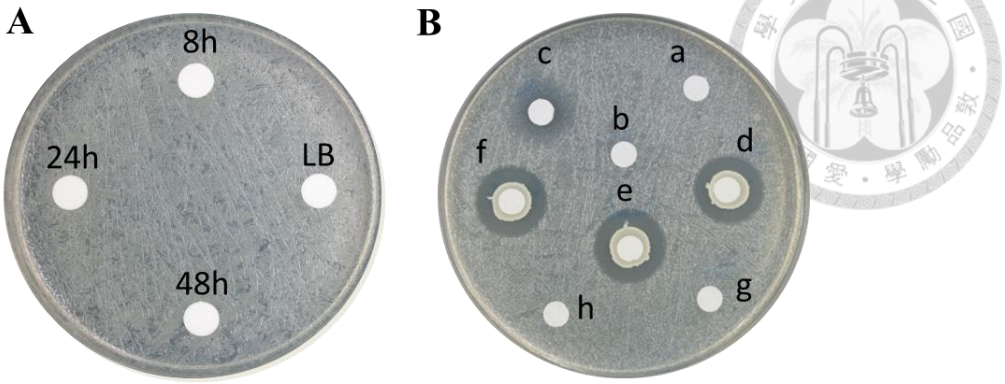


Figure 6. Cell free supernatant of strain WF02, live cell of WF02 and BP *in vitro* antagonistic activity against Aac.

(A) Dual test of cell free supernatant of WF02 (8 h, 24 h, 48 h) against Aac. LB was used as negative control. (B) Antagonistic assay of eight treatments against Aac. a: LB; b: KASUGAMYCIN+COPPER OXYCHLORIDE (commercial pesticide, 1000X 81.3%); c: 100 ppm nalidixic acid; d: WF02; e: WF02+300 ppm BP; f: WF02+600 ppm BP; g: 300 ppm BP; h: 600 ppm BP.

doi:10.6342/NTU201804077

***In vivo* biocontrol efficacy of WF02+BP treatment**

Melon seeds (cv. silver light) were treated individually with various doses of BP, WF02 broth, as well as the combination of WF02 and BP. These seeds were sowed in the horticultural soil and cultivated for 10 days to evaluate their efficacy against Aac. Water-soaked lesion area was usually appeared on the cotyledons after 6 to 10 days of growth (Fig. 7). The germination rates as well as the disease severities of the individual treatment were shown in Fig. 8. The disease severity of individual plant was categorized into five groups (from 0 to 4) according to percentage of the lesion area (Fig. M-1). The percentage of inoculated leaves was represented the disease severity for each treatment according to the lesion area on each leaves (Mutka et al. 2013). Accordingly, the biocontrol effect is considered to be increased in correspondence to the decrease of the value. There was no significant difference in the seed germination rate among the treatments in respective trial (higher than 80%). It indicates that inoculating with WF02 or WF02+BP does not influence the seed germination activity, even those treated with relatively high concentrations of BP (> 25,000 ppm) (Fig. 8A, 8C, 8E).

We noted that additing high concentrations of BP (> 25,000 ppm) to WF02 broth inhibited the growth of this bacterium (Fig. 3). However, while melon seeds were pre-treated with high dose (> 25,000 ppm) of BP, or with the mixed inoculants (WF02 + > 25,000 ppm BP) exhibited decent biocontrol effects on BFB (Fig. 8B). In consideration of disease controlling efficacy as well as the processing expense, we would like to select suitable inoculant formulation with relative low concentration of BP. We found that solely treating 600~5,000 ppm dose of BP failed to inhibit Aac, and around 50%~80% seedlings were dead (Fig. 8D). On the other hand, as long as the above individual dose of BP supplemented with WF02 could effectively reduce the lesion area on the leaves of melon seedlings, suggesting Aac was inhibited. Among the treatments, we found that

doi:10.6342/NTU201804077

either the WF02 or the WF02+600 ppm BP could suppress the disease development more effectively than the other WF02+BP treatments (Fig. 8D). In a different lot of experiment, we found that WF02+300 ppm showed similar biocontrol effect with that of WF02+600 ppm (Fig. 8F). The discrepant values of disease severity in individual treatment, such as the groups of Aac infected as well as those of the WF02 inoculated seedlings shown in Fig. 8D, 8F were resulted from different biological repeat.

We further assessed the biocontrol effects of the eight treatments shown in Fig 8F by three indexes. As shown in Table 2, the commercial pesticide Ksm+oxc treatment showed the highest control efficacy (~99.49%) to suppress the disease development, resulted in the lowest disease incidence (~0.98%) and disease index (~0.36%). Although the control efficacies of the microbial inoculant treatments were not as high as that of the chemical pesticide, those of the WF02 single inoculant as well as the WF02+BP (300 or 600 ppm) mixed inoculant could achieve more than 69%. In addition, these treatments could significantly reduce the disease incidence rate from ~96% to ~53%, as well as reduced the disease index from ~68% to ~21% in comparison with that of the Aac infected group.

doi:10.6342/NTU201804077

Figure 7. Biocontrol efficacy on melon seedlings exerted by different treatments.

Forty seedlings for each treatment were used to evaluate the biocontrol efficacy against BFB. Eight treatments were as following: healthy seedlings, Aac infected seedlings, WF02, 300 ppm BP, 600 ppm BP, WF02+300 ppm BP, WF02+600 ppm BP, Ksm+oxc.

doi:10.6342/NTU201804077

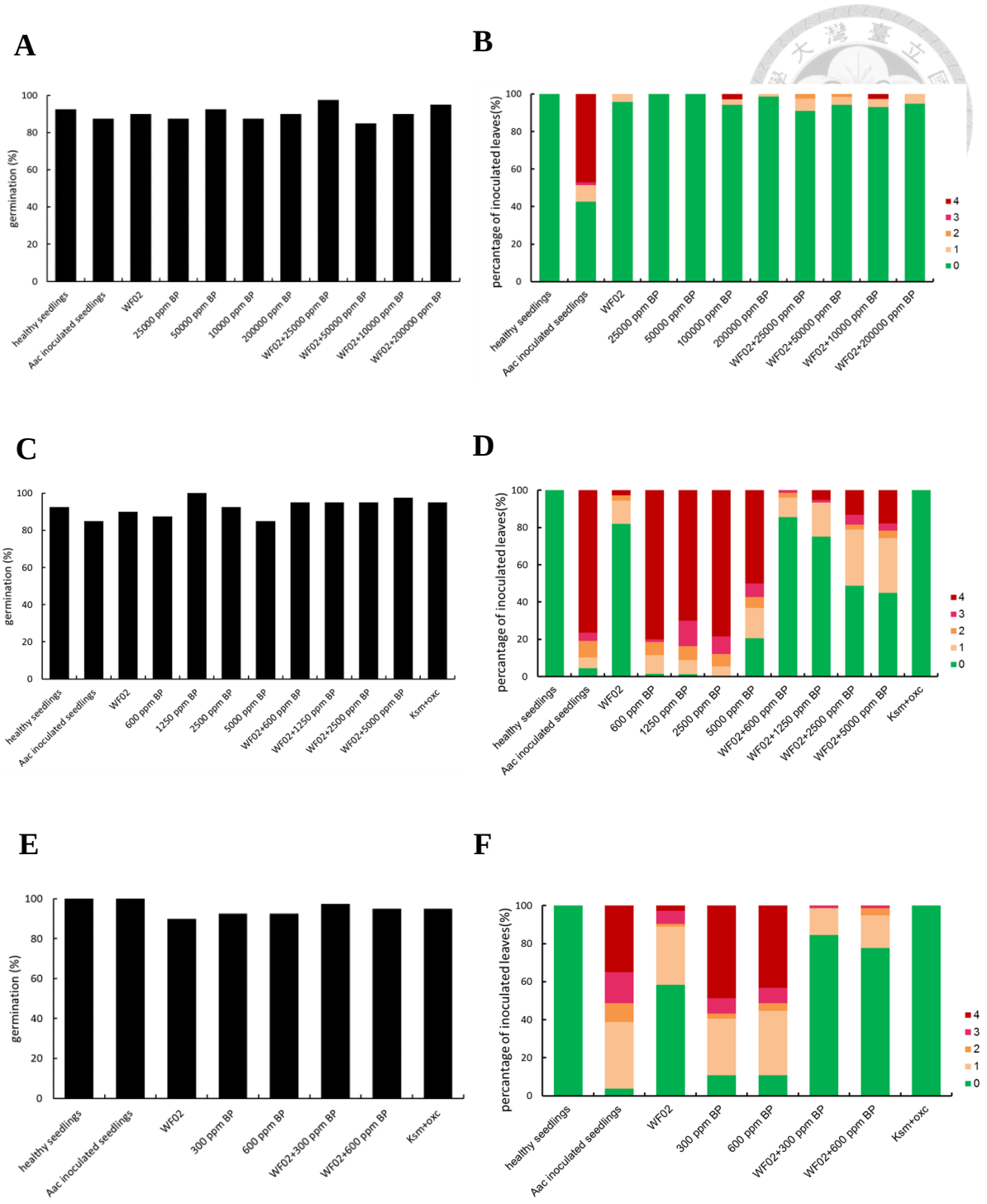


Figure 8. The seed germination and biocontrol effects on melon seedlings exerted by different treatments.

