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評估納豆菌 K1 菌株抑制稻熱病以及促進植物生長之潛力

Evaluate the potential of *Bacillus subtilis* natto K1 for biocontrol activity against rice blast disease as well as for plant growth-promoting traits

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致謝

兩年的碩士班，可以在台灣的最高學府完成，是段可貴的路程。在台灣的這些年，所遇貴人不少，也是造就了今天這本論文的產生，顛覆了我高中以前對自己的期待。其中必須先謝謝啟德老師對學生的接納、包容和實驗上的討論。劉老師對學生的信任，支持我對實驗的想法，積極地協助、討論，跟老師們要求分讓病原菌、撰寫推薦函以及批改論文等，是我完成這本論文的動力。啟德老師積極和謙卑的態度，是學生學習的榜樣。另外，要謝謝共同指導學生的李昆達老師。李老師幽默風趣的個性讓學生在討論實驗的時候更能自在地暢談和闡述想法。李老師對學生的信任讓我更能自由地發揮和嘗試很多有趣的實驗。李老師為人隨和、思緒清晰、想法多元，是學生應多學習的榜樣。

這本論文很多的部份，是得到了不少老師們的幫助方可完成的。謝謝所長兼口試委員的轟睿老師，百忙之中抽空閱讀學生的論文，並於口試時給予很大的鼓勵和信心。期許學生未來可以跟轟睿老師一樣，身負重任之際也從容不迫。感謝提供稻熱病和願意擔任學生口試委員的沈偉強老師。沈老師雖系上事務繁忙，卻在實驗上也盡力協助學生，也同時感謝沈老師實驗室的柏睿，在感染植物實驗中的教導。另外，謝謝楊玉良老師在口試以及 imaging mass 實驗的指導。每次和楊老師討論實驗後，都有種恍然大悟的感覺，總會發現自己想法的不足及對結果現象的侷限解讀。楊老師的研究精神是學生的典範，也謝謝楊老師實驗室的麟傑學長花了不少心力和時間帶我完成 imaging mass 實驗。

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對學生的肯定，建立了學生的信心。謝謝帶我踏上研究這條路的大學專題指導老師，許德賢老師。許老師對人生和研究的見解和分享，培養了學生對研究的熱忱，也讓我意識到了自己在這方面的興趣和可能性。謝謝陳學弘同學在我接觸真菌實驗時的大力相助和分享，以及承泰學長在建構親緣樹圖的幫忙。

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朋友倒是交了不少，各個都生懷絕技。生技所 R04 的大家，是一路最好的陪伴，讓我體會到什麼是真正的正面競爭，大家一路互相幫忙、互相激勵，是多麼可貴的一件事。謝謝晚餐好友團，晚餐吃得好、聊得爽，是結束一日崩壞的最佳方法，也要謝謝 BCT 好朋友，謝謝大學對我不離不棄的同學們，或許就是這樣的正面能量，讓我可以獲得一些平衡，完成了這碩士班的研究。

最後，謝謝我的家人，我的爸媽、外婆和親戚們，對我不時的關心，深怕我承受不住壓力。最終，這條路算是告了一段落，可以堅持到現在，爸媽弟妹是最強力的後盾，永遠支持我做出的每一個決定，從無怨言。您們給予我的太多，我卻無法回饋些什麼。謝謝這一路的鼓勵，這二十六年的養育之恩，期許日後我也可以為這世界、這大自然、這土地，做出奉獻。

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中文摘要

納豆菌因可分泌納豆激酶而被廣泛應用為人類食用益生菌。納豆菌屬枯草桿菌群，具分泌多種抗生物質的能力，因此具有作為植物保護劑的潛力。在本研究中，我們使用一株具分泌多量納豆激酶的納豆菌 K1 菌株，評估其生物防治以及促進植物生長的潛力。我們根據其在 LB 培養基上的菌落型態，挑選出四株彼此具有差異性的納豆菌，分別命名為 WA， WB， WC 及 K1。根據 16S rRNA 與 *gyr*B 序列繪製的親緣演化樹以及碳源代謝活性的結果判斷，四種菌落皆屬相同菌株。在對峙培養實驗發現 BsK1 相較於其他三種株菌落具有更佳的抗稻熱病真菌（MoGuy11）之能力。此外，我們使用枯草桿菌標準菌株 Bs168 及一株台灣本土商用枯草桿菌菌株 BY1336 作為對照比較。BsK1 及 BY1336 處理組相較於 Bs168 能更為有效地抑制稻熱病菌絲生長，而且觀察到菌絲會有異常膨大現象。我們使用對葉稻熱病具不同抗性的水稻品種，包括感病等級的台梗 9 號以及極感等級的台農 67 號來評估上述菌株的抗病能力。 若菌液是施用在根部時，BsK1 或是 BY1336 皆可降低台農 67 號的葉稻熱病罹病指數; 若施用在葉面則只有 Bs168 具抗病效果。此外我們發現 BsK1 及 Bs168 僅有活細胞才具有抑制稻熱病菌絲的作用，而 BY1336 不論是活細胞或是分子量大於 3kDa 的上清液皆具有抑制能力。利用影像質譜分析上述三株菌株所分泌的酯肽類抗生素發現，BY1336 具分泌 iturins, surfactins 以及 fengycins 的能力，Bs168 則完全偵測不到這些抗生物質，而 BsK1 只能偵測到 surfactins。我們發現 BsK1 與稻熱病菌共同培養下所生成的 surfactins 的量較單獨培養多，因此推斷 BsK1 會受到病原菌的誘導而生合成抗生物質。另外，我們也比較了三株菌促進植物生長的能力。三株菌培養在 LB 時所釋放的揮發性氣體可促進阿拉伯芥及菸草小苗的生長。然而若直接將上述菌株施加到小苗根部，

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BY1336 反而會抑制菸草及阿拉伯芥的生長，BsK1 則不影響菸草生長。綜合上述結果， 因 BsK1 具有與商用菌株相似的抗病及促進植物生長能力，且對植物傷害更小，研判具有發展成為植保製劑的潛力。

關鍵字：納豆菌、枯草桿菌、稻熱病菌、揮發性氣體、表面活性素、阿拉伯芥、菸草

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Abstract

Bacillus subtilis natto has been regarded as a kind of human probiotics due to its production of fibrinolytic enzyme (nattokinase). In this study, an elite *Bacillus subtilis* natto strain K1 with high productivity of nattokinase was evaluated for its biocontrol and plant growth-promoting properties. This bacterium displayed at least four distinct colony morphologies, and designated as WA, WB, WC and K1, respectively. Based on 16S rRNA/ *gyr*B constructed phylogenetic trees and carbon utilization traits, these four kinds of colonies belong to the same strain. However, in dual culture assay, BsK1 showed highest inhibition on the mycelia growth of a rice blast fungus, *Magnaporthe oryzae* Guy11 (MoGuy11). In comparison with a commercial strain (*Bacillus* sp. strain Y1336, BY1336) and a model strain of *Bacillus subtilis* (*B. subtilis* 168, Bs168), BsK1 displayed comparable biocontrol activity as that of BY1336. In dual culture plate assay, both BY1336 and BsK1 caused swollen hyphae and growth inhibition on MoGuy11 mycelia. Inoculation of either BsK1 or BY1336 on roots of rice cultivar TNG67 decreased the disease incidence of leaf blast. In contrast, leaf spray of these strains on cultivar TNG67 did not display any biocontrol ability. I found the broth filtrate of BY1336 could inhibit the growth of MoGuy11, however, that of BsK1 could not. Imaging mass analysis was conducted to confirm the synthesis of lipopeptide antibiotics. BY1336 could produce three kinds of lipopeptides (surfactins, fengycins, and iturins) in

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the presence or absence of MoGuy11. In contrast, BsK1 could only synthesize surfactins in the presence of MoGuy11. It suggests that the antibiotics of BsK1 are only synthesized in response to its antagonist. I also found both BsK1 and BY1336 were able to promote the growth of *Arabidopsis thaliana* Col-0 and *Nicotiana tabacum* W38 through volatile compounds emission when they were grown in Luria Bertani agar (LBA). In direct inoculation of BY1336 and BsK1 on both seedlings, growth of AtCol-0 were inhibited by both strains, but only BY1336 suppressed the growth of NtbW38. Taken together, BsK1 just like the commercial strain BY1336, has the potential to serve as elite biological control agent as well as plant growth stimulator.

Keywords: *Bacillus subtilis* natto, *Magnaporthe oryzae* Guy11, Volatile, Surfactin, *Arabidopsis thaliana* Col-0, *Nicotiana tabacum* W38

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Introduction

Rice is one of the most important crops, contributes to more than 20 % of calories consumed by global human population (Thompson and Verdier 2012). International Food Policy Research Institute estimated the increased of 38 % of rice product are needed to feed the expanding human population by 2030 (Wilson and Talbot 2009). However, rice blast disease caused by the filamentous ascomycete fungus *Magnaporthe oryzae* appeared to be a potent threat to rice production (Baker et al. 1997). This disease occurs in more than 85 countries, and causes annual food lost, estimated to feed up to 60 million of peoples (Zeigler et al. 1994). Different approaches are practically used for rice blast control. Low cost strategy such as limiting nitrogen fertilizer, flooding and post harvest burning cannot permanently eliminate this spore spreading disease (Skamnioti and Gurr 2009). Plantation using resistant rice cultivars limited the choices of plantation and resistance rice cultivar with *Pi-ta* R gene was no longer effective (Chuma et al. 2011). Chemical pesticides can effectively suppress rice blast, but they possessed underlying threats on environment and human health (Aktar et al. 2009). Sustainable agriculture thereby gradually drawing attention to agriculture sector worldwide, and subsequently favored the development of effective biopesticides and biofertilizers (Ongena and Jacques 2008).

Microbial agents have been shown to be versatile in their biocontrol activities against several phytopathogens, which provide an alternative to replace chemical pesticides (Cook 1993). *Bacillus* species are spore-forming bacteria that offer a number of advantages as biopestides and biofertilizers (Pérez-García et al. 2011). First, they are

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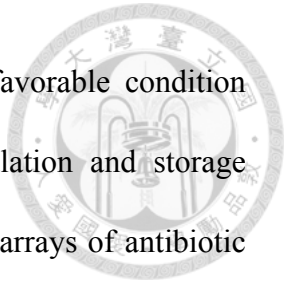

able to survive in harsh environments for latter proliferation in favorable condition owing their spore-forming ability. This also permits easy formulation and storage processes (Schallmey et al. 2004). Second, they can secrete a wide arrays of antibiotic compounds, that act as insecticides, fungicides and microbial pesticides (Francis et al. 2010, Pérez-García et al. 2011). In addition, they were able to induce the systemic resistance of plants through robust roots colonization and related compounds secretion (Rahman et al. 2015, Huang et al. 2016). Furthermore, *Bacillus* species bacteria can directly promote plant growth, play the role of biofertilizers (Xie et al. 2014).

B. subtilis and *B. amyloliquefaciens* groups were the most well studied species for developing biocontrol agents. They devoted up to 5 % of whole genome capacity withholding the gene clusters responsible for antibiotic biosynthesis (Stein 2005). They secrete varieties of compounds that possess bactericidal, fungicidal, and nematicidal activities (Chowdhury et al. 2015). Particularly, cell wall degrading enzymes (such as exoprotease, glucanase and chitinase) (Leelasuphakul et al. 2006, Luo et al. 2013, Tokpah et al. 2016), volatile compounds (Asari et al. 2016, Tahir et al. 2017) and peptide antibiotics (Chowdhury et al. 2015, Molinatto et al. 2017) were reported as their antifungal mechanisms and some of these bacteria have been developed into commercially available *Bacillus*-based biofungicides (Table S1) (Pérez-García et al. 2011).

Lipopeptides secreted by *Bacillus* were identified as one of the key molecules conferring biocontrol properties to this species. *Bacillus* lipopeptides were synthesized by non-ribosomal peptide synthetase (NRPSs) or its hybrid with polyketide synthases (PKSs/NRPSs) (Finking and Marahiel 2004). Amphiphilic cyclic lipopeptide families of

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iturins, surfactins and fengycins were well studied for their ability in controlling plant diseases (Ongena and Jacques 2008). Iturin family possessed a wide range of antifungal and mild antibacterial abilities, and their production seems to be restricted to *B. subtilis* and *B. amyloliquefaciens* species (Bonmatin et al. 2003, Koumoutsi et al. 2004). Fengycins were known to inhibit filamentous fungi, and induce the systemic resistance of host plants (Ongena and Jacques 2008). This cLP family can also be identified in *B. cereus* and *B. thuringiensis* species (Tsuge et al. 1999, Kim et al. 2004). Surfactins and its close variants of lichenysin had been isolated from various species of *Bacillus*, and notable for its ability on biofilm formation, bacteria motility and induce systemic resistance of host plants (Cawoy et al. 2014).

Although surfactin family was rarely to be reported as antifungal compounds (Tendulkar et al. 2007), its presence was crucial in controlling plant diseases (Chen et al. 2013). Surfactin family comprises of structural variants, in which all of them consist of a cyclic lactone ring structure which heptapeptides were interlinked by β－hydroxy fatty acids (Peypoux et al. 1999). They contained a charged side chains to protrude into aqueous phase, while apolar moieties reaching into the hydrophobic phase (Heerklotz et al. 2004). This amphiphilic nature allowed them to affect biological membrane integrity and displayed strong biosurfactant properties, haemolytic, antiviral, antimycoplasma and antibacterial activites (Ongena and Jacques 2008). Its production was mainly modulated by two-component regulatory system of ComA-P (Raaijmakers et al. 2010), with also reportedly regulated by DegS-U (Mäder et al. 2002) and H_2O_2 related PerH regulators (Hayashi et al. 2005). Surfactins also contribute to the biofilm formation of bacteria, establishing effective root colonization process, not only allow the persistence

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of bacteria in plant environment but also benefit host plants (Bais et al. 2004). Studies revealed that the surfactins deficient bacteria showed disrupted biofilm, and decreased in biocontrol ability (Zeriouh et al. 2014, Luo et al. 2015). Additionally, surfactins also induce the systemic resistance of host plants due to acyl moiety and cyclic peptide part in its structure (Jourdan et al. 2009). This molecule activates the defensive response of host plant by triggering disturbance or transient channeling in the plasma membrane, without causing irreversible pore formation on host (Jourdan et al. 2009).

Apart from the nature-born biocontrol ability of *Bacillus* species, this genus of bacteria can also directly regulate and promote the growth of host plants (Ryu et al. 2003). They benefit plants by increasing mineral availability (Idriss et al. 2002), and biosynthesizing various phytohormones (Idris et al. 2007, Shao et al. 2015) and effective compounds such as spermidine (Xie et al. 2014). *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a were first reported by Ryu et al. (2003) that they emit volatiles such as 2,3-butanediol and acetoin to promote the growth of *Arabidopsis thaliana* seedlings. Microarray study revealed volatiles from *B. subtilis* GB03 could promote plant growth through regulation of plant auxin homeostasis and cell expansion mechanism (Zhang et al. 2007). Additionally, *Bacillus* sp B55 emits dimethyl disulfide (DSMS) volatile contributes as sulfur nutrition for the growth of *Nicotiana attenuate* (Meldau et al. 2013). On the other hand, bacteria volatiles also confer biocontrol ability by triggering induce systemic resistance (ISR) in plants (Ryu et al. 2004). Volatiles, 2,3-butanediol and acetoin emitted by *Bacillus* species increase resistance of *A. thaliana* against *Erwinia caratovora* (Ryu et al. 2004), while volatiles from *B. amyloliquefaciens* FZB42 protects tobacco against bacterial wilt caused by *Ralstonia solanacaerum* (Tahir

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et al. 2017). Previously, some reports also revealed the fungicidal and bactericidal activites of volatiles (Arrebola et al. 2010, Asari et al. 2016, Tahir et al. 2017). Within discovered volatiles compounds, compounds such as amines, benzaldehyde, benzothiazole , decanal , cyclohexoanol, dimethyl trisulfide, 2-thyl-1-hexanol and nonanal possessed fungicidal activity (Kai et al. 2009).

