A PRELIMINARY SCREENING OF PHILIPPINE MANGROVE SOIL BACTERIA EXHIBIT SUPPRESSION OF *RALSTONIA SOLANACEARUM* (SMITH) YABUUCHI ET AL. CAUSING MOKO DISEASE OF BANANA (*MUSA ACUMINATA* CAVENDISH SUBGROUP) UNDER LABORATORY CONDITIONS

Armi R. Creencia[1*], Edwin P. Alcantara[1], Rina B. Opulencia[2], Maria Genaleen Q. Diaz[2] and Rosario G. Monsalud[1]
[1]National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB)
[2]Institute of Biological Sciences (IBS), College of Arts and Sciences , UPLB, College, Laguna 4031
*Corresponding author: arcreencia@up.edu.ph

(Received: February 23, 2022; Accepted: May 28, 2022)

ABSTRACT

The study was conducted in 2021 in Los Baños, Laguna, Philippines to determine the potential of the mangrove soil bacteria isolated from Palawan, the Philippines to inhibit local *Ralstonia solanacearum* (Smith) Yabuuchi et al. strains from causing moko disease in Cavendish banana. Out of 55 isolates screened, three bacteria designated as S31 (*Bacillus altitudinis*), S37 (*B. subtilis*) and S38 (*B. thuringiensis*) exhibited antagonism against the four *R. solanacearum* strains based on the cylinder plate assay, with zones of inhibition (ZOIs) ranging from 9.50 ± 0.5 mm to 28.2 ± 0.3 mm. The best isolate, S37, possessed a significant activity and had a minimum inhibitory concentration (MIC) of as low as 78.1 ± 0.0 µg/ml using the broth microdilution method with resazurin as the indicator. Results of the screenhouse assay revealed the ability of S37 to control wilting of the Cavendish banana plants after injection into the pseudostem. Whole genome analysis using antiSMASH indicated the presence of six secondary metabolites in S37 with putative antimicrobial activity. The data showed that the mangrove bacteria may be possible candidates for production of a new biological agent containing non-pathogenic and fast-growing bacterial species to control the said disease.

Key words: antagonism, zones of inhibition, antimicrobial activity, whole genome, secondary metabolites

INTRODUCTION

Banana (*Musa* spp.) and plantains are perennial crops that have been considered as the top traded fruit worldwide and the eighth most important crop in the world in terms of production volume and trade (DA 2018; Dadrasnia et al. 2020; FAOSTAT 2014). The Philippines is one of the major banana exporters and was ranked number 2 next to Ecuador (DA 2018). Area planted to banana was 440,000 ha in 2016, increased to 450,000 ha in 2017 and remained at this level until 2020 (PSA 2016-2020). Banana production has also been threatened by different diseases and thereby posing a risk to global food security. The resulting production losses often cause a major impact on the banana value chain and the socio-economic livelihood as well.

The moko, bugtok and blood diseases are the three main banana bacterial diseases occurring in the tropics. These diseases are closely-related but their distribution is quite distinct. The moko disease, also known as bacterial wilt, is a major systemic disease affecting bananas of the Cavendish cultivar (Blomme et al. 2017). This disease has been recorded in Central and South America and the Caribbean (CABI 2014). In the Philippines, it is rampant in the southern Mindanao region (Raymundo 2001). This disease causes wilting of leaves and necrosis of the vascular tissues. Moko disease can be transmitted through contaminated tools and irrigation water, infested soil, and by contact with roots or vector insects (Alvarez et al. 2015). The bugtok disease is limited to the Philippines and infects the Cardaba and the Saba cultivars (Molina 2006). The symptoms of the bugtok disease include discoloration of the vascular tissue of the peduncle and the fruit pulp of the banana plant. Unlike the moko disease, plants infected with bugtok do not wilt and the suckers remain unaffected. Bugtok disease is commonly transmitted through insects (Molina 1999). Another banana disease transmitted by insects is the blood disease which is caused by *Ralstonia syzygi* subsp. *celebensis*. It was reported to originate from an island off the coast of Sulawesi in Indonesia in the 1900s and affected the dessert bananas (Thwaites et al. 2000). Symptoms of the blood disease include discoloration and shriveling of the male flower bud and peduncle, reddish dry rot of the fruit pulp and reddish discoloration of the vascular tissue throughout the plant. (Blomme et al. 2017).