Melon seeds were treated with WF02 (approximately 10^8 CFU/ml) and BP with different concentration ranges. (A-B) 25,000~200,000 ppm; (C-D) 600 ppm~5,000 ppm BP; and (E-F) 300 ppm and 600 ppm. Ksm+oxc was the commercial pesticide

doi:10.6342/NTU201804077

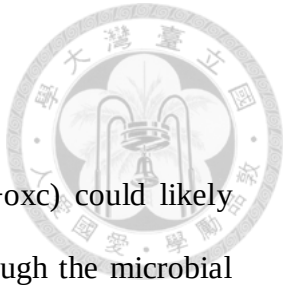
used as a positive control. Categories 0-4 indicate the severity of disease observed in the leaf area (Fig. M-1). 0 = no symptoms; 1 = small necrotic lesions on less than 1/3 of the cotyledon; 2 = necrotic lesions 1/3 to 2/3 of the cotyledon; 3 = large spreading lesions, greater than 2/3 of cotyledon; 4 = dead plant. The percentage of inoculated leaves was exhibiting the lesion covering 0 (healthy plants), < 1/3, 1/3~2/3, > 2/3 of the leaves area and dead plants.

Table 2. Biocontrol effects of different inoculated seed treatments.

	Aac inoculated	WF02	300 ppm BP	600 ppm BP	300 ppm BP +WF02	600 ppm BP +WF02	Ksm+oxc
Biocontrol /Chemical-control efficacy(%)	--	69.51[a] ±17.02	0 [a]	0 [a]	75.27 [a] ±24.33	69 [a] ±26.26	99.49 [a] ±0.87
Disease incidence(%)	96.26 [a] ±4.39	53.65 [b] ±13.57	96.34 [a] ±1.51	96.31 [a] ±4.10	42.40 [bc] ±30.91	53.5 [b] ±19.66	0.98 [c] ±1.69
Disease index(%)	68.34 [a] ±6.45	21.03 [b] ±12.92	72.28 [a] ±8.60	73.93 [a] ±12.42	17.62 [b] ±18.04	21.74 [b] ±19.65	0.36 [b] ±0.63

Biocontrol efficacy/Chemical-control efficacy, disease incidence and disease index were performed to evaluate the biocontrol effects. Values of each indicate were mean ± S.D of three replicates. Values with the different letter within the same line are significantly different at $P < 0.05$ according to Tukey's test.

doi:10.6342/NTU201804077



Reduction of Aac cell numbers by biocontrol treatments

As shown in Fig. 9, the chemical pesticide treatment (Ksm+oxc) could likely completely inhibit the Aac on the leaves. On the other hand, although the microbial inoculants could not inhibit Aac as good as chemical pesticide treatment, the single (WF02) or the mixed inoculants (WF02+300 ppm BP and WF02+600 ppm BP) could significantly reduce the cell number of Aac in comparison with that of Aac infected leaves (Fig. 9). We noticed that although the Aac cell numbers of the microbial inoculant treatments maintained at high value (approximately10^7~10^8 CFU/g leaves), the disease incidences and severities were significantly reduced (Table 2). It suggests that in spite of the high population of Aac was still existed on the leaves of seedlings after treating with the WF02 derivative inoculants, the virulence of Aac is effectively repressed. This finding remains to be elucidated.

doi:10.6342/NTU201804077

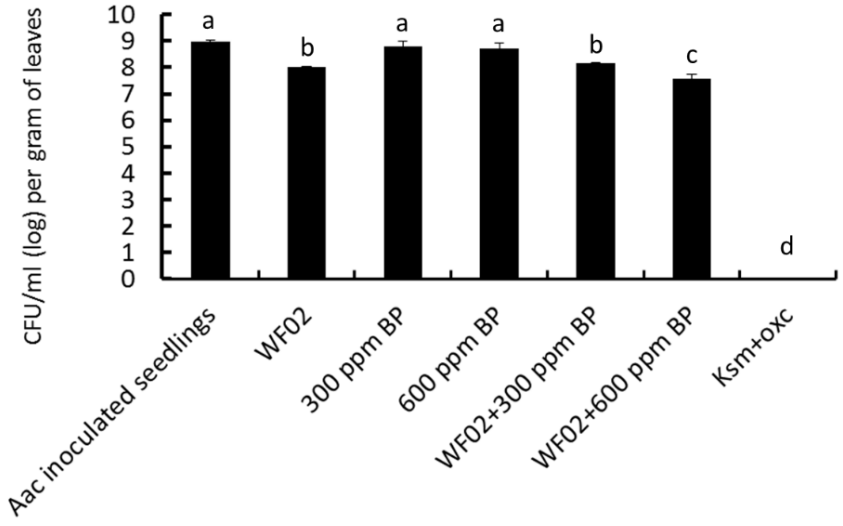


Figure 9. Extraction of Aac cells from inoculated melon leaves.

Twenty cotyledons of each treatment were collected and soaked in 40 ml PBS with shaking for 30 min to extract Aac. The bacterial suspension was diluted and spreaded on AacG semi-selective agar plate. The cell number per gram of cotyledons was acquired from colonies calculating. Values are the mean cell number ± S.D of three replicates. Values with the different letter are significantly different at $P < 0.05$ according to Tukey's test.

doi:10.6342/NTU201804077

General genome features of strain WF02

The general features of WF02 and other relevant strains of *B. amyloliquefaciens* were summarized in Table 3. The complete genome of WF02 comprises one circular chromosome of 4,026,624 bp with a GC content of 46.5% (Figure 10). Genome analysis showed that the genome contained 3,873 protein-coding genes, 9 rRNA operons, 86 tRNA genes. The protein-coding genes cover 88.7% of the genome and have an average length of 922 bp (Table 3). We noted that the genome size of WF02 was somewhat bigger than that of *B. amyloliquefaciens* FZB42 that is a well-known biocontrol bacterium against fungal and bacterial pathogens (Borriss 2011; Chen et al. 2007).

Table 3. Genomic features of the WF02 and the other *B. amyloliquefaciens* strains.