Bacillus species are bacteria with consistent plant growth promotion and biocontrol traits. One of the subspecies of *B. subtilis*, *B. subtilis* natto (formerly designated *Bacillus natto*) was recognized as human probiotic strains. They were commonly used to ferment soybeans in Japan (Sumi et al. 1987). These bacteria can produce nattokinase (formerly designated subtilisin) during fermentation of soybean, and exhibits fibrinolytic (Dubey et al. 2011), thrombolytic (Fujita et al. 1995) and recently, amyloid-degrading activity (Hsu et al. 2008). Therefore, of those features, this bacteria have gained 'generally recognized as safe' status from US Food and Drug Administration and 'qualified presumption of safety' status from European Food Safety Authority (Kobayashi 2015). Thereby, we expect that prescription of this strain in biofertilizer might increase it general acceptance by public for its applications in foods and crops. In addition, *B. subtilis* natto was known for its multiple cell types characteristic, display various morphologies (Lombardía et al. 2006). This proposed that *B. subtilis* natto might react differentially towards various environment cues, further showing its flexibility for survival in different niches (Lopez et al. 2009) .

In this study, we aim to evaluate the biocontrol activities and plant growth-promoting traits of some isolated strains of *Bacillus subilis* natto. We set up a comparative study by incorporating a well established laboratory strain of *B. subtilis*

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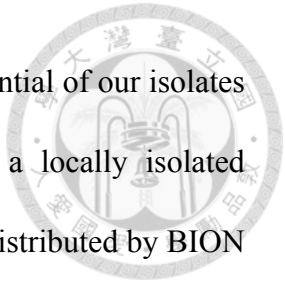

168 and a commercial strain, *Bacillus* sp. Y1336 to evaluate the potential of our isolates of *Bacillus subtilis* natto strains. Commercial strain BY1336 is a locally isolated biocontrol *Bacillus* strain, formulated into product as BioBac®, and distributed by BION TECH, Taiwan, notable for its ability in suppressing wide range of phytopathogen. Meanwhile, *B. subtilis* 168 is a whole genome sequenced type strain of *Bacillus subtilis* species (Koumoutsi et al. 2004), and can promote plant through volatiles emission, comparatively effective as *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a (Ryu et al. 2003), another two commercial available strains. Regarding biocontrol potential evaluation, we employed a devastating rice blast fungus, *M. oryzae* Guy11 as an antagonist, hope to discover the underlying mechanism of selected strain in both *in vitro* and *in vivo* experiments. We also acknowledged the imaging mass platform to investigate lipopeptides secretion of our strains and its interaction with antagonist. Furthermore, *in vitro* volatile emission assay was conducted to test the direct plant growth-promotion potential of isolates. In addition, two model plants, *A. thaliana* Col-0 and *N. tabacum* W38 were used, due to their genomes availability, research and economic importance.

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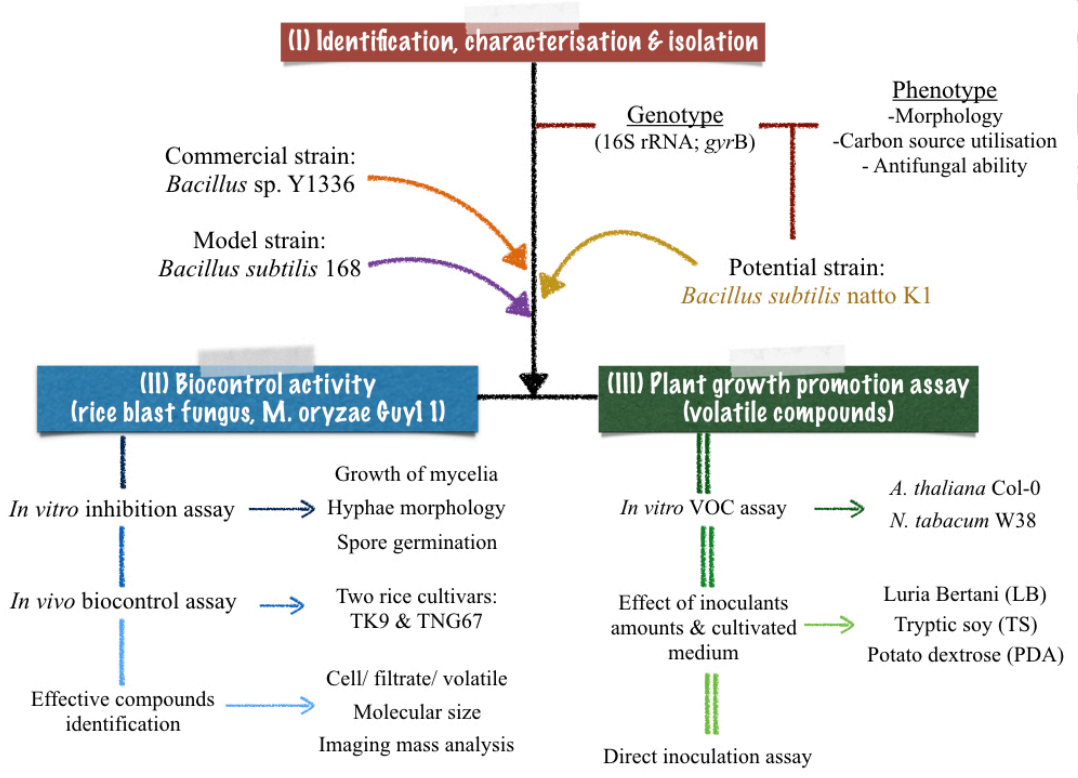

Figure I-1 Scheme of this study. The aim of this study is to evaluate the biocontrol and plant growth stimulating activities of *Bacillus subtilis* natto strains. Rice blast fungus, *Magnaporthe oryzae* Guy11 and rice, *Oryza sativa* were used to evaluate biocontrol activity, while seedlings of model plants, *Arabidopsis thaliana* and *Nicotiana tabacum* were used for analyzing plant growth-promoting traits.

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Materials and Methods

Bacteria isolation and phenotype identification

Bacillus subtilis natto strains WA, WB, WC and K1 in this study were isolated from two lyophilized bacteria powders (W indicates "萬達素", and 'N represents "高密度納豆菌"). Packages of 'W' and 'N' were derived from the products of a single *Bacillus subtilis* natto strain fermented in different strategies. 'W' was a product with high protease activity, while 'N' was a high cell counts product. One gram of respective powder was dissolved in 10 mL of sterilized water. 10^{-3}-10^{-4} serial diluted powder was then spread in Luria-Bertani (LB) agar (Trypton 10 g/L; Yeast extract 5 g/L; NaCl 10 g/L; pH 7.0), and cultivated at 37 ^{o}C for 12 hours before being moved to room temperature to obtain morphologically differentiable single colony. All strains were purified after streak culture of single colony for three times and kept as stock at -80 ^{o}C, added with 50 % sterilized glycerol in ratio of 1:1. *Bacillus subtilis* 168 is one of the most commonly used in laboratories (Patrick and Kearns 2009), which was procured from Dr. Kung-Ta Lee's lab (Department of Biochemical science and Technology, National Taiwan University), and *Bacillus* sp. Y1336 was isolated from a commercial biopesticide, BioBac® (BION TECH, Taiwan). For experiment purpose, all *bacillus* strains including *B. subtilis* 168 and *B.* sp. Y1336 were grown and maintained in LB agar.

The strains cultivated in LB broth at 37 ^{o}C for 12 hours were used for observing cell morphology by light microscope (BX-51, Olympus, Tokyo, Japan). Meanwhile, 10^{-3}-10^{-4} diluted cells were spread on LB agar plate and cultivated at 37 ^{o}C for 12 hours. Plates were then placed at room temperature for 4-8 hours until colony formation is

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observed, and examined by stereomicroscope (VEM-100, Optima, Taipei, Taiwan).

For API50-CH biochemical test, fresh LB plate grown bacteria were scraped and suspended in the API-50CHB medium to yield turbidity of 2.0 McFarland (equivalent to 2x 10^8 CFU/mL). Bacterial cells were grown in individual test strips under 37^{o}C, kept in sterilized box to maintained humidity following manufacturer's protocol (bioMérieux, France). After 12 hours of cultivation, data were collected and identity of each strain was compared with the online database of bioMérieux as following link: http://210.242.211.31/servlet/Identify?action=prepareNew&stripId=9

Genotype identification and phylogenetic analyses

Bacteria genomic DNA were extracted from the 12 hours LB cultivated bacteria using Wizard® genomic DNA purification kit (Promega) following the manufacturer's protocol for gram positive bacteria. Extracted gDNA were quantified using NanoDrop® ND-1000 spectrophotometer (Thermo scientific), and used as template for both 16S rRNA and *gyr*B genes PCR amplification. To amplify 16S rRNA, a total PCR reaction of 25 μL containing 1x GoTaq® PCR master mix (Promega), 1 μL of extracted gDNA (approximately 0.1-0.5 μg of final concentration) and 1 mM of primers (27F & 1492R) (Eden et al. 1991) were used. For *gyr*B genes amplification, a degenerate primer pair (UP-1 & UP-2R) (Yamamoto and Harayama 1995) was used and 100 μL of PCR products were concentrated into 25 μL to yield minimum concentration for sequencing. Both genes were performed in 35 cycles of amplification, with DNA denaturation at 95 ^{o}C for 1 min, primer annealing at 54 ^{o}C for 1 min (60 ^{o}C for *gyr*B) and extension of primer at 72 ^{o}C for 2 min using Veriti® 96-Well Thermal Cycler (Applied Biosystem,

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USA). For sequencing, specific primers (UP-1S & UP-2Sr) were used to sequence *gyr*B genes. All PCR products were purified using QIAquick® PCR purification kit (QIAGEN) and subsequently sent for Sanger sequencing by Genomics®, Taiwan. All primers sequences were listed in Table M-1.

All gene sequences were edited and aligned using Bioedit software (Hall 1999). Identities of isolates were confirmed by submitting particular gene sequences to the Basic Local Alignment Search Tool (BLASTn) search algorithm. Evolutionary distances were calculated and the phylogenetic trees were constructed using the Maximum likelihood methods present in MEGA 7 software (Kumar et al. 2016). Phylogenetic tree of 16S rRNA was constructed based on the Hasegawa-Kishino-Yano model with Gamma distribution (HKG+G) (Hasegawa et al. 1985). For gyrB phylogenetic tree construction, Tamura-Nei model with Gamma distribution (TN93+G) was employed (Tamura and Nei 1993). SplitsTree (www.splitstree.org) was used to combined the sequences of 16S rRNA and *gyr*B, and the phylogenetic tree was generated based on Tamura-Nei model with Gamma distribution and Invariant model (TN93+G+I) (Tamura and Nei 1993). The topologies of all trees were estimated using 1000 bootstraps replicates.

Oatmeal agar (OMA) preparation

For cultivation of *Magnaporthe oryzae* Guy11, 50 g/L of oatmeal agar was used (Shen et al. 1999). 50 g of commercial oatmeal (Good Day® fine oat flakes, 陽光燕麥®) were added with approximately 300 mL of distilled water, and subjected to 70 ^{o}C water bath for 60 minutes. Heated oatmeal broth was then filtered through four layers of gauze

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to remove oatmeal. Collected filtrate were diluted to 1 L and added with 1.5 % of agar before sterilization. Noted that oatmeal agar should be prepared thicker than usual to allow long-term cultivation of fungi without drying out.

Table M- 1 Primer used in this study.

Primer	Sequence (5' to 3')	Target gene	Reference
16S-27F	AGAGTTTGATCCTGGCTCAG	16S ribosome	Eden et al. 1991
16S-1492R	GGTTACCTTGTTACGACTT	16S ribosome	Eden et al. 1991
UP-1	GAAGTCATCATGACCGTTCTG CAYGCNGGNGGNAARTTYGA	DNA gyrase	Yamamoto and Harayama 1995
UP-2r	AGCAGGGTACGGATGTGCGAGC CRTCNACRTCNGCRTCNGTCAT	DNA gyrase	Yamamoto and Harayama 1995
UP-1S	GAAGTCATCATGACCGTTC TGCA	DNA gyrase	Yamamoto and Harayama 1995
UP-2Sr	AGCAGGGTACGGATGTG CGAGCC	DNA gyrase	Yamamoto and Harayama 1995

Culture condition for fungi and collection of spores

Rice blast fungus, *M. oryzae* Guy11 provided by Dr. Wei-Chiang Shen was grown and maintained on OMA and incubated at 26 ^{o}C with 12/12 hours of photoperiod. For mycelia cultivation, approximately 1 cm^2 of mycelia plug from the growing edge of fungi (younger mycelia) was placed on the center of fresh OMA. Plates were then sealed with porous surgical tape (3M™, Micropore™, USA), avoid using paraffin or electrical tape that prevent airflow and generates humidity. Fungi are ready for used after 5th day post inoculation and can be grown until 20th days. For spores collection, 7-14 dpi fungi was added with 5-10 mL of 0.05 % Tween-20. Fungi were gently scraped using triangular bars or tissue scrapers to release spores. Liquids containing spores were filtered through two layers of Miracloth® (Merck) to remove mycelia, and

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spores number was counted using Hemocytometer under light microscope. Noted that 5 mL of 10^5-10^6 of spores can be collected from a single 7 days cultured fungi plate (Figure M-2B).

***In vitro* antagonistic assay**

For bacterial/fungi confrontation assay, *M. oryzae* Guy11 mycelia plug was placed on the center of 15 mL potato dextrose agar plate (PDA) (BD Difco). Subsequently, 10 μL of OD_{600} 0.1 LB cultivated bacterial strains were inoculated on four sterilized filter paper discs placed 2 cm away from the mycelia plug at four cardinal points as described by Wise et al. (2012) (Figure M-1A). For bacteria filtrate inhibition study, 1 mL of LB cultivated bacterial broth was transferred into 6 mL potato dextrose broth (PDB) for 24 hours of cultivation. Broth were then centrifuged at 5000 xg for 10 minutes, filtrate were collected after two times of filtration using 0.22 μM PES membrane (Model: PES017S022I, Startech®, Taiwan). Aliquot of 50 μL filtrate was poured into respective small chamber that was poked by the collar of a 1000 μL blue pipette tip) located 2 cm away from the center mycelia plug as described by Chen et al. (2016) (Figure M-1B). The filtrate was further separated into two parts, by a molecular weight cutoff (3 kDa) usingVivaspin-20 ® 3000 MWCO PES (Sartorius, UK). Volatile inhibition test were studied using partitioned plate as described by Spence et al. (2014) with modification that both fungi and bacteria were inoculated on PDA plate. Fungi mycelia plugs were placed on one side, and 20 μL of OD_{600} 0.1 LB cultivated bacteria were inoculated at the other side of plate (Figure M-1C). All plates were sealed with paraffin and cultured at 26 ^{o}C, 12/12 hours of photoperiod growth chamber for 7 days. Total surface area of *M.*

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oryzae Guy11 was estimated using ImageJ software and used as an indicator to evaluate the inhibition potential of each strains.

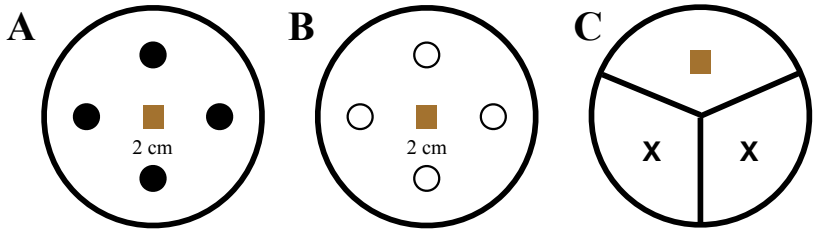

Figure M-1 Schematic diagram for *in vitro* antagonistic assays. Mycelia plug of *M. oryzae* Guy11 was placed on the middle of plate while bacteria or filtrates were inoculated at four corners of (A) sterilized paper discs or (B) chambers, which were located 2 cm away from the mycelia plug. (C) Bacteria were inoculated in different chambers in the Y-plate for volatile inhibition assay.

Spore germination assay

This assay was conducted according to the procedure developed by Spence et al. (2014). Spores were collected from 7-10 days OMA grown *M. oryzae* Guy11 and adjusted to a final concentration of 10^5 spores/mL. Bacteria were first activated in LB broth for 12-16 hours. 1 mL of bacteria were then inoculated into 6 mL PDB and cultivated at 37 ^{o}C for 24 hours. Glass slide was used as a hydrophobic surface for germination, which was sterilized with 70% ethanol before being placed in the petri dish containing water droplets to maintain humidity (Figure M-2). For bacterial treatments, a 1:1 (v:v) solution of spores plus broth were made with a final volume of 10 μL containing 10^5 spores/mL and OD_{600} 0.1 PDB diluted bacteria. For the treatment

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with filtrate, PDB cultivated bacteria were centrifuged and the supernatants were filtered twice through 0.22 μm PES membrane before used. All plates were then sealed with paraffin and kept in dark for 6 hours at room temperature (Spence et al. 2014). Percentage of germination was determined by counting the germinated spores from total of more than 100 spores per replicates, with three replicates for each treatment.