The causative organism of both the moko disease and the bugtok disease, *Ralstonia solanacearum* (Smith) Yabuuchi et al., is a Gram-negative aerobic bacterium that naturally inhabits the soil and is present in all continents (Blomme et al. 2017). This species is genetically diverse in that it has been classified into biovars based on acid production from certain sugars and oxidation of hexose alcohols (Buddenhagen and Kelman 1964). These biovars differ in geographic distribution, epidemiology and physiology. They are also grouped into races based on their host range. Moko disease, in particular, is caused by *R. solanacearum* race 2 biovar 1 (Blomme et al. 2017; Hayward 1994). Several strategies for managing and mitigating moko disease have been practiced. These include biological, physical, chemical and cultural means, among others (Yuliar et al. 2015). In Colombia, clean and thermotherapy-treated seeds are used as part of primary preventive management. Likewise, tools, footwear and vehicle tires are disinfected. Diseased plants are eradicated from the plantation to prevent further spread (Alvarez et al. 2015). To help contain the progression of the disease, soils can be drenched with copper oxychloride (2000 ppm) or antibiotics such as streptomycin or streptocycline (500 ppm) (Thiribhuvanamala et al. 2018). In the Philippines, formalin was used to drench the soil around the infected banana plant to reduce *R. solanacearum* count and the insecticide and nematicide Furadan® was also tested (Pava et al. 2003). The use of chlorine dioxide to control the disease has also been explored (Ramirez et al. 2015). However, there is still no direct chemical treatment that has been identified to treat moko disease. Chemical treatments are also not recommended for long-term use as these could cause harm to humans and the environment, and may increase the risk of antibiotic resistance. Currently, most of the Cavendish banana plantations in Mindanao Island in the Philippines just disinfect the tools, and cut and burn infected plants (personal communication). Thus, there is a need for a safer, more effective and more environment-friendly means to address to such problem.

The main goal of this study is to determine the potential and suitability of the mangrove soil bacteria to be developed into a new biocontrol agent against the moko disease of banana. Specifically, the study aimed to screen for antimicrobial activity of the mangrove bacteria against four local moko disease-causing *R. solanacearum* strains; to identify the three best-performing bacteria through phenotypic and genotypic methods; to determine the antimicrobial secondary metabolites of the most promising isolate through whole genome sequencing (WGS); and to determine the effectiveness of the most promising bacterium to control the disease under screenhouse conditions.

To the best of our knowledge, this is the first published report on mangrove soil bacteria isolated in Palawan, Philippines exhibiting significant activity against four different local strains of pathogenic *R. solanacearum* causing moko disease of Cavendish banana. Through this study, local isolates inherent

to the Philippine soil possessing promising antimicrobial activity against the bacterial wilt-causing pathogen can be made readily available for stakeholders in the country as these have been deposited at the Philippine National Collection of Microorganisms (PNCM) for long-term preservation and distribution.

MATERIALS AND METHODS

Mangrove bacterial isolates. The 55 bacteria from Palawan, Philippines mangrove soil were previously isolated by the lead author and obtained from the Philippine National Collection of Microorganisms (PNCM) for this study. The isolates were grown on Marine Agar (MA) and incubated at 30°C for 24 h.

***Ralstonia solanacearum* test organisms.** The four *R. solanacearum* local isolates were obtained from four different Cavendish banana farms in Davao, Philippines provided by Lapanday Banana Plantation. These were designated as M1, M2, M3 and M4. The test strains were grown on Kelman's Agar (KA) medium supplemented with 0.1% (w/v) 2,3,5-triphenyl tetrazolium chloride (TZC) and incubated at 30°C for 24 h (Kelman, 1954). The cultural, morphological and some of the biochemical and physiological characteristics of the *R. solanacearum* strains were determined.