Features	WF02	SQR9	FZB42
Genbank accession	-	CP006890	CP000560
Genome size (bp)	4,026,648	4,117,023	3,918,589
G+C content (%)	46.5	46.1	46.4
rRNA operons	9	7	10
tRNA	86	72	89
Protein-coding genes	3,873	4,078	3,693
Average CDS size	922	916	933
Biology property	biocontrol characteristics	plant growth promotion / antimicrobial compounds producing	antimicrobial compounds producing
Reference	This study	(Zhang et al. 2015; Chowdhury et al. 2015)	(Chen et al. 2007; Chowdhury et al. 2015)

doi:10.6342/NTU201804077

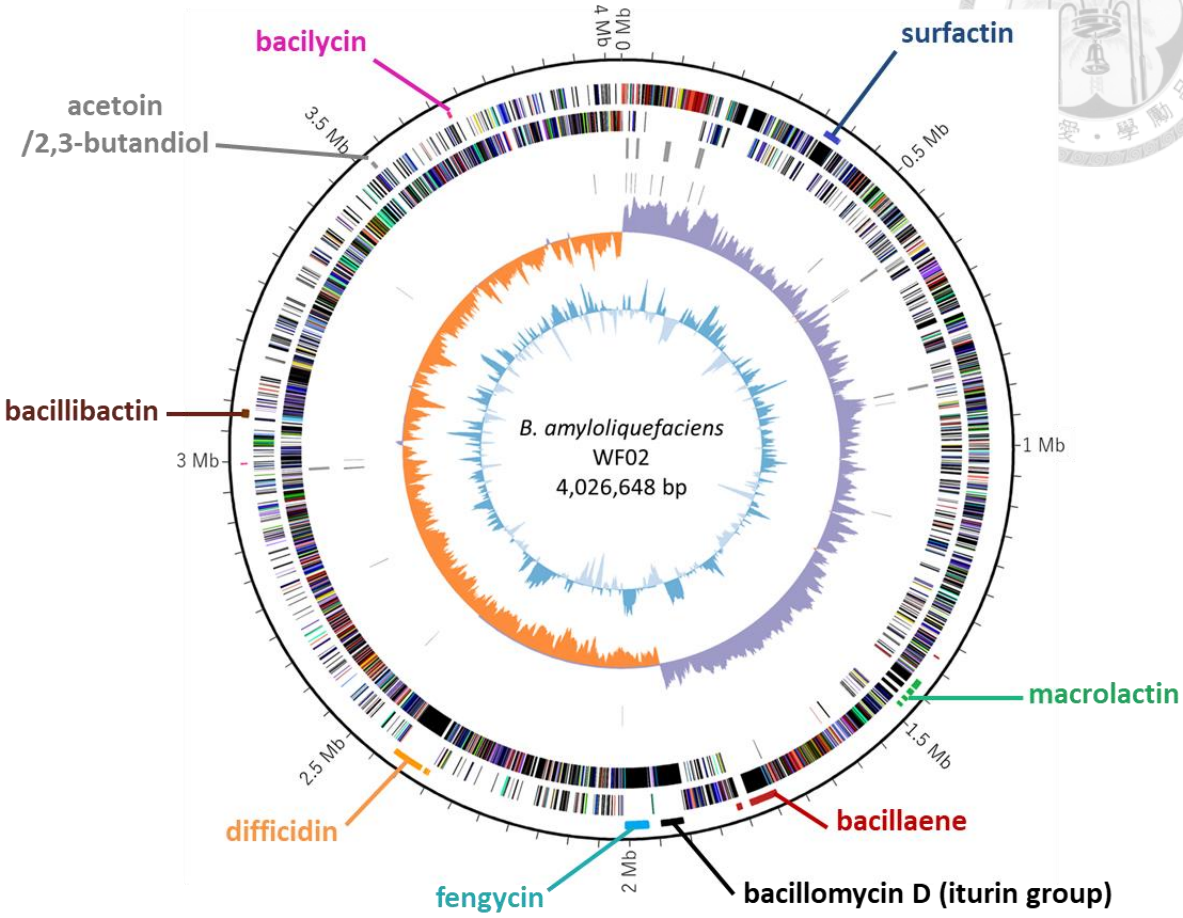


Figure 10. Genome map of *B. amyloliquefaciens* WF02.

From outer to inner circle, (1) antimicrobial compounds related gene clusters (NRPS and PKS) were categorized into different colors; (2) protein-coding regions on the forward strain; (3) protein-coding regions on the reverse strain; (4) rRNA; (5) tRNA; (6) GC skew purple (above average) and orange (below average); (7) GC content in dark blue (above average) and light blue (above average).

doi:10.6342/NTU201804077

Genes encoding secondary metabolites of biocontrol function

B. amloliquefaciens strains are known for their diverse of secondary metabolites production, such as those of cyclic lipopeptides (LPs): surfactin, iturin and fengycin (Ongena and Jacques 2008). These metabolites were reported to be involved in suppressing harmful microbes in the plant rhizosphere (Chen et al. 2007). According to the genomic analysis, WF02 possesses 9 genes clusters which contribute to synthesis of antimicrobial metabolites are approximately 8% of the whole genome (Fig. 10, Table 4). These clusters are also present in the other *B. amyloliqefaciens* strains, FZB42 and SQR9, which are known for the biocontrol characteristics (Chen et al. 2007; Chowdhury et al. 2015; Zhang et al. 2015). The gene clusters of the non-ribosomal peptide synthases (NRPSs) were found, such as the antimicrobial lipopepetides surfactin (BAWF02_03200-03230, BAWF02_03250), bacillomycin D which is an antibiotic of iturin group (BAWF02_17610-17640), fengycin (BAWF02_17870-17910). In particular, the gene cluster of fengycin synthetase (*fenABCDE*) showed low identities (61.6~71.8%) to those in *B. amyloliquefaciens* SQR9 and *B. amyloliquefaciens* FZB42 (61.6~71.9%). Besides, WF02 also harbors the putative iron-siderophore bacillibactin (BAWF02_28790-28830), and antimicrobial compound bacilysin (BAWF02_35690-35720, BAWF02_27980) related genes. The gene cluster of bacilysin except *bacA* gene (BAWF02_27980) showed high identity to that in SQR9 (~99%) and in FZB42 (~98.7%). The *bacA* gene which is within the bacilysin gene cluster displayed low identity to that in *B. amyloliquefaciens* SQR9 (54.1%) and in *B. amyloliquefaciens* FZB42 (53.8%). The gene clusters encode the polyketide synthase (PKS) for difficidin (BAWF02_22040-22180), macrolactin (BAWF02_13880-13960) and bacillaene (BAWF02_13310, BAWF02_16460-16580) were also identified in the genome. The volatile compounds (VOCs) produced by rhizobacteria, such as acetoin

doi:10.6342/NTU201804077

and 2, 3-butanediol were reported to trigger induced systemic system (ISR) in plants (Yi et al. 2016). The gene *ydjL* (BAWF02_05810), nowadays renamed *bdhA*, which is responsible for encoding acetoin reductase/butanediol dehydrogenase (Nicholson 2008), and *alsDRS* (BAWF02_34020-34040) that is involved in the synthesis of acetoin (Renna et al. 1993). In total, we estimated that there was 317.4 kb of genes with respect to the synthesis of the antimicrobial compounds in the genome of *B. amyloliquefaciens* WF02.

doi:10.6342/NTU201804077

Table 4. Genes and gene clusters involved in synthesis of biocontrol metabolites in *B. amyloliquefaciens* WF02.

Compound	Locus tag	gene name	size	Function (Chowdhury et al. 2015)	Identity to SQR9 (%)	Identity to FZB42 (%)
Surfactin	BAWF02_ 03200-03230 /BAWF02_03250	*srfABCD,sfp*	28.3kb	Biofilm formaition , ISR	97.5– 98.2	97.4 - 98.3
Bacillomycin D	BAWF02_ 17610-17640	*bmyCBAD*	37.2kb	Direct suppression , ISR	75.8 – 97.4	75.8 – 97.4
Fengycin	BAWF02_ 17870-17910	*fenABCDE*	37.6kb	Direct suppression , ISR	61.6 – 71.8	61.6 – 71.9
Bacillibactin	BAWF02_ 28790-28830	*dhbABCEF*	11.7kb	Siderophore	96.1 – 97.6	95.9 – 97.5
Difficidin	BAWF02_ 22040-22180	*dfnAYXBCDEFGHIJKLM*	69.5kb	Direct suppression	96.8 – 98.6	97.0 – 98.6
Macrolactin	BAWF02_ 13880-13960	*mlnABCDEFGHI*	53.2kb	Direct suppression	96.8 – 97.8	97.0 – 97.6
Bacillaene	BAWF02_13310 /BAWF02_ 16460-16580	*baeBCDE,acpK, baeGHIJLMNRS*	71.1kb	Direct suppression	97.0 – 98.7	59.4 – 98.7
Bacilysin	BAWF02_ 35690-35720 /BAWF02_27980	*bacABCDE,ywfG*	5.2kb	Direct suppression	54.1 – 99.0	53.8 – 98.7
Acetoin/2,3-butandiol	BAWF02_05810 /BAWF02_ 34020-34040	*bdhA, alsDSR*	3.6kb	Synthesis of VOCs , ISR	98.5 – 99.6	98.3 – 99.1