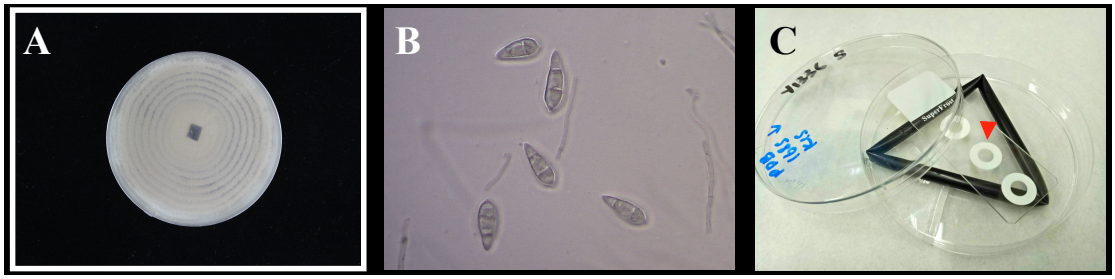

Figure M-2 Growth of rice blast fungus, *Magnaporthe oryzae* Guy11. (A) Growth of *M. oryzae* Guy11 mycelia in PDA for 20 days. (B) Spores (macroconidia) of *M. oryzae* Guy11 harvested at 7 dpi. (C) Set-up of spore germination assay. Stacking of three layers of stickers generated the chamber for inoculation (red arrow). Plastic drinking straw made a triangular base for supporting the glass slides. Approximately 3 mL of water were added at the base of 9 cm petri dish to maintain humidity.

In vivo biocontrol activity of *Bacillus* strains on rice seedlings

Two susceptible rice cultivars (TK9 and TNG67) were used to evaluate the biocontrol activity of bacterial strains against *M. oryzae* Guy11. Rice seeds were provided by Dr. Huu-Sheng Lur's lab. Seeds were first sterilized using 2 % sodium hypochlorite for 20 minutes before rinsed with water for three times to remove excess sodium hypochlorite (Oyebanji et al. 2009). Sterilized seeds were then immersed in

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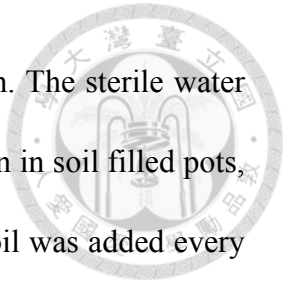

ddH_2O and incubated at 37 ^oC for 48 hours to stimulate germination. The sterile water was changed at 24^{th} and 36^{th} hours. Germinated seeds were then sown in soil filled pots, and covered with ddH_2O. Urea with concentration of 0.27 g/kg of soil was added every seven days as nitrogen source. Seedlings were placed under 16/8 photoperiods chamber at 26 ^oC for 14 days. We inoculated the bacteria and *M. oryzae* Guy11 as described by Shan et al. (2013) with some modifications. Approximately 30 seedlings were set as a treatment group, and treated with 10 mL of OD_{600} 0.1 bacteria at 14^{th} days, LB broth was used as control. After 24 hours, 10 mL of harvested conidia (10^5 spores/mL) were sprayed onto leaves for each treatment using larynx-like sprayer (Zhang et al. 2009). Infected seedlings were moved into box filled with shallow water to generate high humidity (>90 %) and kept in dark to stimulate infection (Tokpah et al. 2016) (Figure M-3). Thereafter, we exposed the seedlings to 16/8 photoperiods (2500- 3500 lux) for up to 4-6 days before harvest for disease incidence (DI) and disease severity (DS) assessment (Rahman et al. 2014). Disease incidence was determined by the percentage of infected seedlings with at least one lesion on second leaves. 8-10 of most infected second leaves were collected and disease severity was assessed using an index of 0 to 5 as described in Tokpah et al. (2016). Disease index and biocontrol efficacy of each strains were calculated as follows (Tokpah et al. 2016):

Disease index = [$\sum$ (The number of diseased plants in each disease rating x the number of plants at corresponding rating)/ (Total number of plants investigated x the highest disease rating)] x 100

Biocontrol efficacy = [(Relative disease index of Control treatment – Disease index of treatment)/ Disease index of control] x 100%

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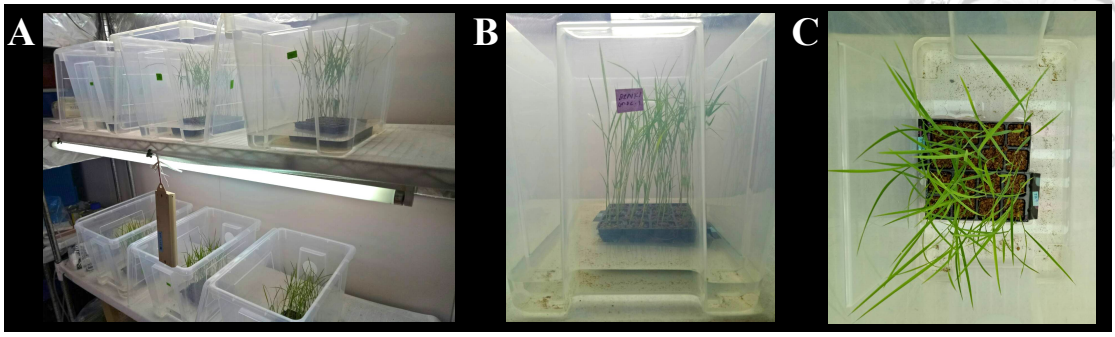

Figure M-3 *In vivo* biocontrol assay (A) Separated cultivated box for different treatments to prevent cross contamination. Front view (B) and top view of set up (C). Experiments were carried out in plant room supplemented with fluorescent lamp (>90 % of humidity; 2500- 3500 lux).

Identification of lipopeptides using imaging mass analysis

A *M. oryzae* Guy11 mycelia plug was placed on one side of 10 mL PDA plate to grow for 24 hours before 10 μL of OD_{600} 1.0 LB cultivated bacteria that were inoculated 3 cm away from the mycelia plug. Plates were then sealed and cultivated at 26 ^{o}C for four days under 12/12 hours photoperiod until mycelia inhibition were observable. At least three repeats per treatment with uniform size of bacteria colony and inhibition distance were chosen for further imaging mass analyses. Region of interest contained single culture or dual culture measuring 7.5 cm x 2.5 cm were excised, and placed on a flat surface metal slide. Excised agar should be handled carefully to prevent any cracking, and the trapped air bubbles in the agar must be removed. A 2.5 mL of universal matrix (Sigma) with concentration of 20 mg/mL was coated on the agar sample, and dried at 37 ^{o}C for 24 hours before subjected to further analysis. Two intact sample slides for each treatment were selected and analyzed using Bruker Autoflex

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Speed MALDI-TOF/TOF MS (Bruker, Bremen, Germany) as mentioned in Liao et al. (2016). IMS data with 200-2000 Da were collected and analyzed using Bruker FlexImaging 3.0 software (Bruker, Bremen, Germany). Lipopeptides including surfactins, iturins and fengycins families were selected and confirmed according to the reported *m/z* ratios (Chen et al. 2008, Liao et al. 2016, Ben Ayed et al. 2017).

In vitro plant growth promotion assay

Arabidopsis thaliana Col-0 (AtCol-0) and *Nicotiana tabacum* W38 (NtbW38) were used to study plant-promoting effects of bacterial strains. Seeds were put into Eppendorf tubes and immersed with 70 % ethanol, and the tubes were inverted several times to ensure that all seeds were fully sterilized before rinsed with MilliQ water. This initial sterilization was repeated for three times. Further sterilization was conducted using 1 % sodium hypochlorite with 20 μL of Tween-20, and rinsed with MilliQ water for ten times. Sterilized seeds were germinated on half-strength Murashigee and Skoog (MS) medium supplemented with 1.5 % of sucrose (Ryu et al. 2003) and solidified with 0.3 % of Gelzan® agar (pH 5.7). Plates with seeds were sealed with paraffin and incubated at 22-25 ^{o}C under 16/8 hours of photoperiod for seven days (AtCol-0) and ten days (NtbW38), respectively.

For direct inoculation assay, plantlets (n=7) were transplanted into 2 cm high petri dish filled with 3 % sucrose half-strength MS medium agar, and then 10 μL of LB diluted bacteria was inoculated on each seedling. Experiment was conducted at least three biological repeats. For evaluation of the plant-promotion trait exerted by volatile organic compounds (VOC), seedlings (n=5 for AtCol-0; n=4 for NtbW38 on each side)

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were placed on two sides of Y-plate containing 3 % sucrose half-strength MS medium agar. 20 μL of sterilized water suspended OD_{600}1.0 bacteria cells were inoculated on the third side of Y-plate filled with LB, tryptic soy (TS) or PDA (1.5 % agar). For dosage assay with different inoculation amounts, one, two or three drops of *B. subtilis* natto K1 were inoculated on LB agar compartment. Plates were sealed with paraffin (for direct inoculation assay) or with electrical tape (for VOC assay), and further arranged in randomized complete block design. Plantlets were harvested at 10 dpi (NtbW38) and 14 dpi (AtCol-0). One side of Y-plate plantlets were pooled together and used as a biological repeat, and total of six biological repeats per treatments were collected. Leaf surface area were quantified using ImageJ software (Kosma et al. 2009), before removing the shoots to collect fresh weight, dry weight were collected after drying at 60 ^{o}C for three days.

Statistical analysis

Statistic analysis software, SAS version 9.4 (SAS Inc. USA) was used to analyze variance in experiments. All data were analyzed by one-way ANOVA before applying Tukey's HSD (honest significant difference) for multiple range analysis to determine the significance differences between groups. Results were considered significant at $P<0.05$.

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Results

Isolation of *Bacillus subtilis* natto strains

Four *Bacillus subtilis* natto strains (K1, WA, WB and WC) were isolated from two commercial packages ('N' and 'W', respectively). Colony morphologies of the strains were shown in Figure 1A. BsK1 and BsWA displayed similar colony morphology, both had irregular shape with undulate margin, raised and rough surface, cream-liked yellow color and opaque colonies. In contrast, colonies of BsWB were comparative round in shape with undulate margin, possesses a smooth surface, cream-colored flat colonies, and comparatively translucent. Strains BsWC possessed opaque colonies with translucent center, and appeared to be cream-like yellow in color, irregular shape with lobate margin and a rough surface raised colony, comparatively smaller than BsK1 and BsWA. The cells of all strains were motile and rod-shaped (Figure 1B). The lengh of BsWB cells were comparatively longer than the others, with approximately 5-10 μm. BsWC were the shortest cells with 2 μm in length. BsK1 and BsWA have intermediate cell length (approximately 3-7μm) among the bacteria. (Figure 1B).

Identification and characterization of isolates

In order to differentiate isolates, we conducted a biochemical assay using bioMérieux API-50CH to characterize the four isolates. However, they utilized almost similar sources for growth, with exception of D-xylose was only utilized by BsK1, and arbutin was utilized by BsWC (Table 1). By comparing their utilization ability with the available database, all isolates were grouped as *Bacillus subtilis*/ *Bacillus amyloliquefaciens* with similarities higher than 94 %.

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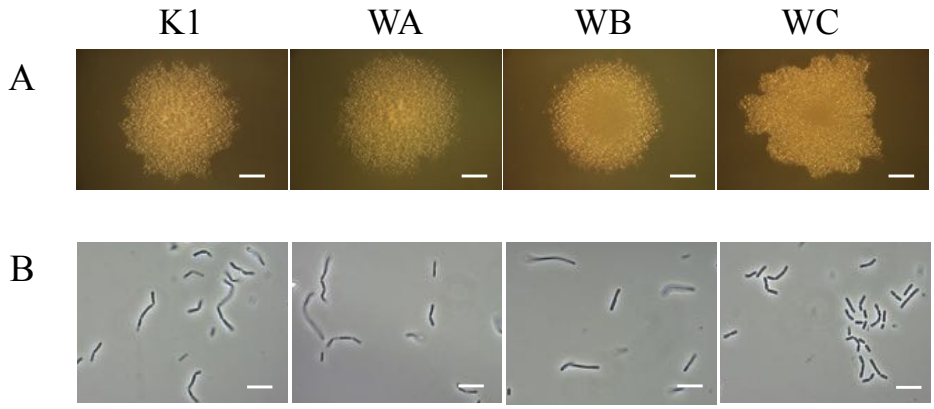

Figure 1 Morphology of four isolated *Bacillus subtilis* natto strains. Morphology of LB cultivated strains using (A) stereomicroscopy for colony observation (scale bar = 0.1 mm) and (B) bright-field microscopy for cell observation (scale bar = 10 μm).

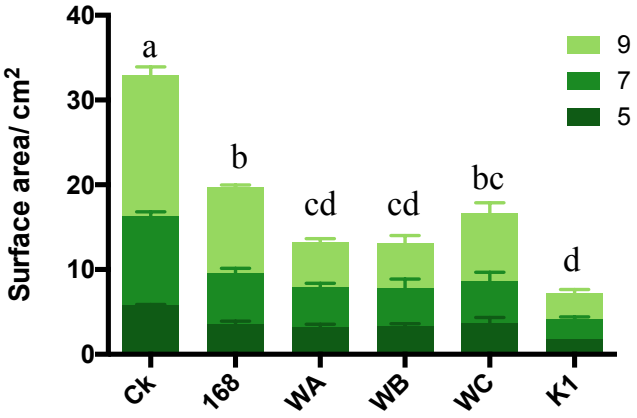

Figure 2 Antifungal activity of four isolated *Bacillus subtilis* natto strains. Total surface area of rice blast fungus, *Magnaporthe oryzae* Guy11 in dual culture assay with isolated strains. Upper right legends indicate the days post inoculations. Error bars indicate standard error of mean (SEM). Different letters indicate statistically significant difference calculated by Tukey's HSD test (P<0.05). For phylogenetic identification, 16S rRNA and *gyr*B sequences of all isolates were analyzed. Four isolates shared identical 16S rRNA sequences and clustered together in *Bacillus subtilis* group in the constructed phylogenetic tree (Figure 3A). Due to the low node value of 16S rRNA

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phylogenetic tree, we employed a more conserved gene, *gyr*B, which encodes the subunit B protein of DNA gyrase, as an alternative phylogenetic marker. Phylogenetic tree based on *gyr*B sequences revealed that isolates were clustered with the *Bacillus subtilis* subspecies *subtilis* with more representative node value (Figure 3B). A single-base nucleotide mutation in the *gyr*B gene resulted in a separation of BsWA from the other isolates. These four isolates were all classified as *Bacillus subtilis* subsp. *subtilis*, although they were unable to be separated from subsp. *subtilis* and subsp. *natto* even by combining two sequences (Figure 3C), In brief, four morphological distinct isolates shared an identical 16S rRNA and higher similar *gyr*B sequences, and classified as *Bacillus subtilis*.

In vitro antagonistic assay against _Magnaporthe oryzae_ Guy11

We employed dual culture assay to evaluate the biocontrol potential of isolates by analyzing the surface area of *M. oryzae* Guy11 (MoGuy11) mycelia at 5, 7 and 9 days post inoculation. Figure 2 showed that all strains, including the laboratory strain, *B. subtilis* 168 (Bs168), inhibited the growth of MoGuy11 at 9 dpi. As shown in Figure 2, MoGuy11was more significantly inhibited by BsK1 than by the others strains. BsWA and BsWB possessed greater inhibitory effect as compared to Bs168 and BsWC. Thereby, BsK1 was selected in our further study.

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Table 1 Carbon sources utilization of four isolated strains using API-50 commercial kits.

Carbon sources	K1	WA	WB	WC
Control	-	-	-	-
Glycerol	+	+	+	+
Erythritol	-	-	-	-
D-Arabinose	-	-	-	-
L-Arabinose	+	+	+	+
D-Ribose	+	+	+	+
D-Xylose	+	-	-	-
L-Xylose	-	-	-	-
D-Adonitol	-	-	-	-
Methyl-βD-Xylopyranoside	-	-	-	-
D-Galactose	-	-	-	-
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	+	+	+	+
L-Sorbose	-	-	-	-
L-Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	+	+	+	+
D-Mannitol	+	+	+	+
D-Sorbitol	+	+	+	+
Methyl-αD-Mannopyranoside	-	-	-	-
Methyl-αD-Glucopyranoside	+	+	+	+
N-Acetylglucosamine	-	-	-	-
Amygdalin	-	-	-	-
Arbutin	-	-	+	-
Esculin ferric citrate	+	+	+	+
Salicin	-	-	-	-
D-Cellobiose	-	-	-	-
D-Maltose	+	+	+	+
D-lactose	-	-	-	-
D-Melibiose	-	-	-	-

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D-Saccharose (sucrose)	+	+	+	+
D-Trehalose	+	+	+	+
Inulin	+	+	+	+
D-Melezitose	-	-	-	-
D-Raffinose	-	-	-	-
Amidone (starch)	-	-	-	-
Glycogen	-	-	-	-
Xylitol	-	-	-	-
Gentiobiose	-	-	-	-
D-Turanose	-	-	-	-
D-Lyxose	-	-	-	-
D-Tagatose	-	-	-	-
D-Fucose	-	-	-	-
L-Fucose	-	-	-	-
D-Arabitol	-	-	-	-
L-Arabitol	-	-	-	-
Potassium gluconate	-	-	-	-
Potassium 2-Ketogluconate	-	-	-	-
Potassium 5-Ketoglutanate	-	-	-	-
B. subtilis/ *amyloliquefaciens*	94.8 %	99.4 %	99.9 %	99.4 %

Symbols of '-' indicates no color changed of medium, while '+' indicates color changed of medium. Percentage of strains identity was gained after compared with kit's available reference strains from online database.