Pathogenicity testing. The *R. solanacearum* cell suspensions (9.0 x 10^8 CFU/ml) were prepared in sterile distilled water from 24-hr old cultures. Five milliliters of each bacterial suspension were injected into the lower pseudostem of four two-month-old tissue-cultured Cavendish banana plants. The control set-ups were injected with uninoculated sterile distilled water. The inoculated banana plants were monitored every day up to 30 days for symptoms of bacterial wilt. The *R. solanacearum* strains were re-isolated from the wilted plants and were used in the succeeding tests. After the experiment, the banana plant set-ups were decontaminated in an autoclave for 30 mins at 15 psi.

Extraction of antimicrobial bioactive compounds. The antimicrobial compounds of the 55 mangrove soil bacterial isolates were extracted following the method of Hayashida-Soiza and group (2008) with modifications, active metabolites from a marine bacterium broth culture were extracted using ethyl acetate. Each bacterial isolate was cultivated in 1L Marine Broth (MB) (Pronadisa, Spain) and incubated at 30°C for 48 h with shaking (150 rpm). After incubation, the culture broth was centrifuged at 8000 *g* for 30 mins. An equal volume of ethyl acetate was added to the cell-free supernatant and shaken gently (120 rpm) overnight. The resulting top organic layer was collected and evaporated at 40°C using the rotary evaporator. The concentrated crude extract was suspended in technical-grade methanol (10,000 µg/ml final concentration) and was immediately used for the antimicrobial assay. Unused extracts were sealed and stored in the laboratory refrigerator (4°C).

Antimicrobial Assay (*in vitro*). The antimicrobial analysis was performed on the 55 bacteria by the cylinder plate assay following the modified Kirby-Bauer Disk Diffusion Method (Hudzicki 2009) which is a standard procedure to determine the susceptibility of the test microorganisms to an antimicrobial agent. The 24-hour old cells of each *R. solanacearum* strain were suspended in 5-ml 0.85% NaCl solution (1.5 x 10^8 CFU/ml). Twenty milliliters of sterile molten basal Kelman's Agar medium (KA) were poured onto glass petri plates and allowed to solidify. Five milliliters of seeded KA (0.05% v/v inoculum) top agar were overlaid onto each basal agar and allowed to solidify. Sterile stainless cylinder cups were carefully positioned on top of the agar. One hundred microliters of the crude extract were dispensed into each cylinder cup. A negative control (100% AR-grade methanol) and a positive control (10,000 µg/ml streptomycin) were also included. The set-ups were incubated at 30°C for up to 24 h. The presence of zone of inhibition (ZOI) was observed and recorded. The experiment was performed in triplicates.

Minimum inhibitory concentration (MIC) of the crude extracts. The MICs of the crude extracts from the three best-performing mangrove bacteria were determined by the broth microdilution method

using resazurin as the bacterial growth indicator, with slight modifications (Bouhdid et al. 2010). The selected method makes use of several two-fold dilutions of the antimicrobial agents in a broth medium placed in a disposable microtiter plate, and visual examination of the plates for inhibition of bacterial growth. For this study, serial two-fold dilutions were prepared in Kelman's broth (KB) in a 96-well microtiter plate. Each sample dilution (50 µl) was inoculated with 50 µl of *R. solanacearum* suspension (1.5 x 10^5 CFU/ml). Final concentrations of the samples were 39.06 to 5,000 µg/ml. Sterile KB was used as the negative control. The experiment was done in triplicates. After incubation of the microtiter plates at 37°C for 18 h, 10 µl of filter-sterilized resazurin solution (0.015% w/v) was added to all wells. The set-ups were incubated for additional two h. The color change was assessed visually. Color changes from blue to pink or colorless indicate growth of the test organism. The lowest concentration that inhibited the growth (no color change) was taken as the MIC value.

Phenotypic characterization of the three best bacteria. The cultural characteristics (color, form, opacity, elevation, margin of colony, presence/absence of soluble pigment) of the bacteria on MA were recorded. The bacterial cells were Gram-stained and the cellular morphology (Gram reaction, shape, size and arrangement of cells) was observed under the oil immersion objective (1,000X) using Olympus BX50 light microscope. Through the VITEK 2 Compact (bioMérieux, France) automated identification system, the substrate utilization, enzyme production and antibiotic resistance profiles of the promising bacteria were determined.