doi:10.6342/NTU201804077

Putative biofilm formation related genes were present in genome of strain WF02

Bacteria are capable of forming structured multicellular communities, such as biofilms (O'Toole et al. 2000). Biofilms produced by rhizobacteria are often associated with plant protection traits (Chen et al. 2013). Genes of the regulatory pathways in controlling biofilm formation were identified in the genome of WF02 (Table 5). The key gene in the regulatory network is biofilm repressor SinR (*sinR*, BAWF02_23050). SinR suppresses the *espA-O* (BAWF02_32370-BAWF02_32510) gene cluster that is associated with the enzymes of exopolysaccharide producing, as well as the *tapA-sipW-tasA* (BAWF02_23060-BAWF02_23080) operon that is responsible for making sugar and protein for synthesis of biofilm matrix (Chu et al. 2006; Kearns et al. 2005). Under biofilm inducing condition, the transcription of the matrix synthesis related genes was depressed in the presence of the anti-repressor SinI (*sinI*, BAWF02_23040). SinI antagonized to SinR and activated by a master regulator (*spo0A*, BAWF02_22670) (Bai et al. 1993; Newman et al. 2013). SinR also acted together with other regulator proteins like AbrB and DegU (*abrB*, BAWF02_00410; *degU*, BAWF02_33490), which were also present in WF02.

doi:10.6342/NTU201804077

Table 5. Putative genes involved in synthesis of biofilm formation in *B. amyloliquefaciens* WF02.

Gene	Locus tag	Start	End	Length (bp)	Definition	Identity to SQR9 (%)	Identity to FZB42 (%)
epsO	BAWF02_32370	3397004	3397969	966	putative pyruvyl transferase	96.3	96.8
epsN	BAWF02_32380	3397948	3399120	1173	aminotransferase	97.4	98.1
epsM	BAWF02_32390	3399125	3399772	648	acetyltransferase	97.0	97.0
epsL	BAWF02_32400	3399769	3400377	609	undecaprenyl-phosphate galactose phosphotransferase	96.7	96.8
epsK	BAWF02_32410	3400374	3401891	1518	putative O-antigen transporter	98.0	97.6
epsJ	BAWF02_32420	3401888	3402922	1035	glycosyl transferase	97.1	97.3
epsI	BAWF02_32430	3402919	3403995	1077	Exopolysaccharide biosynthesis protein	97.4	97.5
epsH	BAWF02_32440	3404000	3405037	1038	putative capsular polysaccharide biosynthesis protein	97.7	97.3
epsG	BAWF02_32450	3405056	3406159	1104	Transmembrane protein	98.2	98.6
epsF	BAWF02_32460	3406163	3407299	1137	glycosyl transferase group 1	97.7	98.0
epsE	BAWF02_32470	3407292	3408134	843	glycosyltransferase	97.9	97.3
epsD	BAWF02_32480	3408131	3409270	1140	group 1 glycosyl transferase	96.5	96.8
epsC	BAWF02_32490	3409286	3411103	1818	caspsular polysaccharide biosynthesis protein	95.8	96.3
epsB	BAWF02_32500	3411324	3412004	681	capsular polysaccharide biosynthesis protein	97.7	98.8
epsA	BAWF02_32510	3412010	3412717	708	protein tyrosine kinase EpsB modulator	98.3	98.1

doi:10.6342/NTU201804077

Table 5. Putative genes involved in synthesis of biofilm formation in *B. amyloliquefaciens* WF02.

Gene	Locus tag	Start	End	Length (bp)	Definition	Identity to SQR9 (%)	Identity to FZB42 (%)
sinR	BAWF02_23050	2504733	2505074	342	master regulator for biofilm formation	99.7	99.7
tapA	BAWF02_23080	2506527	2507198	672	TasA anchoring/assembly protein	98.3	98.0
sipW	BAWF02_23070	2505971	2506555	585	signal peptidase I	97.9	98.6
tasA	BAWF02_23060	2505122	2505907	786	spore coat-associated protein	98.7	98.2
abrB	BAWF02_00410	45960	46244	285	Transition state regulatory protein	100	100
degU	BAWF02_33490	3507920	3508609	690	response regulator	98.9	99.7
sinI	BAWF02_23040	2504532	2504705	174	antagonist of SinR	99.4	99.4
spo0A	BAWF02_22670	2470572	2471372	801	stage 0 sporulation protein	99.1	98.6

doi:10.6342/NTU201804077

Discussion

Several literatures have shown the antagonistic ability of *Bacillus* species against BFB, such as *B. amyloliquafaciens, B. subtilis, B. pumilus* and *B. methylotrophicus* (Fan et al. 2017; Horuz and Aysan 2018; Jiang et al. 2015; Santos et al. 2006). Jiang et al (2015) revealed that *B. amyloliquafaciens* 54 could control BFB by increasing the expression of the defense-related genes *PR1* and accumulating the hydrogen peroxide in the plants. This was the first report for biocontrolling BFB by *B. amyloliquafaciens*. In this study, *B. amyloliquefaciens* strain WF02 showed effective biocontrol activity against Aac both *in vitro* and *in vivo* (Fig. 1 and Fig. 8). We also used *B. subtilis* Y1336 as a positive control strain against BFB for it showed prominent inhibitory effects on Aac in the *in vitro* test, although there was no related report with respect to this trait (Yang et al. 2012; 石 2008) (Fig. 1). *B. amyloliquafaciens* BPD1 was another commercial strain, which was also applied in this study, however, it showed least inhibitory ability among the three *Bacillus* spp. strains (Fig. 1). In a previous study reported by Fan and colleagues, a mutant of *B. subtilus* 9407 (*ΔsrfAB*) that was defected in surfactin-production resulted in a significant decrease in colonization, swarming ability as well as the inhibitory ability of Aac (Fan et al. 2017). It suggests that surfactin plays an important role in suppressing the Aac activity. WF02 also possesses surfactin related genes (BAWF02_03200-03230, BAWF02_03250), whether its antagonism against Aac via surfactin remains to be elucidated.

To enhance the biocontrol efficacies exerted by single BCA, there has been increasing interest recently among researchers in using mixtures of BCAs (Jayakumar et al. 2007; Lopez-Reyes et al. 2016; Xu et al. 2011). As shown in Fig. 8B, we found that high concentrations (> 25,000 ppm) of BP could successfully suppress the disease development of Aac directly, however, they also retarded the growth of WF02 (Fig. 3).

doi:10.6342/NTU201804077

It suggests that too much BP will harm the normal growth of WF02. On the other hand, while treating WF02 with BP improved the amount of biofilm formation of this bacterium (Fig. 4). Sandasi et al. (2010) also reported that the extracts of *Aloe vera*, *Thymus vulgaris*, or *Vaccinium macrocarpon* could enhance the biofilm formation of *Listeria monocytogenes* ATCC 19111. The enhancement of the biofilm formation may due to the presence of certain compounds in the plant extracts, such as lectin (Sandasi et al. 2008). Previous studies have also indicated that supplementation of several kinds of additives, such as skim milk, herbal extracts could improve the cell viability of biocontrol agents (Szczech and Maciorowski 2016; Ting et al. 2009; Xue et al. 2014). As shown in Fig. 5, we also noted that treating WF02 with 600 to 2,000 ppm of BP could effectively improve the cell vitality of this bacterium. Therefore, we deduced that BP could bring synergistic effects on biocontrol efficacy by enhancing the biofilm formation and cell vitality of the BCA WF02.

According to Fig. 6A, the cell-free supernatant of WF02 broth did not show inhibitory effect on pathogen after 8 to 48 h incubation. Since this result was contrary to that shown in Fig. 1, suggesting that the presence of WF02 living cells was necessary to confer the antibacterial effect against Aac. This phenomenon was due to the following possibilities: (i) there was no active ingredient (AI) presented in the supernatant, or the amount of AI in the supernatant was too low to inhibit Aac (Acuña-Fontecilla et al. 2017) (ii) the potential volatile organic compounds (VOCs) or AIs produced by the living cells were eliminated during the preparation of cell-free supernatant. It has been reported that the amounts of VOCs would be lost by 20% to 70% during the freeze-drying process while treating the extraction detergents like alcohol, sulfur, or aldehydes (Her et al. 2015). We have not identified the potential AIs

doi:10.6342/NTU201804077

of WF02, and it would be further investigated for elucidating the inhibitory mechanism against BFB.