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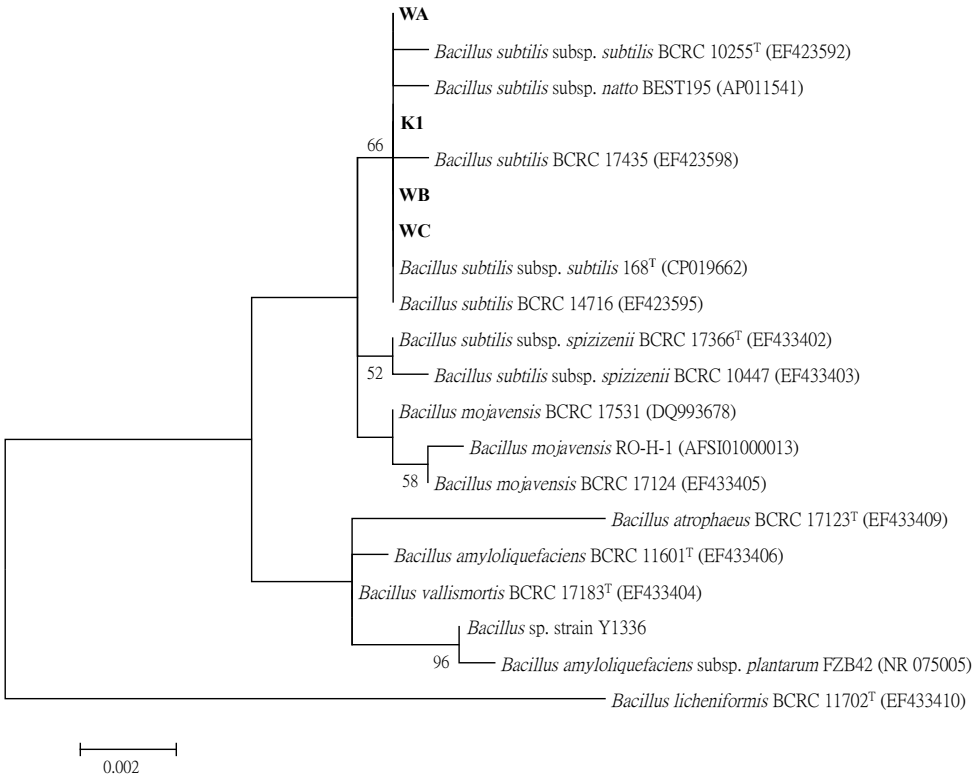
A



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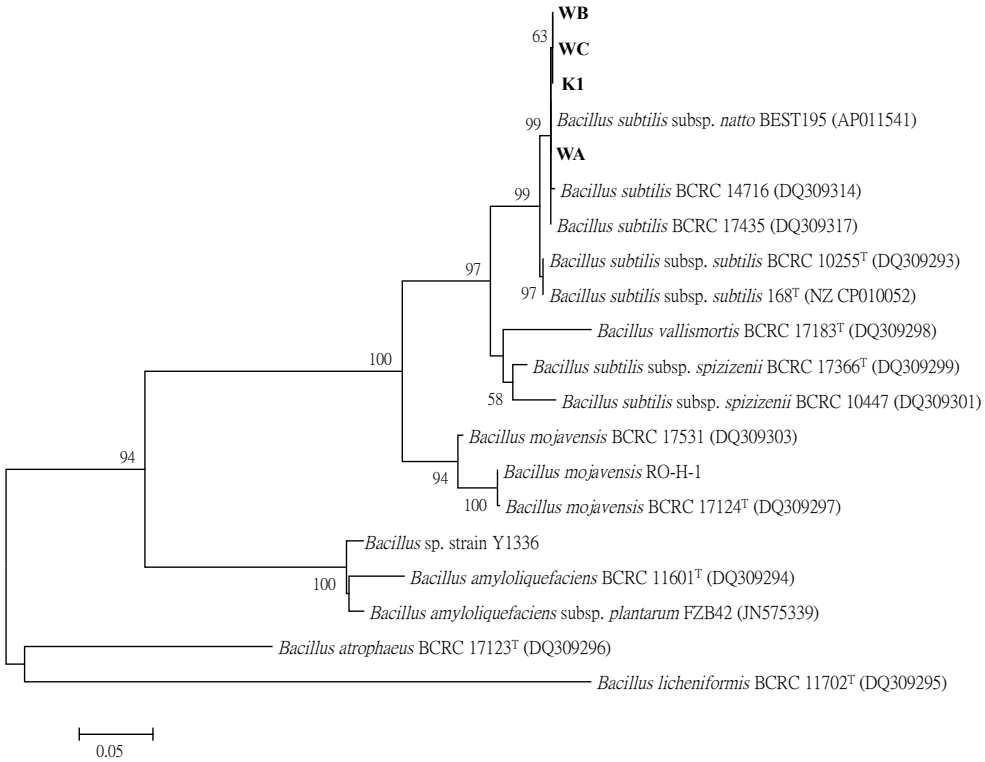

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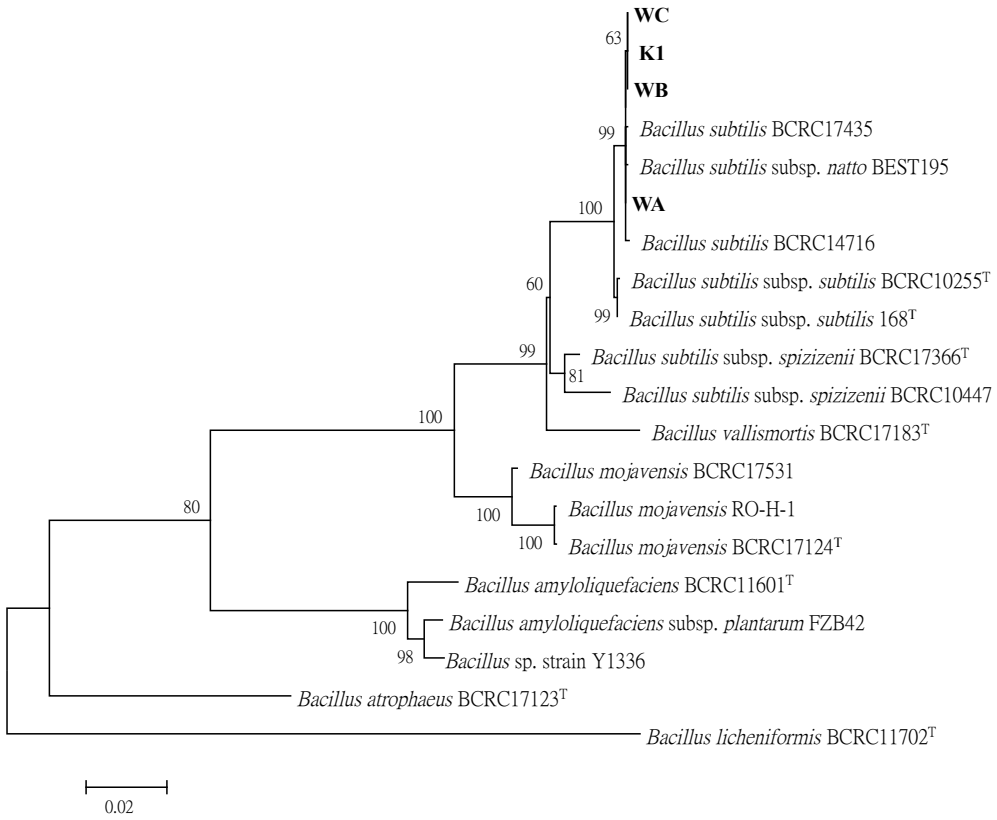

Figure 3 Phylogenetic trees of isolated strains based on partial sequences of (A) 16S rRNA (1388 bp), (B) *gyr*B (1101 bp), and (C) combined gene sequences of 16S rRNA and *gyr*B (2489 bp). The trees were constructed using maximum-likelihood method based on HKG+G model (A), TN93+G model (B) and TN93+G+I model (C) respectively. Strains with superscripted 'T' representing reference type strains and accession numbers for each sequence are indicated in parentheses. Only bootstraps values above 50 % are shown based on 1000 of replications. Bars, 0.002 (A), 0.05 (B), and 0.02 (C) indicate the numbers of substitutions per nucleotides.

Further comparing the inhibitory effects of the potential strain BsK1 with a commercial inoculant *Bacillus* sp. strain Y1336 (BY1336) and the laboratory strain, Bs168, the growth of MoGuy11 mycelia were significantly inhibited by all strains at 7

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dpi (Figure 4A) with observable swollen hyphae (Figure 4B, red arrow). As shown in Figure 4, although the commercial BY1336 grew faster than BsK1, both strains showed almost the same inhibitory activities against MoGuy11. It is worth mentioning that abnormal growth of the fungus hyphae was observed in the presence of all antagonism.

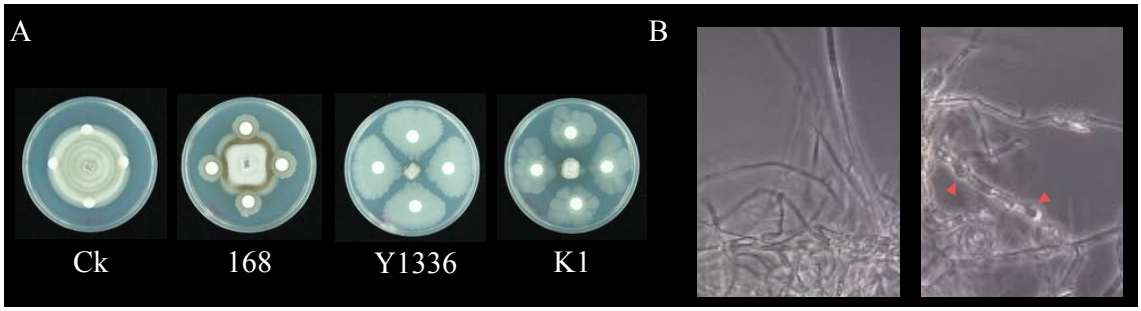

Figure 4 *In vitro* antagonistic effect of *Bacillus* strains against MoGuy11. (A) Representative dual-culture plates of each *Bacillus* strains after 7 days of cultivation. (B) Bright-field microscopic observation of MoGuy11 hyphae from control group (left panel) and *Bacillus* treated groups (right panel). Red arrows indicate the abnormal growth of fungus hyphae in the presence of antagonisms.

Spore germination assay

To initiate rice blast disease, fungal spores must germinate first and penetrate into the host plant, and differentiate into mycelia (Wilson R.A. and Talbot N.J., 2009). Therefore, interfering the spore germination can reduce fungal pathogenicity effectively. In this study, we evaluated the ability of *Bacillus* strains in inhibiting the germination rate of MoGuy11 spores. PDB cultivated *Bacillus* strains were separated into cells and filtrate fractions, and co-cultured with MoGuy11 spores. Total spores and germinated spores were calculated for each treatment. As shown in Figure 5, none of the treatment

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was able to suppress the germination of MoGuy11 spores, indicating these *Bacillus* strains may not spore inhibitors.

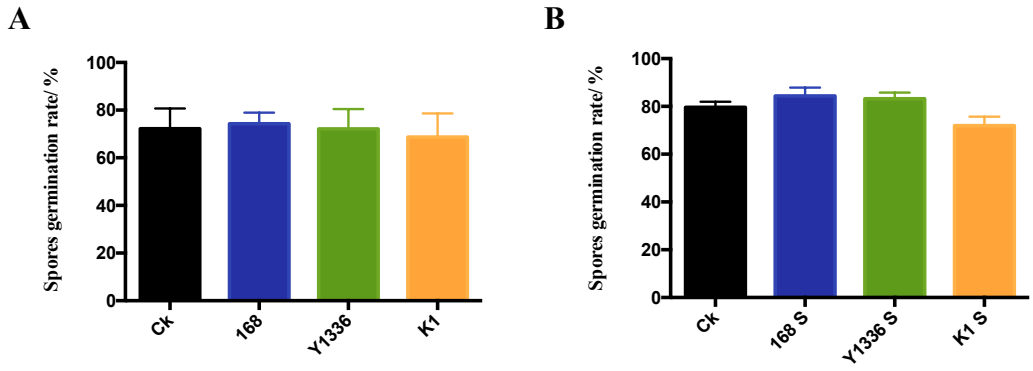

Figure 5 Spores germination rate of MoGuy11 added with cells (A) and filtrate fractions (B) of PDA cultivated *Bacillus* strains.

Figure 6 Leaf blast lesions caused by MoGuy11 in two rice cultivars (TK9, TNG67) with different susceptibility.

In vivo biocontrol activity of *Bacillus* strains

We conducted pot experiments employing two rice cultivars, *Oryza sativa* cv. TK9 and *O. sativa* cv. TNG67 with different leaf rice blast susceptibility to investigate the biocontrol ability of isolated strains. As shown in Figure 6, the cultivar TK9 showed less lesion numbers and size than that of cultivar TNG67. It indicates that the former

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showed more resistance to MoGuy11 than the latter. Two rice cultivars were inoculated with respective *Bacillus* strain on roots or leaves, then were infected by MoGuy11. As shown in Table 2A, the disease indexes were all raised in the cultivar TK9. On the other hand, the disease indexes of the TNG67 plants were decreased while they were inoculated with BY1336 or BsK1 in the roots. Both strains displayed relatively higher biocontrol efficacy (14.78 %) than that of Bs168. This result is in consistence with its inability to inhibit MoGuy11 in dual culture plate assay shown in Figure 4B. However, in the leaves inoculation assay, only the treatment of Bs168 in cultivar TNG67 showed decreased disease index. Leaves showed more severe infection by rice blast fungus while treating with BY1336 and BsK1 (Table 2B). This indicates that biocontrol ability of tested strains is dependent on rice cultivar and treatment method.

Mechanism of *B. subtilis* K1 inhibiting rice blast fungus

Reported *Bacillus* species were able to inhibit pathogen via antibiotics secretion, enzyme activity and volatile emission. As shown in Figure 7A, the surface areas of MoGuy11 were not significantly suppressed during airspace sharing with the three tested strains, even treated with double inoculation of BsK1. Therefore, we excluded the possibility of inhibition through volatile compounds.

We further decreased the initial inoculation of BsK1 up to 10^4 folds of dilution and investigated the MoGuy11 inhibition activity. Figure 8C showed that dilution of BsK1 did not significantly weakened in inhibition, while the filtrates were not effectively against MoGuy11. Even the cultivation time for BsK1 was prolonged until 72 hours, there was no inhibition by culture filtrates. BsK1 cultivation was then switched to PDB

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as dual culture plate assay using PDA showed effective mycelia inhibition by *Bacillus* strains.

Table 2 *In vivo* biocontrol activity of *Bacillus* strains in two rice cultivars with varied susceptibility against rice blast fungus. Plants treated with *Bacillus* strains through roots priming (A) or leaves spray (B) methods before infection with *M. oryzae* Guy11.

A

	Disease index		Biocontrol efficacy/ %	
	TK9	TNG67	TK9	TNG67
Untreated control	12.5	80.0	-	-
Bs168	38.7	87.5	-209.7	-9.4
BY1336	14.3	68.2	-14.3	14.8
BsK1	25.9	68.2	-107.4	14.8

B

	Disease index		Biocontrol efficacy/ %	
	TK9	TNG67	TK9	TNG67
Untreated control	14.3	86.4	-	-
Bs168	33.3	80.8	-133.2	6.5
BY1336	70.0	96.4	-389.9	-11.7
BsK1	22.6	89.3	-58.0	-3.4

Total culture broth of three *Bacillus* strains were separated into cells and filtrates fractions. Cells of three strains inhibit rice blast fungus at different level, supporting the previous results (Figure 4B), while only autoclaved BY1336 cells showed mild inhibition effect (Figure 8A). In terms of culture filtrates, total filtrates and large molecular weight fractions (>3KDa) of BY1336 inhibit mycelia of MoGuy11 (red arrow) (Figure 8B). Peculiarly, filtrates or autoclaved BsK1 did not show any inhibition under tested condition.