Whole genome sequencing of the three best bacteria. The genomic DNAs of the three best-performing bacteria were isolated using the Quick-DNA™ Fungal/Bacterial Miniprep kit (Zymo Research, USA). The DNA samples were submitted to Macrogen Korea for *de novo* whole genome sequencing on HiSeq X Ten platform with 150 bp paired-end setting and 1G throughput. The sequencing library was prepared through the TruSeq Nano DNA kit (Illumina, USA). Prokka v.1.12 was used for functional annotation (Seeman 2014). The annotated genome was analyzed using the Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) v.5.0. (Blin et al. 2019). To ascertain the identity of the bacteria, digital DNA-DNA (dDDH) hybridization was performed through the Type Strain Genome Server (Meier-Kolthoff and Göker 2019). Average Nucleotide Identity (ANI) was estimated using the Kostas Lab ANI calculator (Rodriguez and Konstantinidis 2016).

Screenhouse assay for biocontrol potential. The antimicrobial activity of the most promising bacterial isolate against the *R. solanacearum* M1 strain was confirmed using actual banana plants in a screenhouse set-up. The treatments were: 1 – Positive control (M1 strain cell suspension), 2 – Negative control (sterile distilled water), 3 – S37 crude extract only (10,000 µg/ml), 4 – S37 whole cell suspension, 5 – M1 combined with S37 crude extract (1:1), and 6 – M1 combined with S37 whole cells (1:1). For the whole cell treatments, the cultures were centrifuged at 8000 *g* to collect the cells, washed with sterile distilled water and the concentration adjusted to 9.0 x 10^8 CFU/ml. Each plant was injected with a 5 ml sample (suspended in sterile water) on the lower part of the pseudostem as described by Ramirez and group (2015) with modifications for treatments 5 and 6. For the 5 and 6 set-ups, 5 ml of the mixture (M1 with S37 extract/cells) were injected for two consecutive days. Each sample was tested on five two-month-old Cavendish banana plants. Injections for all treatments were repeated on the 7^{th}, 14^{th} and 21^{st} days. Symptoms of moko disease were monitored for up to 30 days.

Statistical analysis. The one-way Analysis of Variance (ANOVA) using the Tukey post-hoc test was performed to analyze results for the antimicrobial assay and MIC determination by SPSS Statistics v.23.0 (IBM Corp., USA).

RESULTS AND DISCUSSION

Morphological, biochemical and physiological characteristics. The results of the morphological, biochemical and physiological tests performed on the four locally isolated *R. solanacearum* strains are

presented in Table 1. The tests include Gram reaction, oxidase, catalase, utilization of carbohydrates and optimum growth temperature. The *R. solanacearum* strains M1, M2, M3 and M4 were Gram-negative, rod-shaped and oxidase- and catalase-positive which are important characteristics of the genus *Ralstonia*. All four strains were not capable of utilizing disaccharides (cellobiose, lactose and maltose) and hexose alcohols (dulcitol, mannitol and sorbitol). Their sugar utilization profiles suggest that they belong to biovar 1 which causes moko disease. The strains grow only at 30° to 37°C, and are thus mesophilic. This indicates that they can proliferate in tropical regions.

Table 1. Morphological, biochemical and physiological characteristics of *R. solanacearum* M1, M2, M3 and M4 strains.

TESTS	M1	M2	M3	M4
Gram-reaction	Gram-negative	Gram-negative	Gram-negative	Gram-negative
Cell shape	Rods	rods	rods	rods
Oxidase	+	+	+	+
Catalase	+	+	+	+
Cellobiose	-	-	-	-
Lactose	-	-	-	-
Maltose	-	-	-	-
Dulcitol	-	-	-	-
Mannitol	-	-	-	-
Sorbitol	-	-	-	-
Growth at 4°C	-	-	-	-
Growth at 30°C	+	+	+	+
Growth at 37°C	+	+	+	+
Growth at 45°C	-	-	-	-