Since BFB is a seed-borne disease, early protection is effective to prevent breakouts before sowing the seeds in soil (Latin 1996). Seed treatments are treating the biological, physical and chemical agents and techniques applied to seed to provide protection and improve the establishment of healthy crops (Govindaraj et al. 2017), which are convenient, generally inexpensive, and familiar applying methods for farmers (Leggett et al. 2011). When bioprotectants applied in seed treatment trials, the biocontrol strains have the potential to colonize on the emerging roots and produce antibiotics against pathogens (O'Callaghan 2016). In the pot experiments in this study, the melon seeds pre-treated with WF02 individually, as well as with combination of them showed significant inhibitory effects on Aac infection (Fig. 8, Table 2). As shown in Table 2, the biocontrol efficacies (%) were ranging approximately 70% to 75%. These values were much higher than those exerted by *B. amyloliquefaciens* 54 against Aac FC440 on watermelon plants (61.3%), as well as *B. subtilis* 9407 against *A. avenae* subsp. *citrulli* MH21 on melon plants (61.7%), respectively (Fan et al. 2017; Jiang et al. 2015). We noticed that the disease incidence (%) and disease severity (%) were enormously decreased in comparison with that of the Aac infected seedlings (Table 2), and the lesion areas on the cotyledons were reduced significantly (Fig. 7 and Fig. 8). According to the results with respect to cell vitality and pot experiments (Fig. 5 and Fig. 8), we selected 600 ppm BP for developing effective biocontrol agent. Intriguingly, although the cell numbers of Aac were significantly reduced by WF02 and the combined treatments (Fig. 9), the pathogen number still maintain at 10^7~10^8 CFU/g leaves. It seems like the pathogenicity of Aac would be reduced by these treatments, and further studies are necessary.

doi:10.6342/NTU201804077

The genome of WF02 has giant gene clusters that are linked to the features of biocontrol, such as those with respect to non-ribosomal peptide synthetases like surfactin, bacillomycin D, fengycin, secondary compound bacilysin, difficidin, bacillaene, macrolactin and volatile organic compounds acetoin and 2, 3-butandiol (Fig. 10, Table 4). The genes comprised in the fengycin cluster (*fenABCDE*) showed low identity to those in strain SQR9 (61.6~71.8%) and in strain FZB42 (61.6~71.9%) while displayed high identity (~99%) to those in *Bacillus velezensis* NJN-6. *bacA* (BAWF02_27980) which is comprised in the bacilysin cluster also displayed low identity to that in *B. amyloliquefaciens* SQR9 (54.1%) or in *B. amyloliquefaciens* FZB42 (53.8%). However, this gene was closely related to that in *B. amyloliquefaciens* LFB112 (100% identity), which was identified as being *B. amyloliquefaciens* subsp. *plantarum* and applied as probiotics to animal production and welfare (Borriss et al. 2011; Cai et al. 2014). The total size of these gene clusters accounted for 8% of genome, which is smaller than that of *B. amyloliquefaciens* strain FZB42 (8.5%) and SQR9 (9.9%) (Chen et al. 2007; Zhang et al. 2015). However, that of *B. subtilis* was only 5% (Chowdhury et al. 2015). These gene clusters could produce the secondary metabolites like NRPS, PKS, peptide antibiotics and VOCs like acetoin and 2, 3-butandiol, revealing the antifungal and antibacterial activities (Chen et al. 2007; Ongena and Jacques 2008; Yi et al. 2016; Zhang et al. 2015). However, except acetoin and 2, 3-butandiol, the genes responsible for volatiles synthesis are still unknown (Wu et al. 2015). Besides, In addition, we also identified the putative genes involved in biofilm formation (Table 5). The biofilm formation ability of WF02 was also demonstrated in Fig. 4, indicating the potential for root colonization. However, the additional analysis is necessary to understand how conserved they are relative to FZB42 and SQR9 as well

doi:10.6342/NTU201804077

as the similarity to biocontrol related genes in FZB42 and SQR9 which are already known for biocontrol features.

Summary

We evaluated the biocontrol effects of WF02 in combination with the phytogenic additive BP for controlling BFB. BP can bring the synergistic effects for WF02 on biofilm formation and cell vitality improvement. WF02 genome contains the predicted antimicrobial-related genes, indicating the potential for antimicrobial characteristics. However, the antagonistic mechanism of WF02 still needs further researches to clarify. Our study showed the first report of *B. amyloliquefaciens* strain with plant extract additives applied as seed treatment to control BFB.

doi:10.6342/NTU201804077

Conclusions and Future Prospects

In this study, we developed a potential biocontrol agent in combination with the elite biocontrol bacterium WF02 and the phytogenic additive BP to confer antagonistic effects against Aac for suppressing the Bacterial fruit blotch (BFB) in melon. WF02 showed antagonistic activities against Aac both *in vitro* and *in vivo*. The presence of living WF02 cells was necessary to suppress the growth of Aac. According to the pot experiments, the single inoculant (WF02) and the combined treatment (WF02+BP) showed high biocontrol efficacies and could reduce the disease incidence and index significantly. Moreover, the population of Aac was also remarkably decreased by the biological treatments. Although low concentrations of BP did not have antagonistic effects on controlling the BFB disease, it brought the positive effects on the physiology of WF02, such as enhancing the formation of biofilm and increasing its cell vitality. We identified the antimicrobial and biofilm related genes in the genome of WF02. Due to the complete genome sequence of WF02, it allows us to further investigate the antimicrobial mechanisms of WF02 and the physiological effects of BP on WF02 in the future. To extend the application, biocontrol efficacies in the field and antagonistic ability against various kinds of plant pathogens are necessary to be evaluated.

doi:10.6342/NTU201804077

References

石佳平。2008。枯草桿菌 *Bacillus subtilis* Y1336 及放線菌 *Streptomyces candidus* Y21007 菌種之分子鑑定技術及其抑菌效果之研究。碩士論文。高雄：國立高雄師範大學生物科技系。

胡正榮。2017。甜瓜種苗產業發展現況與展望。種苗科技專訊 99: 24-28.

唐致仁。1997。西瓜細菌性果斑病之研究。碩士論文。台中：國立中興大學。

陳鈺楨。2014。瓜類細菌性果斑病菌新選擇性培養基之研發。碩士論文。台中：國立中興大學。

趙佳鴻。2007。瓜類病毒病害防治策略與展望。臺中區農業改良場特刊，88:101-109。

謝奉家、李美珍、高穗生。2003。枯草桿菌菌體及其代謝產物對病原真菌之抑菌效果評估。植物保護學會會刊，45:155-162。

關政平、張凱為、吳明哲。2015。日本西瓜種子檢驗方法在果斑病之應用概況—佐藤仁敏博士專題演講心得與整理。技術服務，101:19-22。

Acuña-Fontecilla, A., Silva-Moreno, E., Ganga, M. A., and Godoy, L. 2017. Evaluation of antimicrobial activity from native wine yeast against food industry pathogenic microorganisms. CYTA J. Food 15:457-465.

Agrios, G. N. 2005. Plant Pathology. 5th eds., Department of Plant Pathology, University of Florida, United States of America.

Ambrus, A., Hamilton, D., Kuiper, H., and Racke, K. 2003. Significance of impurities in the safety evaluation of crop protection products (IUPAC technical report). Pure Appl. Chem. 75:937-973.

doi:10.6342/NTU201804077

Armenta, S., Quintas, G., Garrigues, S., and De la Guardia, M. 2005. Mid-infrared and Raman spectrometry for quality control of pesticide formulations. Trac Trend Anal. Chem. 24:772-781.

Bacon, C. W., and Hinton, D. M. 2002. Endophytic and biological control potential of *Bacillus mojavensis* and related species. Biol. Control 23:274-284.

Bahar, O., and Burdman, S. 2010. Bacterial fruit blotch: a threat to the cucurbit industry. Isr. J. Plant Sci. 58:19-31.

Bai, U., Mandic-Mulec, I., and Smith, I. 1993. SinI modulates the activity of SinR, a developmental switch protein of *Bacillus subtilis*, by protein-protein interaction. Genes Dev. 7:139-148.