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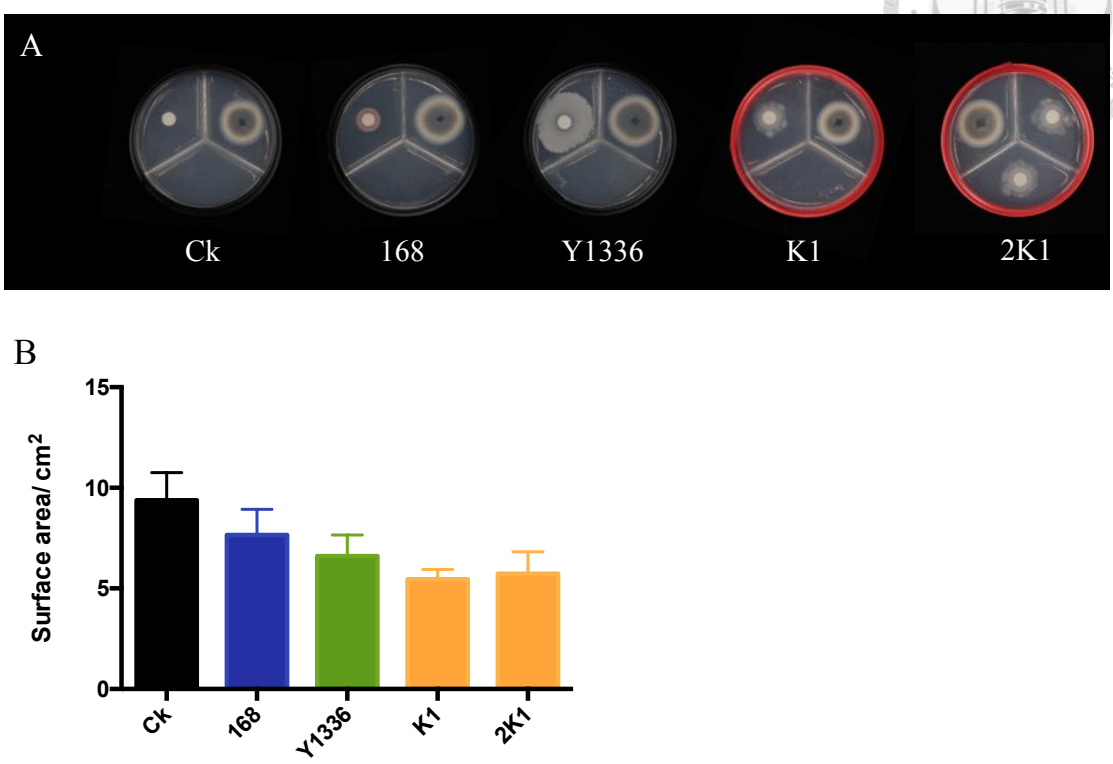

Figure 7 Volatiles inhibition assay of *Bacillus* strains against MoGuy11. (A) *Bacillus* inoculants were separated with MoGuy11 in airspace sharing partitioned plate. '2K1' indicates double dosage of BsK1 inoculant. (B) Surface area of MoGuy11 was collected at 7 dpi. Error bars indicate standard error of mean (SEM). No statistically significant difference was observed using Tukey's HSD test (P<0.05).

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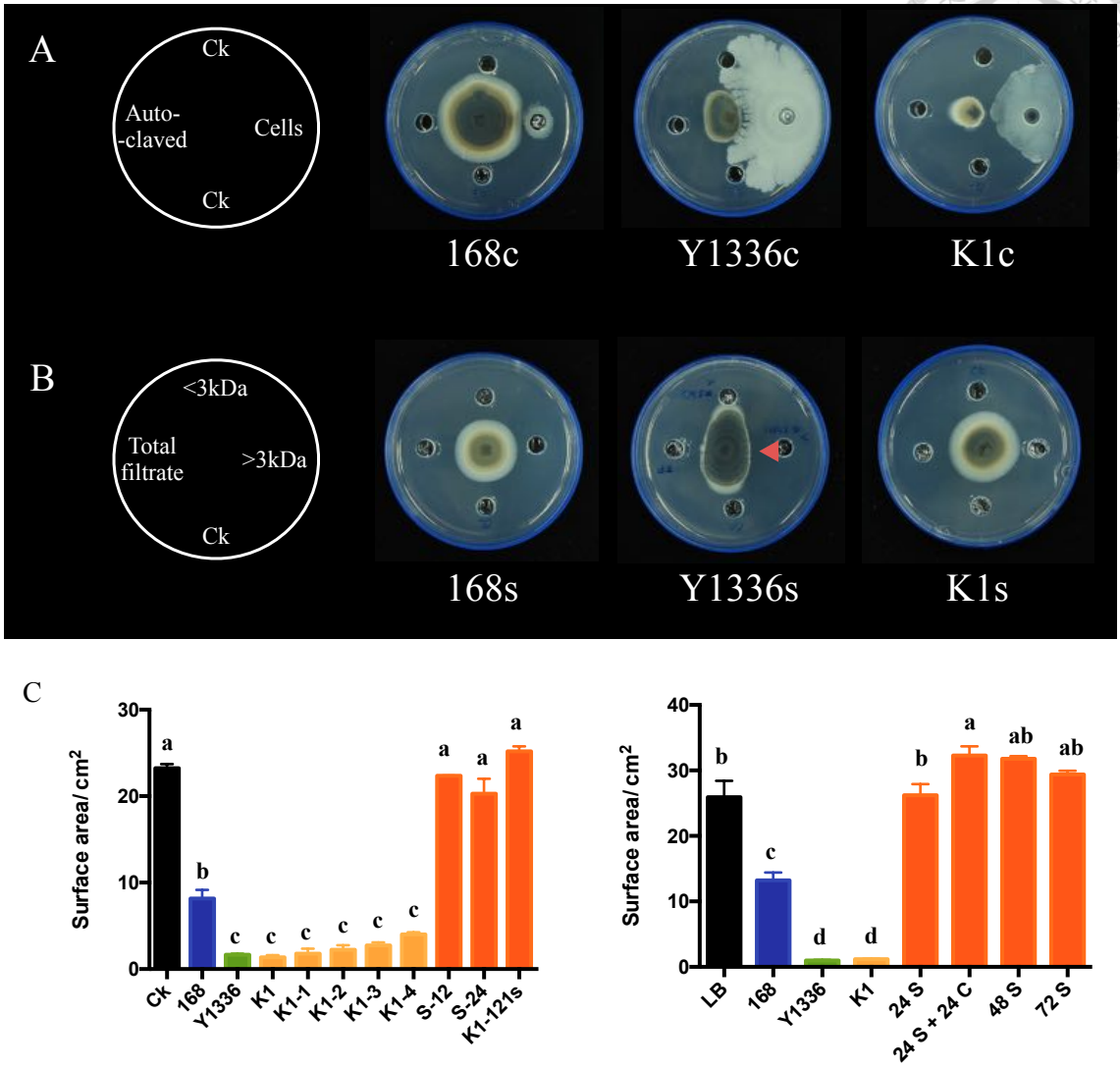

Figure 8 Antagonistic effect of *Bacillus* strains fractions against MoGuy11. (A) Fractions of cells and autoclaved total broth of PDA cultivated *Bacillus* strains. (B) Total filtrate and 3kDa filtrate fractions were harvested from PDA cultivated strains. (C) Dilution of BsK1 inoculation (left) and time course collection of LB cultivated BsK1 filtrate against MoGuy11. 'S' indicates filtrate of BsK1, '24 C' indicates additional 4°C incubation of BsK1 before collected for its filtrate. Error bars indicate standard error of mean (SEM). Different letters indicate statistically significant difference calculated by Tukey's HSD test (P<0.05).

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Imaging mass analyses of lipopeptides secretion

Bacillus species secreted wide range of antibiotics, particularly surfactins, iturins and fengycins families were the most reported lipopeptides to indirectly or directly inhibit of pathogens (Ongena M. and Jacques P., 2007). Single and dual culture assay of *Bacillus* strains and MoGuy11 were set up and imaging mass platform was applied to analyze the secretion of surfactins, iturins and fengycins. As shown in Figure 9, there was no lipopeptide detected in Bs168, in contrast, all three lipopeptides were detected in the commercial BY1336 at both single and dual culture systems. In the case of BsK1, only the lipopeptides of surfactin family were detected, however, the well-known antifungal lipopeptides, such as iturins and fengycins were not detected (Figure 9). Interestingly, I noted that the surfactins secreted by BsK1 were increased in the presence of *M. oryzae* Guy11 (Figure 9).

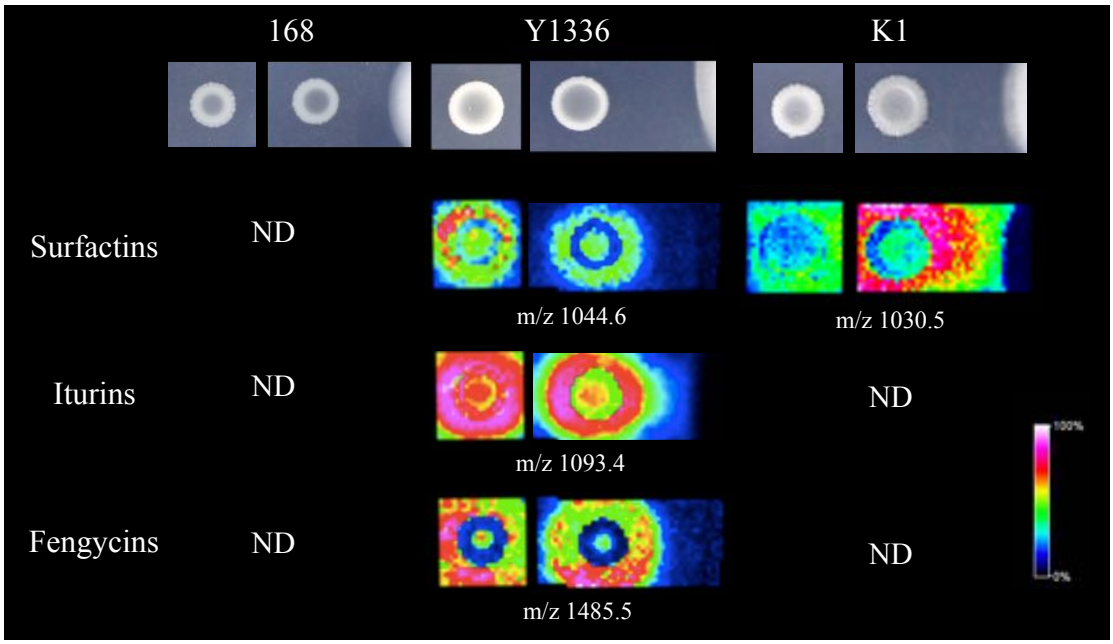

Figure 9 MALTI-TOF IMS profiles of *Bacillus* strains secreted lipopeptides (surfactins, iturins and fengycins) in single (left panel) and dual culture with

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MoGuy11 (right panel). Only a representative of each lipopeptide was selected and respective m/z ratio was shown. 'ND' indicates no particular lipopeptide was detected.

Plant growth promotion of *Bacillus* strains through volatile emission

Bacillus can promote plant growth through robust roots colonization and effective volatile emission (Ali et al. 2014; Asari et al. 2016). In this study, partitioned plate was used to evaluate the plant growth promotion ability of three *Bacillus* strains through volatile compounds. Obvious growth promotion effects on two model plants, *Nicotiana tabacum* W38 (NtbW38) and *Arabidopsis thaliana* Col-0 (AtCol-0), by the three strains were shown in Figure 10A and C, respectively. The total leaf surface area, foliar fresh weights and dry weights of seedlings were all significantly increased, and these effects are likely due to *volatile* emission of the LB-cultivated *Bacillus* strains. There is no difference in promotion ability among these three strains on the both plants, although they varied significantly in the inhibitory activity against MoGuy11.

Effects of inoculating dose of *Bacillus* strains on plant growth promotion

Although seedlings of AtCol-0 exposed to *Bacillus*'s volatiles showed increased growth parameters in overall (Figure 10D), but we noticed that some seedlings located nearer to the inoculants exhibited chlorosis symptom (data not shown). To verify this phenomenon, we increased the inoculating dose by treating up to three folds of BsK1 broth. As shown in Figure 10C, the chlorosis symptoms in AtCol-0 seedlings were indeed more apparent while treating with higher amounts of inoculants. However, neither two nor three folds of BsK1 inoculation significantly affect the plant growth

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paramaters (i.e., total leaf surface area, foliar fresh weight and dry weight) of the AtCol-0 seedlings.

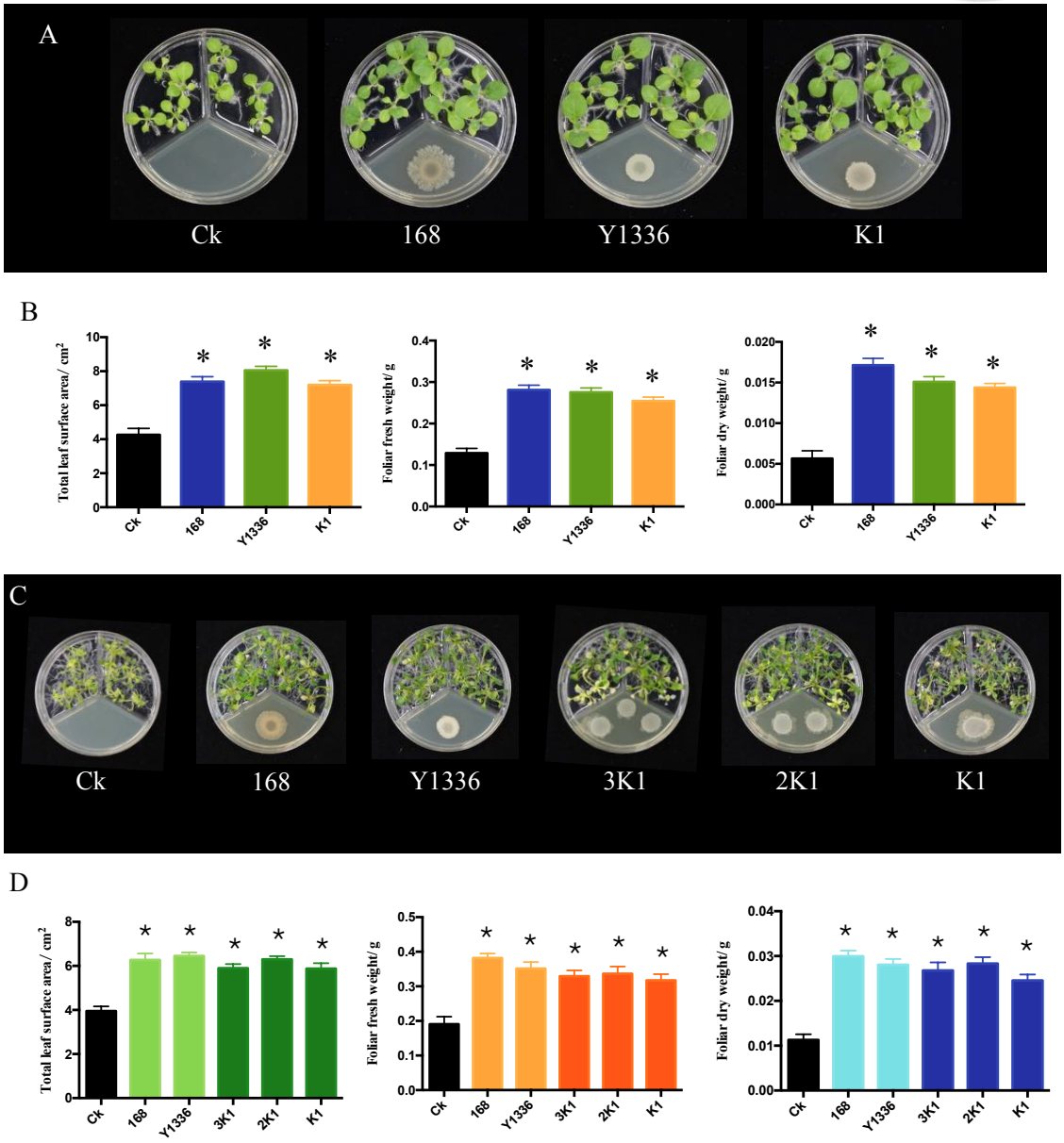

Figure 10 Promotion effects of *Bacillus* strains on *Nicotiana tabacum* W38 and *Arabidopsis thaliana* Col-0 through volatile emission. Growth of NtbW38 (A) and AtCol-0 (C) seedlings when exposed to volatiles emitted by the *Bacillus* strains and the effect of different inoculation dosages of BsK1 on AtCol-0 (C). Total leaf surface area, foliar fresh weight, and dry weight of NtbW38 (B) and AtCol-0 seedlings (C) were

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collected at 10 and 14 days post inoculation, respectively. Error bars indicate standard error of mean (SEM). Asterisks indicate statistically significant difference compared to control group, as calculated by Tukey's HSD test (P<0.05).