+ = positive reaction; - = negative reaction

Pathogenicity of the *R. solanacearum* strains. The four test strains (M1, M2, M3 and M4) produced pinkish mucoidal colonies on KA supplemented with TZC with varying colony sizes and consistencies. The KA medium is particularly useful in distinguishing virulent wild-type *R. solanacearum* strains from avirulent mutant ones. Colonies of virulent strains on KA are white with pink centers and fluidal whereas those of the avirulent strains are smaller, rounder, a darker red with a drier consistency. The cultural characteristics of the four test strains indicate virulence (Figure 1). This has been confirmed by the pathogenicity test using Cavendish banana plant as the host (Figure 2). The leaves of the test plants started to exhibit yellowing two weeks after inoculation and eventually wilted by the 30th day. In addition, their pseudostems turned soft and wet due to the oozing of bacterial growth. The most pronounced symptoms of bacterial wilt were observed for the M1 strain which implied that it was the most pathogenic strain of the four. M1 was followed by M2, M3 then M4 in decreasing virulence. On the other hand, the uninoculated control set-up remained green and healthy after incubation.

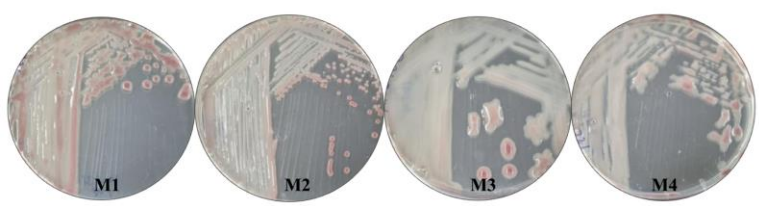

Fig. 1. Colonies of *R. solanacearum* M1, M2, M3 and M4 on KA with TZC after incubation at 30°C for 24 h.

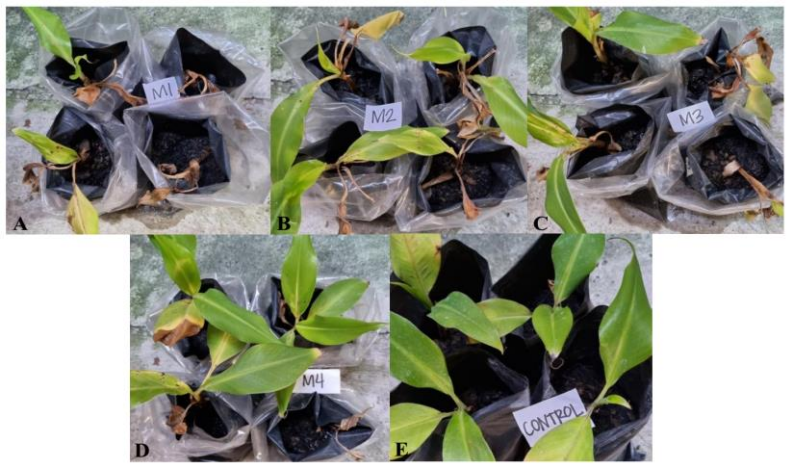

Fig. 2. Wilting of Cavendish banana plants inoculated with *R. solanacearum* M1, M2, M3 and M4 (A,B,C and D) after 30 days. A healthy banana plant in the negative control set-up (E).

Antimicrobial activity of the mangrove bacterial isolates (*in vitro*). Out of 55 bacterial extracts tested, only three exhibited antagonistic activity against the four pathogenic *R. solanacearum* strains. The three mangrove isolates designated as S31, S37 and S38 produced ZOIs against *R. solanacearum* M1, M2, M3 and M4 ranging from 9.50 ± 0.5 to 28.2 ± 0.3 mm in diameter (Table 2). Isolate S37 exhibited the highest ZOI among the three mangrove bacteria which is significantly higher than S31 and S38 against all four *R. solanacearum* strains. The most susceptible *R. solanacearum* strain was M3 where the ZOIs of the extract measured up to 28.2 ± 0.3 mm in diameter. This ZOI is about three times higher than the report of Hasinu et al. (2021) in his study on the antimicrobial activity of his *Bacillus subtilis* strains against *R. solanacearum* bacterial wilt of banana.