Bais, H. P., Fall, R., and Vivanco, J. M. 2004. Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol. 134:307-319.

Baker, K. F. 1987. Evolving concepts of biological control of plant pathogens. Annu. Rev. Phytopathol. 25:67-85.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., and Prjibelski, A. D. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19:455-477.

Bargabus, R., Zidack, N., Sherwood, J., and Jacobsen, B. 2002. Characterisation of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. Physiol. Mol. Plant Pathol. 61:289-298.

doi:10.6342/NTU201804077

Bargabus, R., Zidack, N., Sherwood, J., and Jacobsen, B. 2004. Screening for the identification of potential biological control agents that induce systemic acquired resistance in sugar beet. Biol. Control 30:342-350.

Bargabus, R. L., Zidack, N. K., Sherwood, J. E., and Jacobsen, B. J. 2003. Oxidative burst elicited by *Bacillus mycoides* isolate Bac J, a biological control agent, occurs independently of hypersensitive cell death in sugar beet. Mol. Plant Microbe Interact. 16:1145-1153.

Bartolome, A. P., Villaseñor, I. M., and Yang, W. C. 2013. *Bidens pilosa* L. (Asteraceae): botanical properties, traditional uses, phytochemistry, and pharmacology. Evid. Based Complement. Alternat. Med. 2013:1-51.

Benson, D. A., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., and Sayers, E. W. 2015. GenBank. Nucleic Acids Res. 43:D25-D30.

Borriss, R. 2011. Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents in agriculture. Springer, Berlin, Heidelberg.

Borriss, R., Chen, X. H., Rueckert, C., Blom, J., Becker, A., Baumgarth, B., Fan, B., Pukall, R., Schumann, P., and Spröer, C. 2011. Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM 7^T and FZB42^T: a proposal for *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* subsp. nov. and *Bacillus amyloliquefaciens* subsp. *plantarum* subsp. nov. based on complete genome sequence comparisons. Int. J. Syst. Evol. Microbiol. 61:1786-1801.

Borriss, R. 2015. *Bacillus*, a plant-beneficial bacterium. Pages 379-391 in: Principles of plant-microbe interactions. Springer, Cham.

Breed, R. S., and Dotterrer, W. 1916. The number of colonies allowable on satisfactory agar plates. J. Bacteriol. 1:321-331.

doi:10.6342/NTU201804077

Broadbent, P., Baker, K., and Waterworth, Y. 1971. Bacteria and actinomycetes antogonistic to fungal root pathogens in Australian soils. Aust. J. Biol. Sci. 24:925-944.

Bull, C., Shetty, K., and Subbarao, K. 2002. Interactions between myxobacteria, plant pathogenic fungi, and biocontrol agents. Plant Dis. 86:889-896.

Burdman, S., and Walcott, R. 2012. *Acidovorax citrulli*: generating basic and applied knowledge to tackle a global threat to the cucurbit industry. Mol. Plant Pathol. 13:805-815.

Cai, J., Liu, F., Liao, X., and Zhang, R. 2014. Complete genome sequence of *Bacillus amyloliquefaciens* LFB112 isolated from Chinese herbs, a strain of a broad inhibitory spectrum against domestic animal pathogens. J. Biotechnol. 175:63-64.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T. L. 2009. BLAST+: architecture and applications. BMC Bioinformatics 10:421.

Chen, X. H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., Morgenstern, B., Voss, B., Hess, W. R., and Reva, O. 2007. Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat. Biotechnol. 25:1007-1014.

Chen, Y., Yan, F., Chai, Y., Liu, H., Kolter, R., Losick, R., and Guo, J. h. 2013. Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. Environ. Microbiol. 15:848-864.

doi:10.6342/NTU201804077

Cheng, A., Hsu, Y., Huang, T., and Wang, H. 2000. Susceptibility of cucurbits to *Acidovorax avenae* subsp. *citrulli* and control of fruit blotch on melon. Plant pathology bulletin 9:151-156.

Chiang, Y. M., Chuang, D. Y., Wang, S. Y., Kuo, Y. H., Tsai, P. W., and Shyur, L. F. 2004. Metabolite profiling and chemopreventive bioactivity of plant extracts from *Bidens pilosa*. J. Ethnopharmacol. 95:409-419.

Choi, J. Y., Shin, J. S., Chung, Y. S., and Hyung, N.-I. 2012. An efficient selection and regeneration protocol for Agrobacterium-mediated transformation of oriental melon (*Cucumis melo* L. var. *makuwa*). Plant Cell Tiss. Organ Cult. 110:133-140.

Choudhary, D. K., and Johri, B. N. 2009. Interactions of *Bacillus* spp. and plants–with special reference to induced systemic resistance (ISR). Microbiol. Res. 164:493-513.

Chowdhury, S. P., Hartmann, A., Gao, X., and Borriss, R. 2015. Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42–a review. Front. Microbiol. 6:780.

Chu, F., Kearns, D. B., Branda, S. S., Kolter, R., and Losick, R. 2006. Targets of the master regulator of biofilm formation in *Bacillus subtilis*. Mol. Microbiol. 59:1216-1228.

Cook, R. J. 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. Annu. Rev. Phytopathol. 31:53-80.

Costerton, J. 1995. Overview of microbial biofilms. J. Ind. Microbiol. 15:137-140.

Das, T., Sehar, S., and Manefield, M. 2013. The roles of extracellular DNA in the structural integrity of extracellular polymeric substance and bacterial biofilm development. Environ. Microbiol. Rep. 5:778-786.

doi:10.6342/NTU201804077

Deba, F., Xuan, T. D., Yasuda, M., and Tawata, S. 2007. Herbicidal and fungicidal activities and identification of potential phytotoxins from *Bidens pilosa* L. var. *radiata* Scherff. Weed Biol. Manag. 7:77-83.

Doornbos, R. F., van Loon, L. C., and Bakker, P. A. 2012. Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. Agron. Sustain. Dev. 32:227-243.

Dulbecco, R., and Vogt, M. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. 99:167-182.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792-1797.

Elliott, M. L., Des Jardin, E. A., Batson, W. E., Caceres, J., Brannen, P. M., Howell, C. R., Benson, D. M., Conway, K. E., Rothrock, C. S., and Schneider, R. W. 2001. Viability and stability of biological control agents on cotton and snap bean seeds. Pest Manag. Sci. 57:695-706.

Essghaier, B., Fardeau, M. L., Cayol, J. L., Hajlaoui, M., Boudabous, A., Jijakli, H., and Sadfi-Zouaoui, N. 2009. Biological control of grey mould in strawberry fruits by halophilic bacteria. J. Appl. Microbiol. 106:833-846.

Fan, H., Zhang, Z., Li, Y., Zhang, X., Duan, Y., and Wang, Q. 2017. Biocontrol of bacterial fruit blotch by *Bacillus subtilis* 9407 via surfactin-mediated antibacterial activity and colonization. Front. Microbiol. 8:1973.

Felsenstein, J. 1989. PHYLIP–Phylogeny Interference Package (Version 3.2). Cladistics 5:164-166.

Fessehaie, A., and Walcott, R. 2005. Biological control to protect watermelon blossoms and seed from infection by *Acidovorax avenae* subsp. *citrulli*. Phytopathology 95:413-419.

doi:10.6342/NTU201804077

Govindaraj, M., Masilamani, P., Albert, V. A., and Bhaskaran, M. 2017. Effect of physical seed treatment on yield and quality of crops: A review. Agric. Rev. 38:1-14.

Guzman, V., Burdine, H., Forsee, W., Harris, E., and Orsenigo, J. 1991. Chemical control of bacterial fruit blotch of watermelon. Proc. Fla. State Hort. Soc. 104:270-272.

Harman, G., Jin, X., Stasz, T., Peruzzotti, G., Leopold, A., and Taylor, A. 1991. Production of conidial biomass of *Trichoderma harzianum* for biological control. Biol. Control 1:23-28.

Her, J. Y., Kim, M. S., Kim, M. K., and Lee, K. G. 2015. Development of a spray freeze-drying method for preparation of volatile shiitake mushroom (*Lentinus edodes*) powder. Int. J. Food. Sci. Technol. 50:2222-2228.

Hopkins, D. 1995. The Hypothetical Exam Question Becomes Reality. Plant Dis. 79:761-765.