Effects of different culture media for *Bacillus* strains on plant growth

The volatile compounds released by bacteria are dependent on the composition of culture medium (Asari et al. 2016). Our previous results confirmed the promotion effect of three *Bacillus* strains cultivated on LB agar. To investigate the effects of culture media, we further cultivated the three *Bacillus* strains on tryptic soy agar (TSA) and potato dextrose agar (PDA, used for antagonistic test). As shown in Figure 11, the growths of AtCol-0 seedlings were dramatically inhibited while the *Bacillus* strains were cultivated under TSA. This deleterious effect was most pronounced in the BsK1 strain treatment as all the three plant growth parameters were significantly decreased. In the case of PDA, the growths of AtCol-0 seedlings treated with *Bacillus* strains were not increased significantly in comparison with that of CK. Only the BY1336 treatment showed significant increment on leaf surface area and dry weight. Besides, as the growth parameters in the control group of PDA were higher than those of LBA or TSA, suggesting the composition of PDA seemly can promote the growth of seedlings.

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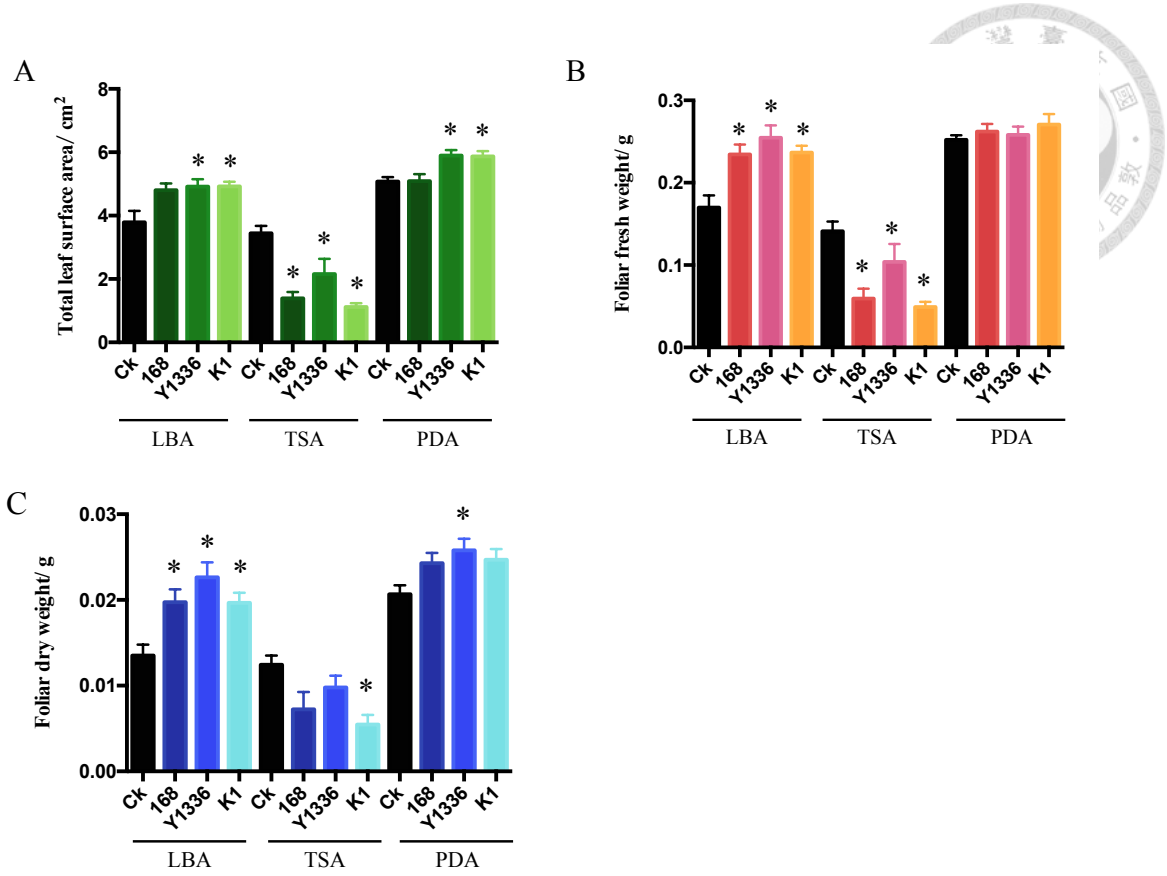

Figure 11 Effects of bacterial culture media on the plant growth. Total leaf surface area (A), foliar fresh weight (B) and dry weight (C) of *Arabidopsis thaliana* Col-0 seedlings were collected at 14 days after treating. Error bars indicate standard error of mean (SEM). Different letters indicate statistically significant difference calculated by Tukey's HSD test (P<0.05).

Effects of dual culture on plant growth

During dual culture at antagonistic assay, presence of MoGuy11 resulted in increased surfactins secretion by BsK1 (Figure 9). Herein, we co-culture the MoGuy11 with *Bacillus* strains to evaluate whether the presence of antagonist affect the volatile emission of *Bacillus* strains as well as the plant growth (Figure 12A). All the experiments were conducted on PDA. As shown in Figure 12B, inhibitory effects of

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Bacillus strains were observed in the BsK1+ MoGuy11 or in the BY1336 + MoGuy11 group, whereas Bs168 failed to outcompete the growth of MoGuy11. Intriguingly, co-culture of the three *Bacillus* strains with MoGuy11 did not affect the growth of AtCol-0 seedlings.

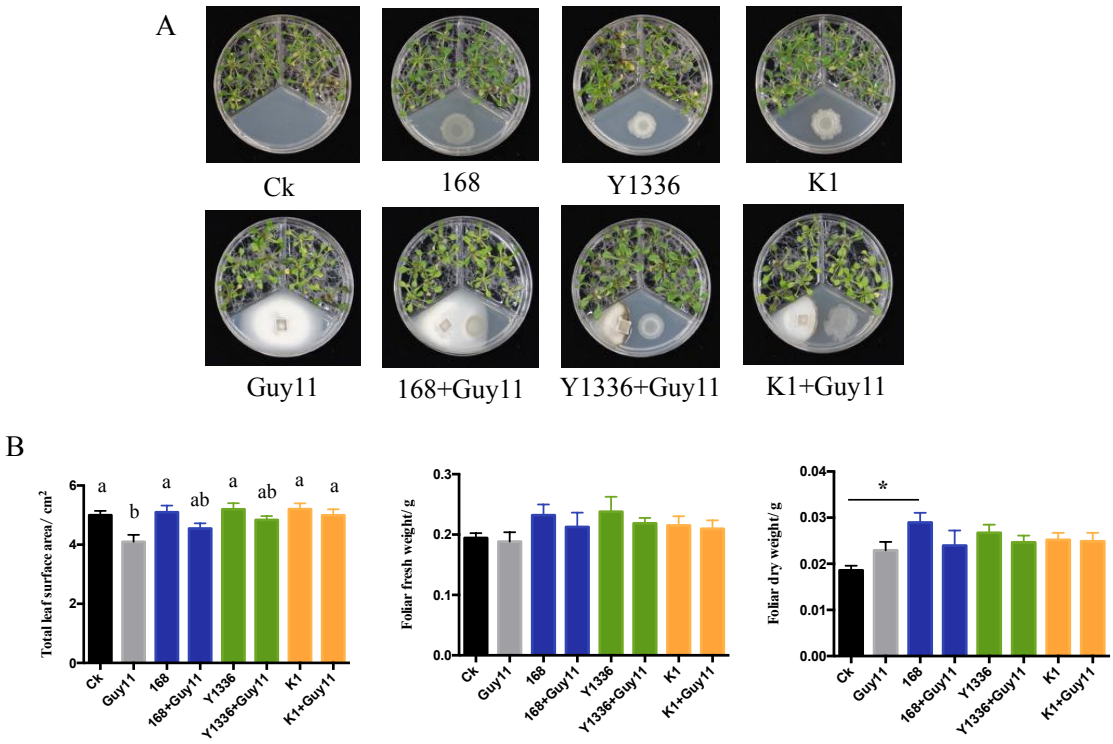

Figure 12 Promoting effect of *Bacillus* strains on AtCol-0 in single (upper panel) and dual culture with MoGuy11 (lower panel). (A) Growth of AtCol-0 at 14 dpi after treating. (B) Total leaf surface area, fresh weight, and dry weight were collected. Error bars indicate standard error of mean (SEM). Different letters indicate statistically significant difference calculated by Tukey's HSD test (P<0.05).

Effects of direct inoculation of *Bacillus* strains on seedlings

Bacillus can form robust biofilm and confer protection on host plants through roots

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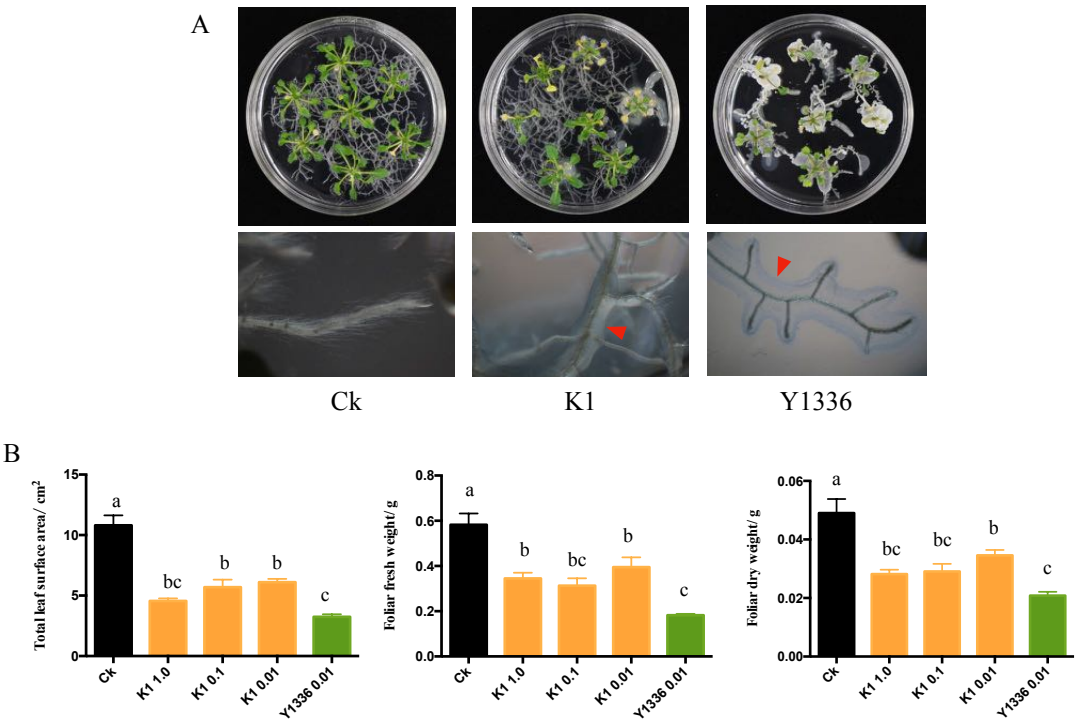
colonization (Chen et al. 2013). We conducted an *in vitro* experiment to investigate the effects of direct inoculation of BsK1 and BY1336 on seedlings. Their effects on host plants were different when inoculating bacteria on the two euphylla stage of NtbW38 or AtCol-0 seedlings. As shown in Figure 13A and C, both BsK1 and BY1336 colonized the roots of seedlings in different manner, in which BY1336 displayed comparatively strong colonization ability. However, both strains did not promote the growth of seedlings, but inhibited the growth of AtCol-0 (Figure 13A and B). Figure 14B showed that the inhibition of BY1336 on AtCol-0 seedlings was significantly more severe than that of low concentration of BsK1 in all analyzed growth parameters. Interestingly, BsK1 did not affect the growth of NtbW38 seedlings in all tested concentration, in contrast, BY1336 possess significantly inhibition (Figure 13D).



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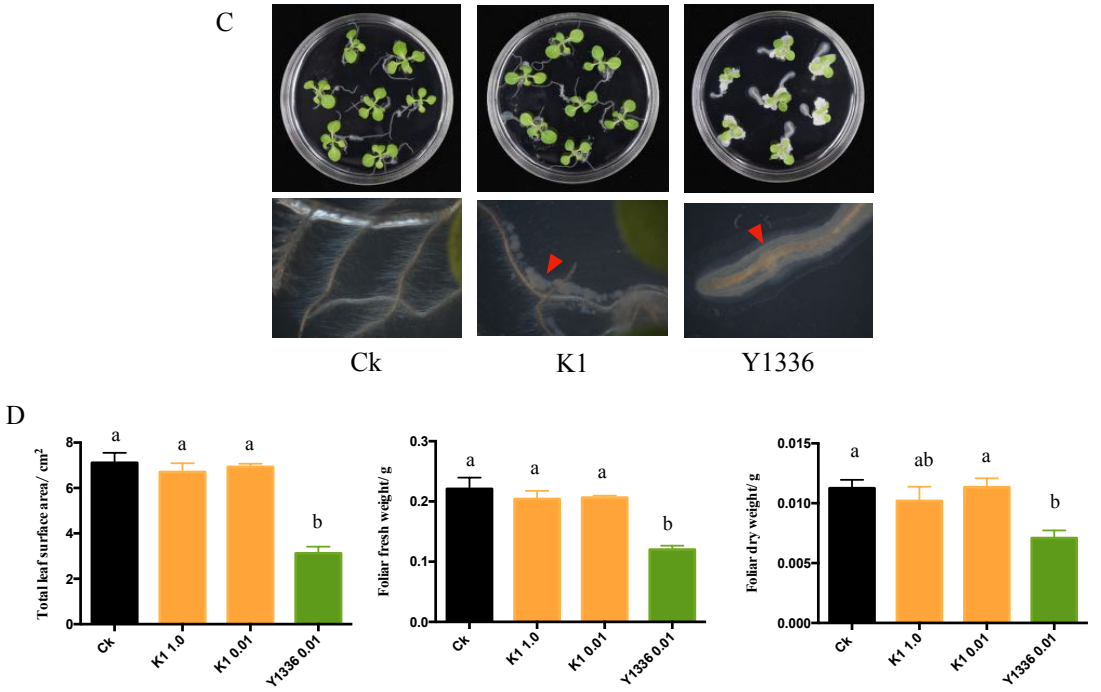

Figure 13 Direct inoculation of *Bacillus* strains on seedling growth. Growth of AtCol-0 seedlings and roots colonization by strains (A) at 14 dpi inoculated with OD_{600}0.01 of BsK1 and BY1336. (B) Total leaf surface area, fresh weight and dry weight of AtCol-0 were collected. Growth of NtbW38 seedlings and root colonization by strains (C) at 10 dpi inoculated with OD_{600}0.01 of BsK1 and BY1336. (D) Total leaf surface area, fresh weight and dry weight of NtbW38 were collected. Error bars indicate standard error of mean (SEM). Different letters indicate statistically significant difference calculated by Tukey's HSD test (P<0.05).

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Discussion

Isolation, identification and characterization of isolates

We identified three types of colony morphologies in four isolates (Figure 1A). In which, particularly, these colony morphologies originated from a single package of 'W'. Initially, packages of 'W' and 'N' were a single strain fermentation product with different fermented strategies. 'W' was designed to increase the protease biosynthesis of strain, while 'N' was a high cell counts products. In fact, the nature of an undomesticated *Bacillus subtilis* strain to produce multiple colony morphologies had been widely reported, thereby regarded as a model species to study its development traits as a multicellular organism (Aguilar et al. 2007). In addition, *Bacillus subtilis* species especially subspecies *natto*, possess a wide range of colony morphologies in a strain (Lombardía et al. 2006). Veening et al. (2006) suggesting that different cultivation parameters, such as cultivation time, medium, and environmental cues can also resulted in various bacterial morphologies. Thereby, the appearance of distinct colony morphologies in 'W', might aroused during fermentation process or medium used as it is a same strain that undergoes different process from 'N'.