Table 2. Antimicrobial activity of S31, S37 and S38 extracts against *Ralstonia solanacearum* M1, M2, M3 and M4 determined by the cylinder plate assay.

SAMPLE	Diameter of zone of inhibition (mm)			
	M1	M2	M3	M4
S31	9.50 ± 0.5b	10.3 ± 0.6c	11.2 ± 0.3c	10.2 ± 0.3c
S37	16.8 ± 1.0a	21.0 ± 1.0a	28.2 ± 0.3a	25.3 ± 0.6a
S38	10.5 ± 0.5b	14.0 ± 0.0b	15.5 ± 0.5b	11.5 ± 0.5b
Positive Control	20.2 ± 0.8	25.3 ± 0.6	30.3 ± 0.6	27.7 ± 0.6
Negative Control	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0

Values that are not significantly different are marked with the same letter ($\alpha = 0.05$, Tukey test).

Minimum Inhibitory Concentration (MIC). Results of the microtiter plate broth dilution assay confirmed that the S37 extract significantly had the best activity among the three bacteria against the four pathogenic *R. solanacearum* strains. Its MIC ranged from 78.12 ± 0.0 to 312.5 ± 0.0 µg/ml. As shown in Table 3, the MIC of S37 against M3 was significantly low and very promising compared to S31 and S38 when tested against all four *R. solanacearum* strains. It is at least 16 times more potent than the other two extracts when tested against the most virulent strain of *R. solanacearum* (M1) having an MIC of only 312.5 ± 0.0 µg/ml. Its MIC against M3 is only 78.12 ± 0.0 µg/ml. Isolate S31 had the highest MIC (≥ 5,000 ± 0.0 µg/ml) and thus the weakest antimicrobial activity. The MIC of S38 ranged from 2,500 ± 0.0 to 5,000 ± 0.0 µg/ml.

Table 3. Minimum Inhibitory Concentration (MIC) of S31, S37, S38 against *Ralstonia solanacearum* M1, M2, M3 and M4 using the broth micro-dilution method.

SAMPLE	MIC (µg/ml)			
	M1	**M2**	**M3**	**M4**
S31	> 5000 ± 0.0c	> 5000 ± 0.0c	5000 ± 0.0c	> 5000 ± 0.0c
S37	312.5 ± 0.0a	156.2 ± 0.0a	78.12 ± 0.0a	156.2 ± 0.0a
S38	5000 ± 0.0b	5000 ± 0.0b	2500 ± 0.0b	2500 ± 0.0b
Positive Control	≤ 39.06 ± 0.0	≤ 39.06 ± 0.0	≤ 39.06 ± 0.0	≤ 39.06 ± 0.0
Negative Control	NA	NA	NA	NA

NA = No antimicrobial activity

Characteristics and identities of the three best mangrove bacteria

Isolate S31. S31 is a Gram-positive rod-shaped spore-forming bacterium. It produces cream to beige small, circular, raised, opaque colonies with entire margin on MA. Based on the results of VITEK 2 Compact identification using BCL card (Table 4), it was able to produce arylamidases (five types), beta-xylosidase, beta-galactosidase, alpha-mannosidase and beta-glucosidase. It utilizes D-mannose, D-tagatose, D-trehalose, D-glucose and D-ribose. S31 can grow at 6.5% NaCl and can also hydrolyze esculin. The VITEK 2 system identified S31 as *Bacillus pumilus* (96% similarity) based on phenotypic characteristics. The pairwise dDDH (85.8%) and ANI (98.4%) analyses identify S31 as *B. altitudinis*. The generally accepted species boundary for dDDH and ANI values are 70.0% and 95.0%, respectively (Chun et al. 2018; Goris et al. 2007). Results in comparison with the *B. pumilus* type strain were just 36.1% for dDDH and 87.7% for ANI. These results strongly suggest that S31 belongs to *B. altitudinis* and not to *B. pumilus.*