Hopkins, D., Cucuzza, J., and Watterson, J. 1996. Wet seed treatments for the control of bacterial fruit blotch of watermelon. Plant Dis. 80:529-532.

Hopkins, D. L., Thompson, C. M., Hilgren, J., and Lovic, B. 2003. Wet seed treatment with peroxyacetic acid for the control of bacterial fruit blotch and other seedborne diseases of watermelon. Plant Dis. 87:1495-1499.

Horuz, S., and Aysan, Y. 2018. Biological control of watermelon seedling blight caused by *Acidovorax citrulli* using antagonistic bacteria from the genera curtobacterium, microbacterium and pseudomonas. Plant Prot. Sci. 54:138-146.

Huang, C. N., Lin, C. P., Hsieh, F. C., Lee, S. K., Cheng, K. C., and Liu, C. T. 2016. Characterization and evaluation of *Bacillus amyloliquefaciens* strain WF02

doi:10.6342/NTU201804077

regarding its biocontrol activities and genetic responses against bacterial wilt in two different resistant tomato cultivars. World J. Microbiol. Biotechnol. 32:183.

Hyatt, D., Chen, G. L., LoCascio, P. F., Land, M. L., Larimer, F. W., and Hauser, L. J. 2010. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics 11:119.

Isakeit, T., Black, M., Barnes, L., and Jones, J. 1997. First report of infection of honeydew with *Acidovorax avenae* subsp. *citrulli*. Plant Dis. 81:694-694.

Islam, M. T., Hashidoko, Y., Deora, A., Ito, T., and Tahara, S. 2005. Suppression of damping-off disease in host plants by the rhizoplane bacterium *Lysobacter* sp. strain SB-K88 is linked to plant colonization and antibiosis against soilborne Peronosporomycetes. Appl. Environ. Microbiol. 71:3786-3796.

Jayakumar, V., Bhaskaran, R., and Tsushima, S. 2007. Potential of plant extracts in combination with bacterial antagonist treatment as biocontrol agent of red rot of sugarcane. Can. J. Microbiol. 53:196-206.

Jiang, C. H., Wu, F., Yu, Z. Y., Xie, P., Ke, H. J., Li, H. W., Yu, Y. Y., and Guo, J. H. 2015. Study on screening and antagonistic mechanisms of *Bacillus amyloliquefaciens* 54 against bacterial fruit blotch (BFB) caused by *Acidovorax avenae* subsp. *citrulli*. Microbiol. Res. 170:95-104.

Kadaikunnan, S., Rejiniemon, T. S., Khaled, J. M., Alharbi, N. S., and Mothana, R. 2015. *In vitro* antibacterial, antifungal, antioxidant and functional properties of *Bacillus amyloliquefaciens*. Ann. Clin. Microbiol. Antimicrob. 14:9.

Kanehisa, M., Sato, Y., and Morishima, K. 2016. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. J. Mol. Biol. 428:726-731.

doi:10.6342/NTU201804077

Kearns, D. B., Chu, F., Branda, S. S., Kolter, R., and Losick, R. 2005. A master regulator for biofilm formation by *Bacillus subtilis*. Mol. Microbiol. 55:739-749.

Keel, C., Wirthner, P., Oberhänsli, T., Voisard, C., Haas, D., and Défago, G. 1990. Pseudomonads as antagonists of plant pathogens in the rhizosphere: role of the antibiotic 2, 4-diacetylphloroglucinol in the suppression of black root rot of tobacco. Symbiosis 9:327-341.

Khan, M., Kihara, M., and Omoloso, A. 2001. Anti-microbial activity of *Bidens pilosa*, *Bischofia javanica*, *Elmerillia papuana* and *Sigesbekia orientalis*. Fitoterapia 72:662-665.

Krzywinski, M. I., Schein, J. E., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S. J., and Marra, M. A. 2009. Circos: an information aesthetic for comparative genomics. Genome Res. 19:1639-1645.

Lagesen, K., Hallin, P., Rødland, E. A., Stærfeldt, H.-H., Rognes, T., and Ussery, D. W. 2007. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res. 35:3100-3108.

Langston Jr, D., Walcott, R., Gitaitis, R., and Sanders Jr, F. 1999. First report of a fruit rot of pumpkin caused by *Acidivorax avenae* subsp. *citrulli* in Georgia. Plant Dis. 83:199-199.

Lanteigne, C., Gadkar, V. J., Wallon, T., Novinscak, A., and Filion, M. 2012. Production of DAPG and HCN by *Pseudomonas* sp. LBUM300 contributes to the biological control of bacterial canker of tomato. Phytopathology 102:967-973.

Latin, R. 1996. Bacterial fruit blotch. Compendium of cucurbit diseases. APS Press, St. Paul, Minn:34-35.

doi:10.6342/NTU201804077

Lee, S. K., Lur, H. S., Lo, K. J., Cheng, K. C., Chuang, C. C., Tang, S. J., Yang, Z. W., and Liu, C. T. 2016. Evaluation of the effects of different liquid inoculant formulations on the survival and plant-growth-promoting efficiency of *Rhodopseudomonas palustris* strain PS3. Appl. Microbiol. Biotechnol. 100:7977-7987.

Leeman, M., Van Pelt, J., Den Ouden, F., Heinsbroek, M., Bakker, P., and Schippers, B. 1995. Induction of systemic resistance by *Pseudomonas fluorescen*s in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. Eur. J. Plant Pathol. 101:655-664.

Leggett, M., Leland, J., Kellar, K., and Epp, B. 2011. Formulation of microbial biocontrol agents–an industrial perspective. Can. J. Plant Pathol. 33:101-107.

Li, L., Stoeckert, C. J., and Roos, D. S. 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. Genome Res. 13:2178-2189.

Lightbown, J. 1954. An antagonist of streptomycin and dihydrostreptomycin produced by *Pseudomonas aeruginosa*. Microbiology 11:477-492.

Lima Silva, F., Fischer, D. C. H., Fechine Tavares, J., Sobral Silva, M., Filgueiras de Athayde-Filho, P., and Barbosa-Filho, J. M. 2011. Compilation of secondary metabolites from *Bidens pilos*a L. Molecules 16:1070-1102.

Lopez-Reyes, J. G., Gilardi, G., Garibaldi, A., and Gullino, M. L. 2016. *In vivo* evaluation of essential oils and biocontrol agents combined with hot water treatments on carrot seeds against *Alternaria radicina*. J. Phytopathol. 164:131-135.

Lowe, T. M., and Eddy, S. R. 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 25:955-964.

doi:10.6342/NTU201804077

Martin, H., O'Brien, R., and Abbott, D. 1999. First report of *Acidovorax avenae* subsp. *citrulli* as a pathogen of cucumber. Plant Dis. 83:965-965.

Merriman, P., Price, R., Kollmorgen, J., Piggott, T., and Ridge, E. 1974. Effect of seed inoculation with *Bacillus subtilis* and *Streptomyces griseus* on the growth of cereals and carrots. Aust. J. Agric. Res. 25:219-226.

Merritt, J. H., Kadouri, D. E., and O'Toole, G. A. 2011. Growing and analyzing static biofilms. Curr. Protoc. Microbiol. 22:1B.1.1-1B.1.18.

Michael, M., Phebus, R. K., and Schmidt, K. A. 2010. Impact of a plant extract on the viability of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in nonfat yogurt. Int. Dairy J. 20:665-672.

Miles, A. A., Misra, S., and Irwin, J. 1938. The estimation of the bactericidal power of the blood. Epidemiol. Infect. 38:732-749.

Milner, J. L., Silo-Suh, L., Lee, J. C., He, H., Clardy, J., and Handelsman, J. 1996. Production of kanosamine by *Bacillus cereus* UW85. Appl. Environ. Microbiol. 62:3061-3065.

Mutka, A. M., Fawley, S., Tsao, T., and Kunkel, B. N. 2013. Auxin promotes susceptibility to *Pseudomonas syringae* via a mechanism independent of suppression of salicylic acid-mediated defenses. Plant J. 74:746-754.

Newman, J. A., Rodrigues, C., and Lewis, R. J. 2013. Molecular basis of the activity of SinR, the master regulator of biofilm formation in *Bacillus subtilis*. J. Biol. Chem. 288:10766-10778.