Multiple traits of *Bacillus* species resulted in finely tuned genetic coordination in a colony (Aguilar et al. 2007, Arnaouteli et al. 2016). As that, in a cluster of *B. subtilis*, part of cells may undergo sporulation process, some adjusted to produce extracellular matrix for biofilm architectural, while others may turned into competence cells, readily to incorporate critical genes (Lopez et al. 2009). From that, during isolation of four strains, cells may still possibly retain their genetic modification from previous process, thereby resulted in different cell growth speed, further causing the varied cells length

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observed (Figure 1B).

Although BsK1 and BsWA shared most similar colony morphology (Figure 1A), but BsWA and BsWC utilized identical carbon sources for growth (Table 1). D-xylose and arbutin was solely utilized by BsK1 and BsWB, respectively (Table 1). Among the carbon sources tested, all four strains used differently only two carbon sources. By comparing carbon source utilization to available database, they were all clustered into *Bacillus subtilis*/ *amyloliquefaciens* group under high value of similarities (Table 1), suggesting this level of difference was not enough to separate the identities of four strains. Moreover, we collected only 12 hours and 24 hours of incubation results for this experiment. Since our isolated strains might differ in growth, the sources utilization might also be differed in longer time of cultivation.

We constructed three phylogenetic trees to identify the isolates (Figure 3). A typical 16S rRNA gene sequences provided a lower resolution for separating *Bacillus* groups (Figure 3A). Therefore, we employed *gyr*B gene sequence, in which can effectively classify *Bacillus* genus bacteria to subspecies level (Wang et al. 2007, Rooney et al. 2009). Although both *gyr*B and the combined sequences of 16S rRNA and *gyr*B phylogenetic analysis were able to separate subsp. *spizizenii* from subsp. *subtilis*, subsp. *natto*, they were remained inseparable with subsp. *subtilis*. This suggests that more conserved genes, such as quorum sensing *com*Q sequences, should be incorporated to further classify the subsp. *natto* strains, (Oslizlo et al. 2015). A nucleotide difference in BsWA *gyr*B sequence resulted in separation of it from three of others isolates. However, the node value was too low (i.e., 63) (Figure 3B, Figure 3C) to support this separation. Accordingly, all of the isolates were originated from a pure

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strain of *Bacillus subtilis*.

Biocontrol ability of *Bacillus* strains

Bacillus strains can regulate their cellular differentiation in a coordination manner via three main transcriptional regulators, Spo0A, DegU and ComK (Kobayashi 2007, Lopez et al. 2009). Regulators Spo0A and DegU participate in *Bacillus* biocontrol activities, in which Spo0A regulates the biofilm matrix production, while DegU regulates the exoprotease production by *Bacillus* species. When cells reach high densities, Spo0A and DegU were induced (Lopez et al. 2009). In the *in vitro* antagonistic assay, the biocontrol potential of BsK1 was significantly surpassed the other three strains (Figure 2). As BsK1 was derived from the product with high-cell-density, we deduced that BsK1 could reach stationary phase earlier than the other strains, and triggered the matrix production genes to lead to the accumulation of higher concentration of exoprotease and antibiotics, thereby exhibiting greater inhibition on MoGuy11.

In this study, we compared the antagonistic ability of BsK1 with the well-studied *Bacillus subtilis* type strain Bs168, and the commercial available strain *Bacillus* sp. BY1336 (Figure 4A). These two strains were not yet reported to inhibit rice blast fungus. In Figure 4B, swollen hyphae of MoGuy11 were observed in the presence of *Bacillus* strains, indicating mycelia inhibition. This antifungal trait was also reported in a previous study with respect to *B. amyloliquefaciens* BPD-1 for rice blast disease control (Liao et al. 2016).

In the studies of pot experiment using two rice blast susceptible cultivars, TK9 and

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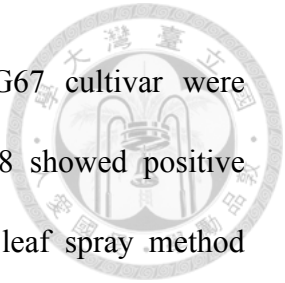

TNG67, biocontrol abilities of BsK1 and BY1336 in the TNG67 cultivar were demonstrated through root inoculation method. In contrast, Bs168 showed positive effect in suppressing leaf rice blast in the TK9 cultivar through leaf spray method (Table 2). Since cultivar TK9 is reported to be more resistant towards leaf rice blast (Chen et al. 2004), no typical leaf blast lesion was formed (Figure 6), thus infected seedlings were classified based on the appearance of small black spots. However, use this selected criterion to assess the efficacy of biological control of rice blast on the resistant cultivar TK9 may be misleading. To initiate rice blast disease, fungal spores must germinate first and penetrate into the host plant, and differentiate into mycelia (Wilson and Talbot 2009). BsK1 and BY1336 did not inhibit the spore germination of MoGuy11 (Figure 5), however, they still exhibited mild biocontrol efficacy in TNG67 through root inoculation method (Table 2A). This phenomenon suggests that both strains may induce the defense mechanisms of rice seedlings, and indirectly suppressed the leaf rice blast as mentioned by (Rahman et al. 2014). Therefore, seed dressing or prolonged the inoculation period might increase the biocontrol efficacy of both strains. For raising the infection rate, the infected seedlings were kept in dark for 24 hours and cultivated under high humidity before harvest in this study. This condition was perfected for MoGuy11 infection (Wilson and Talbot 2009), but rarely found in natural condition. Thereby, field studies on rice blast disease could be conducted to evaluate the biocontrol potential of BsK1 and BY1336 under natural condition.

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Mechanism of BsK1 in suppressing MoGuy11

Bacillus strains secreted a wide range of antifungal compounds, including lipopeptides, polyketides, degradative enzymes and volatiles compounds (Chowdhury et al. 2015, Wu et al. 2015, Tokpah et al. 2016, Tahir et al. 2017). As shown in Figure 4A, BsK1 and BY1336 inhibited the mycelia growth of MoGuy11 without direct contact, suggesting the effective inhibitory compounds may be released by these two strains, but not merely through growth territory competition. As shown in Figure 8A, BY1336 didn't secrete low molecular weight (<3kDa) antifungal lipopeptides, such as iturins or fengycins (Romero et al. 2007, Chowdhury et al. 2015), instead they secreted large molecular weight compounds that are more likely to be degradative enzymes. However, the filtrate of BsK1 did not suppress MoGuy11 mycelia (Figure 8B). Although the specific triggers for *Bacillus* to secrete antibiotic remained unclear, inhibition by *Bacillus* filtrate may be dependent on the medium composition and cultivation time (Akpa et al. 2001, Al-Ajlani et al. 2007). In the case of BsK1, two tested medium (LB and PDB) (Figure 8B, Figure 8C), addition of chitin or MoGuy11 filtrate (data not shown), and prolonged cultivation time (Figure 8C; right) did not result in effective inhibition through culture filtrate. Bacteria reacted towards presence of antagonist, and eventually alter self-physiological and metabolic change (Blacutt et al. 2016, Kulimushi et al. 2017). Since culture filtrate of BsK1 failed to inhibit MoGuy11, we deduced that complex metabolites crosstalk between MoGuy11 and BsK1 during confrontation might induce the antibiotic secretion of bacteria.

Recently, imaging mass platform had been widely acknowledged for molecular interaction studies (Debois et al. 2014, Shih et al. 2014). Three deduced lipopeptide

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groups, including surfactins, iturins and fengycins families were identified in BY1336 and BsK1 (Figure 9). In contrast, Bs168 did not seem to secrete any lipopeptide (Figure 9). According to a whole genome study of Bs168, it derived a mutation in *sfp* gene and *deg*Q promoter causing the lost of above mentioned lipopeptides biosynthesis (Koumoutsi et al. 2004, Chen et al. 2007). Thereby, the lipopeptide-deficient Bs168 showed less pronounced antifungal activity compared to BY1336 and BsK1 (Figure 4A). Meanwhile, the commercial BY1336 could secrete all three lipopeptides in either single or dual culture, demonstrating its ability to secrete antibiotics with or without the presence of antagonist (i.e., MoGuy11 in this study). Interestingly, BsK1 did not secrete detectable antifungal iturins and fengcycins, but only surfactins in both single and dual culture assays (Figure 9). Although surfactins are mostly involved in biofilm formation, roots colonization and induction of systemic resistance of plants (Cawoy et al. 2014), they are rarely reported in direct inhibition of phytopathogen (Tendulkar et al. 2007). Recent studies have demonstrated that, surfactin production by *Bacillus* species played a critical role in controlling several plant diseases indirectly (Chen et al. 2013), and also served as intra- and interspecific communication signals between organisms in environments (Aleti et al. 2016). Accordingly, the surfactins secreted by BsK1 might participate in regulating bacterial community within a shared niche, and provide protection against attack by phytopathogen. This remains to be elucidated. In addition, higher concentration of surfactins were observed in dual culture of BsK1 and MoGuy11 (Figure 9). This phenomenon suggests that BsK1 may react towards antagonist, and possibly triggers the secretion of other non-detected antifungal compounds to counteract MoGuy11. Kulimushi et al. (2017) also mentioned the triggering of antibiotics secretion

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of *Bacillus* in the presence of antagonist. However, no particular compounds or underlying mechanism were clearly stated. Therefore, retrieving compounds from dual culture agar plate and separation using chromatography and mass analysis could be carried out to identify the specific antifungal compounds produced by BsK1 in suppressing MoGuy11 mycelia.

Plant growth promotion by BsK1

Bacillus species were recognized for its promotion effect on plant through volatile emissions (Ryu et al. 2003, Meldau et al. 2013). In our study, Bs168 was set as a positive control strain. It had been reported to be effective in promoting the growth of AtCol-0 seedlings as comparable to a successful commercial strain, *B. subtilis* GB03, the first reported *Bacillus subtilis* strain to promote plants through volatile emission (Ryu et al. 2003). Meanwhile, the commercial strain, BY1336 was also claimed to be able to promote plants growth. Our results indicated that, the isolated BsK1 promoted the seedlings of AtCol-0 and NtBW38 as that of Bs168 and BY1336 (Figure 10). Different amounts of inoculants and cultivation medium were reported to affect the growth of AtCol-0 seedlings (Asari et al. 2016). In contrast to our results, triple units of BsK1 did not affect the promotion effects on AtCol-0. This may due to the low amounts of inoculants in our study, while Asari et al. discovered the mild suppression only observed in 15 units of inoculants. However, we discovered the increased of chlorosis leaves near to double and triple inoculants (Figure 10C). Chlorosis on plant leaves can be caused by oxidative stress and antioxidant defense in *A. thaliana* Col-0 exposed to high concentration of metals ions (Drążkiewicz et al. 2004). This suggesting that high

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amounts of BsK1 might emits volatiles that trigger plant oxidative response or contains metals ions.

We evaluated the promotion effect of strains cultivated under tryptic soy agar and potato dextrose agar compared to LB agar. We also used half strength of Murashigee and Skoog medium to cultivate bacteria, however, three of the tested strains were unable to grow under this medium (data not shown). Our strains emitted deleterious volatiles during cultivation under tryptic soy agar, which is not consistent with Asari et al. results, as they did not observe negative impact on AtCol-0 seedlings. This may due to different strains of bacteria applied. In addition, they also reported that sole medium without bacteria could also produce detectable volatiles that were affected by the composition of medium (Asari et al. 2016). This explained the promotion effect of PDA control group in Figure 11, as this medium might in fact released volatiles that promote the growth of AtCol-0 without the presence of bacteria. Our results first report on the promotion effect by volatiles produced by bacteria cultivated under PDA. Thus, the volatiles profile of PDA calls for a further investigation. During our study, the promotion effect of bacteria on the growth of AtCol-0 through volatiles slightly differed in each repeats. Promoting effect of PDA cultivated BsK1 and BY1336 on AtCol-0 surface area were not significant in Figure 12, but significant in Figure 11A. This unstable effect might be caused by the promoting effect of control itself, or the variation of seeds used.

Not all strains of *Bacillus* with effective volatile are able to promote plants during direct contact (Asari et al. 2016). In our experiments of direct inoculation, the commercial strain BY1336 showed inhibition activity on both seedlings, while BsK1

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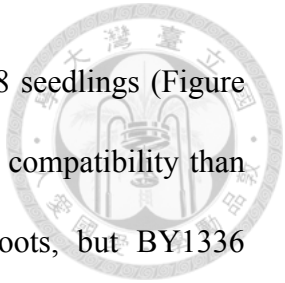

less severely inhibited AtCol-0, and possessed no effect on NtbW38 seedlings (Figure 13). From there, BsK1 displayed broader host selection, and higher compatibility than that of BY1336. Both strains were able to colonize seedling roots, but BY1336 colonized in a greater extend (Figure 13). This may due to the overgrown of BY1336, further lead to a negative impact on plant growth. In addition, AtCol-0 originated as a weed, while NtbW38 is an economic crop. The promotion effect of BsK1 on NtbW38 and deleterious effect on AtCol-0 showing its potential as a weeds controller and crops promoting bacteria.

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Conclusions and future prospects

This study demonstrated the potential of *Bacillus subtilis* natto K1 in suppressing mycelia growth of rice blast fungus and promoting the seedlings growth of *Arabidopsis thaliana* Col-0 and *Nicotiana tabacum* W38. BsK1 cells inhibited the surface area of MoGuy11 mycelia on plate, and inoculation of BsK1 decreased the disease index of leaf rice blast in cultivar TNG67. The volatiles emission of LB cultivated BsK1 promoted the growth of seedlings as that of commercial and positive control *Bacillus subtilis* strains. Moreover, direct inoculation of BsK1 did less harm on AtCol-0 seedlings compared to the commercial strain BY1336, and did not affect the growth of NtBW38, showing its potential as an applicable biofertilizer.

In terms of biocontrol ability, we proposed that BsK1 might secrete antibiotics other than fengycins and iturins to inhibit the growth of MoGuy11 as both antifungal lipopeptides were not detected in the imaging mass analysis of BsK1. Identify the culture condition to optimize the production of antibiotics of BsNK1 will be helpful in formulating BsK1 as a more successful biocontrol products. Besides, BsK1 also reacted towards the presence of MoGuy11, as surfactins concentration was comparatively higher in dual culture in imaging mass analysis. We hope to study the interaction between phytopathogen and BsK1 and identify the compounds or condition that trigger the response of BsK1. In addition, roots inoculation of BsK1 relieved leaf blast disease in cultivar TNG67. We thereby proposed that prolonged inoculation periods or inoculation methods such as seeds dressing might increase the biocontrol efficacy of BsK1 in controlling rice blast disease. Together with the plant-growth promotion ability

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of BsK1, we also hope that seeds dressing of BsK1 able to increase the yield and health of crops. Through performing relative quantitation of gene expression using real-time PCR could investigate the effect of BsNK1 inoculation on the transcriptional level of genes responsible for growth and phytopathogen resistance genes in plants.

Some *Bacillus* strains were discovered to be endophytic bacteria and proliferate inside plants tissues, in future, we hope to identify the colonization niche of BsK1 in rice cultivar. As MoGuy11 invade leaf rice tissues in late stage of infection, endophytic colonization of BsK1 might be able to inhibit hyphae of MoGuy11 *in vivo*. Lastly, BsK1 were confirmed to secrete surfactins, which one of its function was to serve as intra- and inter specific communication signals. Accompanied by its wide arrays of compounds secretion, its roles in shaping the soil or plant microbiome might worth for future study.

doi:10.6342/NTU201704205

References

Aguilar, C., et al. (2007). "Thinking about *Bacillus subtilis* as a multicellular organism." *Current Opinion in Microbiology* **10**(6): 638-643.

Akpa, E., et al. (2001). "Influence of culture conditions on lipopeptide production by *Bacillus subtilis*." *Applied Biochemistry and Biotechnology* **91**(1): 551-561.

Aktar, W., et al. (2009). Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary Toxicology*. **2:** 1-12.

Al-Ajlani, M. M., et al. (2007). "Production of surfactin from *Bacillus subtilis* MZ-7 grown on pharmamedia commercial medium." *Microbial Cell Factories* **6**(1): 17.

Aleti, G., et al. (2016). "Surfactin variants mediate species-specific biofilm formation and root colonization in *Bacillus*." *Environmental Microbiology* **18**(8): 2634-2645.

Arnaouteli, S., et al. (2016). "Just in case it rains: building a hydrophobic biofilm the *Bacillus subtilis* way." *Current Opinion in Microbiology* **34**: 7-12.