Isolate S37. S37 is also a Gram-positive rod-shaped bacterium capable of producing endospores. Its colonies on MA appear off-white to cream, circular to irregular in form, raised, opaque with undulating margin. This isolate produces alpha-galactosidase, beta-galactosidase, beta-glucosidase and six types of arylamidases. It can utilize D-glucose, D-ribose, inositol, glycogene, maltotriose, D-mannose, palatinose and inulin. It also acidifies methyl-A-D-pyranoside, hydrolyzes esculin and grows at 6.5% NaCl (Table 4). S37 was identified as *Bacillus subtilis* based on phenotypic characteristics (94% similarity). The pairwise dDDH and ANI analyses results between the genomes of S37 and the *B. subtilis* type strain were 89.1% and 98.7%, respectively. Both phenotypic and genotypic methods indicate that S37 belongs to *B. subtilis*.

Isolate S38. This is a spore-forming Gram-positive rod-shaped bacterium, produces off-white to cream, circular, raised, opaque colonies with entire to undulating margin on MA. Its enzymes include beta-N-acetyl-glucominidase, alpha-glucosidase and six types of arylamidases. This bacterium assimilates glycogen, maltotriose, N-acetyl-D-glucosamine, D-trehalose, D-glucose, D-ribose. It likewise hydrolyzes esculin and grows at 6.5% NaCl. S38 is the only bacterial isolate that has resistance to kanamycin, oleandomycin and polymyxin B (Table 4). S38 was identified as *Bacillus thuringiensis* based on phenotypic characteristics (95% similarity). The pairwise dDDH and ANI values between the whole genomes of S38 and the *B. thuringiensis* type strain were 84.7% and 98.6%, respectively. Phenotypic and genotypic analyses agree that S38 belongs to the said species.

As members of the *Bacillus* group, the promising bacteria grow fast and very easy to cultivate in large quantities with very minimal nutritional requirements. This group is known to harbor biosynthetic gene clusters that have been reported to have antimicrobial activities against a wide range of microorganisms. These promising bacteria produce endospores which render them relatively resistant to harsh environmental conditions, and thus will have comparably better survival when

applied in the field. Isolates S31, S37 and S38 were found to belong to non-pathogenic *Bacillus* species and can be safely utilized by humans.

Table 4. Substrate assimilation, enzyme production, antibiotic resistance profile and identity of S31, S37 and S38 determined by the VITEK 2 Compact Identification System (BCL card).

TESTS	S31	S37	S38
Beta- Xylosidase	+	+	-
L-Lysine-Arylamidase	-	-	-
L-Aspartate Arylamidase	(-)	+	+
Leucine Arylamidase	+	+	+
Phenylalanine Arylamidase	+	+	+
L-Proline Arylamidase	-	-	-
Beta-Galactosidase	+	(+)	-
L-Pyrrolidonyl-Arylamidase	-	+	+
Alpha-Galactosidase	-	+	-
Alanine Arylamidase	+	-	+
Tyrosine Arylamidase	+	+	+
Beta-N-Acetyl-Glucosaminidase	-	-	+
Ala-Phe-Pro Arylamidase	-	-	-
Cyclodextrine	-	-	-
D-Galactose	-	-	-
Glycogene	-	+	+
myo-Inositol	-	+	-
Methyl-A-D-Glucopyranoside	-	+	-
Acidification			
Ellman	+	-	+
Methyl-D-Xylosidase	-	-	-
Alpha-Mannosidase	+	-	-
Maltotriose	-	(+)	+
Glycine Arylamidase	+	+	-
D-Mannitol	-	-	-
D-Mannose	+	+	-
D-Melezitiose	-	-	-
N-Acetyl-D-Glucosamine	-	-	+
Palatinose	-	+	-
L-Rhamnose	-	-	-
Beta-Glucosidase	+	+	-
Beta-Mannosidase	-	-	-
Phosphoryl Choline	-	-	-
Pyruvate	+	-	+
Alpha-Glucosidase	-	-	+
D-Tagatose	+	-	-
D-Trehalose	+	-	+
Inulin	-	+	-
D-Glucose	+	+	+
D-Ribose	+	+	+
Putrescine Assimilation	-	-	-
Growth in 6.5% NaCl	+	+	+
Kanamycin Resistance	(-)	-	+
Oleandomycin Resistance	-	-	+
Esculin Hydrolysis	+	+	+
Tetrazolium Red	+	+	(-)
Polymyxin B Resistance	-	-	+
IDENTITY	*Bacillus pumilus* (96%)	*B. subtilis* (94%)	*B. thuringiensis* (95%)