Nicholson, W. L. 2008. The *Bacillus subtilis ydjL* (*bdhA*) gene encodes acetoin reductase/2,3-butanediol dehydrogenase. Appl. Environ. Microbiol. 74:6832-6838.

doi:10.6342/NTU201804077

O'Callaghan, M. 2016. Microbial inoculation of seed for improved crop performance: issues and opportunities. Appl. Microbiol. Biotechnol. 100:5729-5746.

O'Toole, G., Kaplan, H. B., and Kolter, R. 2000. Biofilm formation as microbial development. Annu. Rev. Microbiol. 54:49-79.

Ongena, M., and Jacques, P. 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol. 16:115-125.

Palumbo, J. D., Yuen, G. Y., Jochum, C. C., Tatum, K., and Kobayashi, D. Y. 2005. Mutagenesis of *β*-1, 3-glucanase genes in *Lysobacter enzymogenes* strain C3 results in reduced biological control activity toward Bipolaris leaf spot of tall fescue and Pythium damping-off of sugar beet. Phytopathology 95:701-707.

Pozharitskaya, O., Shikov, A., Makarova, M., Kosman, V., Faustova, N., Tesakova, S., Makarov, V., and Galambosi, B. 2010. Anti-inflammatory activity of a HPLC-fingerprinted aqueous infusion of aerial part of *Bidens tripartita* L. Phytomedicine 17:463-468.

Pieterse, C. M., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C., and Bakker, P. A. 2014. Induced systemic resistance by beneficial microbes. Annu. Rev. Phytopathol. 52:347-375.

Priest, F., Goodfellow, M., Shute, L., and Berkeley, R. 1987. *Bacillus amyloliquefaciens* sp. nov., nom. rev. Int. J. Syst. Evol. Microbiol. 37:69-71.

Rane, K. K., and Latin, R. X. 1992. Bacterial fruit blotch of watermelon: Association of the pathogen with seed. Plant Dis. 76:509-512.

Renna, M. C., Najimudin, N., Winik, L., and Zahler, S. 1993. Regulation of the *Bacillus subtilis alsS*, *alsD*, and *alsR* genes involved in post-exponential-phase production of acetoin. J. Bacteriol. 175:3863-3875.

doi:10.6342/NTU201804077

Romero, D., de Vicente, A., Rakotoaly, R. H., Dufour, S. E., Veening, J.-W., Arrebola, E., Cazorla, F. M., Kuipers, O. P., Paquot, M., and Pérez-García, A. 2007. The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Mol. Plant Microbe Interact. 20:430-440.

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Kloepper, J. W., and Paré, P. W. 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiol. 134:1017-1026.

Sandasi, M., Leonard, C., and Viljoen, A. 2008. The effect of five common essential oil components on *Listeria monocytogenes* biofilms. Food Control 19:1070-1075.

Sandasi, M., Leonard, C., and Viljoen, A. 2010. The *in vitro* antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*. Lett. Appl. Microbiol. 50:30-35.

Sansinenea, E., and Ortiz, A. 2011. Secondary metabolites of soil *Bacillus* spp. Biotechnol. Lett. 33:1523-1538.

Santos, E. R., Gouveia, E. R., Mariano, R. L. R., and Souto-Maior, A. M. 2006. Controle biológico da mancha-aquosa do melão por compostos bioativos produzidos por *Bacillus* spp. Summa Phytopathol. 32:376-378.

Shafi, J., Tian, H., and Ji, M. 2017. *Bacillus* species as versatile weapons for plant pathogens: a review. Biotechnol. Biotechnol. Equip. 31:446-459.

Silo-Suh, L. A., Lethbridge, B. J., Raffel, S. J., He, H., Clardy, J., and Handelsman, J. 1994. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. Appl. Environ. Microbiol. 60:2023-2030.

doi:10.6342/NTU201804077

Sinclair, J. B., and Dhingra, O. D. 1995. Basic plant pathology methods, 2nd Ed. CRC press, Boca Raton, FL, USA.

Somodi, G. C., Jones, J., Hopkins, D., Stall, R., Kucharek, T., Hodge, N., and Watterson, J. 1991. Occurrence of a bacterial watermelon fruit blotch in Florida. Plant Dis. 75:1053-1056.

Suzuki, S., Aono, T., Lee, K. B., Suzuki, T., Liu, C. T., Miwa, H., Wakao, S., Iki, T., and Oyaizu, H. 2007. Rhizobial factors required for stem nodule maturation and maintenance in Sesbania rostrata-*Azorhizobium caulinodans* ORS571 symbiosis. Appl. Environ. Microbiol. 73:6650-6659.

Szczech, M., and Maciorowski, R. 2016. Microencapsulation technique with organic additives for biocontrol agents. J. Hort. Res. 24:111-122.

Ting, A., Fang, M., and Tee, C. 2009. Assessment on the effect of formulative materials on the viability and efficacy of *Serratia marcescens*—a biocontrol agent against *Fusarium oxysporum* f. sp. *cubense* race 4. Am. J. Agric. Biol. Sci. 4:283-288.

Tjamos, E. C., Tsitsigiannis, D. I., Tjamos, S. E., Antoniou, P. P., and Katinakis, P. 2004. Selection and screening of endorhizosphere bacteria from solarized soils as biocontrol agents against *Verticillium dahliae* of Solanaceous hosts. Eur. J. Plant Pathol. 110:35-44.

Tjamos, S. E., Flemetakis, E., Paplomatas, E. J., and Katinakis, P. 2005. Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent K-165 and pathogenesis-related proteins gene expression. Mol. Plant Microbe Interact. 18:555-561.

Tobinaga, S., Sharma, M. K., Aalbersberg, W. G., Watanabe, K., Iguchi, K., Narui, K., Sasatsu, M., and Waki, S. 2009. Isolation and identification of a potent

doi:10.6342/NTU201804077

antimalarial and antibacterial polyacetylene from *Bidens pilosa*. Planta Med. 75:624-628.

Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26:379-407.

Wu, L., Wu, H.-J., Qiao, J., Gao, X., and Borriss, R. 2015. Novel routes for improving biocontrol activity of *Bacillus* based bioinoculants. Front. Microbiol. 6:1395.

Xu, X.-M., Jeffries, P., Pautasso, M., and Jeger, M. J. 2011. Combined use of biocontrol agents to manage plant diseases in theory and practice. Phytopathology 101:1024-1031.

Xue, J. J., Hou, J. G., Zhang, Y. A., Wang, C. Y., Wang, Z., Yu, J. J., Wang, Y. B., Wang, Y. Z., Wang, Q. H., and Sung, C. K. 2014. Optimization of storage condition for maintaining long-term viability of nematophagous fungus *Esteya vermicola* as biocontrol agent against pinewood nematode. World J. Microbiol. Biotechnol. 30:2805-2810.

Yang, W., Xu, Q., Liu, H. X., Wang, Y. P., Wang, Y. M., Yang, H. T., and Guo, J. H. 2012. Evaluation of biological control agents against *Ralstonia* wilt on ginger. Biol. Control 62:144-151.

Yi, H. S., Ahn, Y. R., Song, G. C., Ghim, S. Y., Lee, S., Lee, G., and Ryu, C. M. 2016. Impact of a bacterial volatile 2, 3-butanediol on *Bacillus subtilis* rhizosphere robustness. Front. Microbiol. 7:993.

Yoshihisa, H., Zenji, S., Fukushi, H., Katsuhiro, K., Haruhisa, S., and Takahito, S. 1989. Production of antibiotics by *Pseudomonas cepacia* as an agent for biological control of soilborne plant pathogens. Soil Biol. Biochem. 21:723-728.

doi:10.6342/NTU201804077

Yuan, J., Raza, W., Shen, Q., and Huang, Q. 2012. Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. *cubense*. Appl. Environ. Microbiol. 72:5942-5944.

Zhang, N., Yang, D., Wang, D., Miao, Y., Shao, J., Zhou, X., Xu, Z., Li, Q., Feng, H., and Li, S. 2015. Whole transcriptomic analysis of the plant-beneficial rhizobacterium *Bacillus amyloliquefaciens* SQR9 during enhanced biofilm formation regulated by maize root exudates. BMC Genomics 16:685.

doi:10.6342/NTU201804077