Arrebola, E., et al. (2010). "Effect of volatile compounds produced by *Bacillus* strains on postharvest decay in citrus." *Biological Control* **53**(1): 122-128.

Asari, S., et al. (2016). "Multiple effects of *Bacillus amyloliquefaciens* volatile compounds: plant growth promotion and growth inhibition of phytopathogens." *FEMS Microbiology Ecology* **92**(6).

Bais, H. P., et al. (2004). "Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production." *Plant Physiology* **134**(1): 307-319.

Baker, B., et al. (1997). "Signaling in plant-microbe interactions." *Science* **276**(5313): 726-733.

doi:10.6342/NTU201704205

Ben Ayed, H., et al. (2017). "Identification and natural functions of cyclic lipopeptides from *Bacillus amyloliquefaciens* An6." *Engineering in Life Sciences* **17**(5): 536-544.

Blacutt, A., et al. (2016). "*Bacillus mojavensis* RRC101 lipopeptides provoke physiological and metabolic changes during antagonism against *Fusarium verticillioides*." *Molecular Plant-Microbe Interactions* **29**(9): 713-723.

Bonmatin, J.-M., et al. (2003). "Diversity among microbial cyclic lipopeptides: iturins and surfactins. Activity-structure relationships to design new bioactive agents." *Combinatorial Chemistry & High Throughput Screening* **6**(6): 541-556.

Cawoy, H., et al. (2014). "Plant defense stimulation by natural isolates of *Bacillus* depends on efficient surfactin production." *Molecular Plant-Microbe Interactions* **27**(2): 87-100.

Chen, H., et al. (2008). "Isolation and characterization of lipopeptide antibiotics produced by *Bacillus subtilis*." *Letters in Applied Microbiology* **47**(3): 180-186.

Chen, L.-C., et al. (2004). "Test of rice varieties and strains resistant to rice blast in blast nurseries during 1990-2002." *Journal of Agricultural Research of China*(53): 269-283.

Chen, W.-J., et al. (2016). "Involvement of type VI secretion system in secretion of iron chelator pyoverdine in *Pseudomonas taiwanensis*." *Scientific Reports* **6**: 32950.

Chen, X. H., et al. (2007). "Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42." *Nature Biotechnology* **25**(9): 1007.

Chen, Y., et al. (2013). "Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation." *Environmental Microbiology* **15**(3): 848-864.

Chowdhury, S. P., et al. (2015). "Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42 - a review." *Frontiers in Microbiology* **6**: 780.

doi:10.6342/NTU201704205

Chuma, I., et al. (2011). "Multiple translocation of the AVR-Pita effector gene among chromosomes of the rice blast fungus *Magnaporthe oryzae* and related species." *PLOS Pathogens* **7**(7): e1002147.

Cook, R. J. (1993). "Making greater use of introduced microorganisms for biological control of plant pathogens." *Annual Review of Phytopathology* **31**(1): 53-80.

Debois, D., et al. (2014). "Spatiotemporal monitoring of the antibiome secreted by *Bacillus* biofilms on plant roots using MALDI mass spectrometry imaging." *Analytical Chemistry* **86**(9): 4431-4438.

Drążkiewicz, M., et al. (2004). "Copper-induced oxidative stress and antioxidant defence in *Arabidopsis thaliana*." *Biometals* **17**(4): 379-387.

Dubey, R., et al. (2011). "Isolation, production, purification, assay and characterization of fibrinolytic enzymes (Nattokinase, Streptokinase and Urokinase) from bacterial sources." *African Journal of Biotechnology* **10**(8): 1408-1420.

Eden, P. A., et al. (1991). "Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA." *International Journal of Systematic and Evolutionary Microbiology* **41**(2): 324-325.

Finking, R. and M. A. Marahiel (2004). "Biosynthesis of nonribosomal peptides." *Annual Review of Microbiology* **58**(1): 453-488.

Francis, I., et al. (2010). "The Gram-positive side of plant–microbe interactions." *Environmental Microbiology* **12**(1): 1-12.

Fujita, M., et al. (1995). "Thrombolytic effect of nattokinase on a chemically induced thrombosis model in rat." *Biological and Pharmaceutical Bulletin* **18**(10): 1387-1391.

Hall, T. A. (1999). *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT*. Nucleic acids symposium series, [London]:

doi:10.6342/NTU201704205

Information Retrieval Ltd., c1979-c2000.

Hasegawa, M., et al. (1985). "Dating of the human-ape splitting by a molecular clock of mitochondrial DNA." *Journal of Molecular Evolution* **22**(2): 160-174.

Hayashi, K., et al. (2005). "The H_2O_2 stress-responsive regulator PerR positively regulates *srf*A expression in *Bacillus subtilis*." *Journal of Bacteriology* **187**(19): 6659-6667.

Heerklotz, H., et al. (2004). "Membrane perturbation by the lipopeptide surfactin and detergents as studied by deuterium NMR." *The Journal of Physical Chemistry B* **108**(15): 4909-4915.

Hsu, R.-L., et al. (2008). "Amyloid-degrading ability of nattokinase from *Bacillus subtilis* natto." *Journal of Agricultural and Food Chemistry* **57**(2): 503-508.

Huang, C.-N., et al. (2016). "Characterization and evaluation of *Bacillus amyloliquefaciens* strain WF02 regarding its biocontrol activities and genetic responses against bacterial wilt in two different resistant tomato cultivars." *World Journal of Microbiology and Biotechnology* **32**(11): 183.

Idris, E. E., et al. (2007). "Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42." *Molecular Plant-Microbe Interactions* **20**(6): 619-626.

Idriss, E. E., et al. (2002). "Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effecta." *Microbiology* **148**(7): 2097-2109.

Jourdan, E., et al. (2009). "Insights into the defense-related events occurring in plant cells following perception of surfactin-type lipopeptide from *Bacillus subtilis*." *Molecular Plant-Microbe Interactions* **22**(4): 456-468.

Kai, M., et al. (2009). "Bacterial volatiles and their action potential." *Applied*

doi:10.6342/NTU201704205

Microbiology and Biotechnology **81**(6): 1001-1012.

Kim, P., et al. (2004). "Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26." *Journal of Applied Microbiology* **97**(5): 942-949.

Kobayashi, K. (2007). "*Bacillus subtilis* pellicle formation proceeds through genetically defined morphological changes." *Journal of Bacteriology* **189**(13): 4920-4931.

Kobayashi, K. (2015). "Plant methyl salicylate induces defense responses in the rhizobacterium *Bacillus subtilis*." *Environmental Microbiology* **17**(4): 1365-1376.

Kosma, D. K., et al. (2009). "The impact of water deficiency on leaf cuticle lipids of *Arabidopsis*." *Plant Physiology* **151**(4): 1918-1929.

Koumoutsi, A., et al. (2004). "Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42." *Journal of Bacteriology* **186**(4): 1084-1096.

Kulimushi, P. Z., et al. (2017). "Stimulation of fengycin-type antifungal lipopeptides in *Bacillus amyloliquefaciens* in the presence of the maize fungal pathogen *Rhizomucor variabilis*." *Frontiers in Microbiology* **8**.

Kumar, S., et al. (2016). "MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets." *Molecular Biology and Evolution* **33**(7): 1870-1874.

Leelasuphakul, W., et al. (2006). "Purification, characterization and synergistic activity of β-1,3-glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight." *Enzyme and Microbial Technology* **38**(7): 990-997.

Liao, J.-H., et al. (2016). "Clarification of the antagonistic effect of the lipopeptides produced by *Bacillus amyloliquefaciens* BPD1 against *Pyricularia oryzae* via In Situ MALDI-TOF IMS Analysis." *Molecules* **21**(12): 1670.

doi:10.6342/NTU201704205

Lombardía, E., et al. (2006). "A LuxS-dependent cell-to-cell language regulates social behavior and development in *Bacillus subtilis*." *Journal of Bacteriology* **188**(12): 4442-4452.

Lopez, D., et al. (2009). "Generation of multiple cell types in *Bacillus subtilis*." *FEMS Microbiology Reviews* **33**(1): 152-163.

Luo, C., et al. (2015). "Bacillomycin L and surfactin contribute synergistically to the phenotypic features of *Bacillus subtilis* 916 and the biocontrol of rice sheath blight induced by *Rhizoctonia solani*." *Applied Microbiology and Biotechnology* **99**(4): 1897-1910.

Luo, Y., et al. (2013). "Identification and characterization of an anti-fungi *Fusarium oxysporum f.* sp. *cucumerium* protease from the *Bacillus subtilis* strain N7." *Journal of Microbiology* **51**(3): 359-366.

Mäder, U., et al. (2002). "*Bacillus subtilis* functional genomics: genome-wide analysis of the DegS-DegU regulon by transcriptomics and proteomics." *Molecular Genetics and Genomics* **268**(4): 455-467.

Meldau, D. G., et al. (2013). "Dimethyl disulfide produced by the naturally associated bacterium *Bacillus* sp B55 promotes *Nicotiana attenuata* growth by enhancing sulfur nutrition." *The Plant Cell* **25**(7): 2731-2747.

Molinatto, G., et al. (2017). "Key impact of an uncommon plasmid on *Bacillus amyloliquefaciens* subsp. *plantarum* S499 developmental traits and lipopeptide production." *Frontiers in Microbiology* **8**: 17.

Ongena, M. and P. Jacques (2008). "*Bacillus* lipopeptides: versatile weapons for plant disease biocontrol." *Trends in Microbiology* **16**(3): 115-125.

Oslizlo, A., et al. (2015). "Exploring ComQXPA quorum-sensing diversity and biocontrol potential of *Bacillus* spp. isolates from tomato rhizoplane." *Microbial Biotechnology* **8**(3): 527-540.

doi:10.6342/NTU201704205

Oyebanji, O., et al. (2009). "Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds." *African Journal of Biotechnology* **8**(20).

Patrick, J. E. and D. B. Kearns (2009). "Laboratory strains of *Bacillus subtilis* do not exhibit swarming motility." *Journal of Bacteriology* **191**(22): 7129-7133.

Pérez-García, A., et al. (2011). "Plant protection and growth stimulation by microorganisms: biotechnological applications of bacilli in agriculture." *Current Opinion in Biotechnology* **22**(2): 187-193.

Peypoux, F., et al. (1999). "Recent trends in the biochemistry of surfactin." *Applied Microbiology and Biotechnology* **51**(5): 553-563.

Raaijmakers, J. M., et al. (2010). "Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics." *FEMS Microbiology Reviews* **34**(6): 1037-1062.

Rahman, A., et al. (2014). "Induction of salicylic acid-mediated defense response in perennial ryegrass against infection by *Magnaporthe oryzae*." *Phytopathology* **104**(6): 614-623.

Rahman, A., et al. (2015). "Induced systemic resistance responses in perennial ryegrass against *Magnaporthe oryzae* elicited by semi-purified surfactin lipopeptides and live cells of *Bacillus amyloliquefaciens*." *Molecular Plant Pathology* **16**(6): 546-558.

Romero, D., et al. (2007). "The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*." *Molecular Plant-Microbe Interactions* **20**(4): 430-440.

Rooney, A. P., et al. (2009). "Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov." *International Journal of Systematic and Evolutionary Microbiology* **59**(10):

doi:10.6342/NTU201704205

2429-2436.

Ryu, C.-M., et al. (2003). "Bacterial volatiles promote growth in *Arabidopsis*." *Proceedings of the National Academy of Sciences* **100**(8): 4927-4932.

Ryu, C.-M., et al. (2004). "Bacterial volatiles induce systemic resistance in *Arabidopsis*." *Plant Physiology* **134**(3): 1017-1026.

Schallmey, M., et al. (2004). "Developments in the use of *Bacillus* species for industrial production." *Canadian Journal of Microbiology* **50**(1): 1-17.

Shan, H., et al. (2013). "Biocontrol of rice blast by the phenaminomethylacetic acid producer of *Bacillus methylotrophicus* strain BC79." *Crop Protection* **44**: 29-37.

Shao, J., et al. (2015). "Contribution of indole-3-acetic acid in the plant growth promotion by the rhizospheric strain *Bacillus amyloliquefaciens* SQR9." *Biology and Fertility of Soils* **51**(3): 321-330.

Shen, W.-C., et al. (1999). "Isolation of pheromone precursor genes of *Magnaporthe grisea*." *Fungal Genetics and Biology* **27**(2-3): 253-263.

Shih, C.-J., et al. (2014). "Bringing microbial interactions to light using imaging mass spectrometry." *Natural Product Reports* **31**(6): 739-755.

Skamnioti, P. and S. J. Gurr (2009). "Against the grain: safeguarding rice from rice blast disease." *Trends in Biotechnology* **27**(3): 141-150.

Spence, C., et al. (2014). "Natural rice rhizospheric microbes suppress rice blast infections." *BMC Plant Biology* **14**(1): 130.

Stein, T. (2005). "*Bacillus subtilis* antibiotics: structures, syntheses and specific functions." *Molecular Microbiology* **56**(4): 845-857.

Sumi, H., et al. (1987). "A novel fibrinolytic enzyme (nattokinase) in the vegetable

doi:10.6342/NTU201704205

cheese Natto; a typical and popular soybean food in the Japanese diet." *Cellular and Molecular Life Sciences* **43**(10): 1110-1111.

Tahir, H. A. S., et al. (2017). "*Bacillus* volatiles adversely affect the physiology and ultra-structure of *Ralstonia solanacearum* and induce systemic resistance in tobacco against bacterial wilt." *Scientific Reports* **7**: 40481.

Tamura, K. and M. Nei (1993). "Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees." *Molecular Biology and Evolution* **10**(3): 512-526.

Tendulkar, S., et al. (2007). "Isolation, purification and characterization of an antifungal molecule produced by *Bacillus licheniformis* BC98, and its effect on phytopathogen *Magnaporthe grisea*." *Journal of Applied Microbiology* **103**(6): 2331-2339.

Thompson, R. D. and J. Verdier (2012). Networks of seed storage protein regulation in cereals and legumes at the dawn of the omics era. *Seed Development: OMICS technologies toward improvement of seed quality and crop yield: OMICS in seed biology*. G. K. Agrawal and R. Rakwal. Dordrecht, Springer Netherlands**:** 187-210.

Tokpah, D. P., et al. (2016). "An assessment system for screening effective bacteria as biological control agents against *Magnaporthe grisea* on rice." *Biological Control* **103**: 21-29.

Tsuge, K., et al. (1999). "The genes *degQ*, *pps*, and *lpa-8* (*sfp*) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production." *Antimicrobial Agents and Chemotherapy* **43**(9): 2183-2192.

Veening, J.-W., et al. (2006). "Effects of phosphorelay perturbations on architecture, sporulation, and spore resistance in biofilms of *Bacillus subtilis*." *Journal of Bacteriology* **188**(8): 3099-3109.

Wang, L.-T., et al. (2007). "Comparison of *gyr*B gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group." *International*

doi:10.6342/NTU201704205

Journal of Systematic and Evolutionary Microbiology **57**(8): 1846-1850.

Wilson, R. A. and N. J. Talbot (2009). "Under pressure: investigating the biology of plant infection by *Magnaporthe oryzae*." *Nature Reviews Microbiology* **7**(3): 185-195.

Wise, C., et al. (2012). "Production and antimicrobial activity of 3-hydroxypropionaldehyde from *Bacillus subtilis* strain CU12." *Journal of Chemical Ecology* **38**(12): 1521-1527.

Wu, L., et al. (2015). "Novel routes for improving biocontrol activity of *Bacillus* based bioinoculants." *Frontiers in Microbiology* **6**.

Xie, S.-S., et al. (2014). "Plant growth promotion by spermidine-producing *Bacillus subtilis* OKB105." *Molecular Plant-Microbe Interactions* **27**(7): 655-663.

Yamamoto, S. and S. Harayama (1995). "PCR amplification and direct sequencing of *gyr*B genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains." *Applied and Environmental Microbiology* **61**(3): 1104-1109.

Zeigler, R., et al. (1994). "Lineage exclusion: a proposal for linking blast population analysis to rice breeding." *The Rice Blast Disease, John Hopkins Press, Baltimore, Maryland, USA*: 267-291.

Zeriouh, H., et al. (2014). "Surfactin triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity." *Environmental Microbiology* **16**(7): 2196-2211.

Zhang, H., et al. (2007). "Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis*." *Planta* **226**(4): 839.

Zhang, H., et al. (2009). "MgCRZ1, a transcription factor of *Magnaporthe grisea*, controls growth, development and is involved in full virulence." *FEMS Microbiology Letters* **293**(2): 160-169.

doi:10.6342/NTU201704205