+ = positive reaction; - = negative reaction; (+) = weak positive, reaction slightly below detection threshold; (-) = weak negative, reaction slightly above detection threshold

Secondary metabolites produced by the most promising bacteria. The functional annotation indicated that the genes in S37 encoding biosynthesis of secondary metabolites account for only 1.2% of the genome. About 20% of the genes are responsible for general functions, while around 23% correspond to genes with unknown functions. The rest of the genes of the promising bacteria are involved in vital cellular processes such as transcription, translation, replication, cell wall-, membrane- and envelope biogenesis, motility, energy production, and carbohydrate and amino acid transport.

The antiSMASH analysis predicted a total of nine secondary metabolite biosynthetic gene clusters (smBGCs) from the S37 genome (Table 5). However, only six have been found to encode specific compounds. These include bacillaene, fengycin, bacillibactin, subtilosin A, bacilysin and surfactin.

Table 5. Secondary metabolites detected from the S37 genome by antiSMASH (v.5.0).

Location (Region)	Most similar known cluster	Percent Similarity
1.1	terpene-type	-
1.2	bacillaene	100
1.3	fengycin	93
1.4	terpene-type	-
1.5	T3PKS-type	-
2.1	bacillibactin	100
2.2	subtilosin A	100
2.3	bacilysin	100
3.1	surfactin	78

- = Not determined; T3PKS = Type III Polyketide Synthase

Bacillaene is a polyketide that has been found to exhibit antimicrobial activity against *Staphylococcus aureus* and *Fusarium* spp. (Patel et al. 1995; Um et al. 2013). Fengycin is an NRP lipopeptide which has been reported to have antifungal activity against the plant pathogen *Colletotrichum gloeosporioides* (Kim et al. 2010) and antibacterial activity against the human pathogen *Listeria monocytogenes* (Lin et al. 2020). It has also been reported to inhibit the growth of other pathogenic bacteria such as *Aeromonas hydrophila*, *Xanthomonas axonopidis pv. vesicatoria* and *Pseudomonas aeruginosa* PA01 by producing alterations on the cell surface of the target bacteria (Medeot et al. 2020). Bacillibactin is a siderophore type of NRP that exhibited antimicrobial activity against the plant pathogen *Pseudomonas syringae* pv. *tomato*, and human pathogens *Staphylococcus aureus, Enterococcus faecalis, P. aeruginosa* and *Klebsiella pneumoniae* (Chakraborty et al. 2022; Dimopoulou et al. 2021). Subtilosin A, an antibiotic peptide produced by *B. subtilis*, had antagonistic activity against Gram-positive bacteria such as *S. aureus* and *Streptococcus faecium* (Babasaki et al. 1985). Bacilysin, a type of non-thiotemplate NRP, has been reported to have antagonistic activities against the pathogenic bacteria *Erwinia amylovora* and *Microcystis aeruginosa*, and fungi *Aspergillus fumigatus* and *Candida albicans* (Chen et al. 2009; Milewski et al 1986; Wu et al. 2014). Surfactin is a cyclic lipopeptide that acts as a potent biosurfactant (Santos et al. 2018). It has been explored in the development of animal feed additives as it possesses antibacterial activity against *Clostridium perfringens* and *Brachyspira hyodysenteriae* (Horng et al. 2019). One or more of the six mentioned compounds may be responsible for the antagonistic activity of S37 against the pathogenic *R. solanacearum* strains.

Biological activity of the most promising bacteria (*in planta*). The biological activity of the most promising bacterial isolate, S37, was tested in a screenhouse assay. On Day 0, all Cavendish banana plants from all treatments were healthy (Fig.3 - 1A to 6A). On the 2nd week of incubation, plants

inoculated with the pathogen M1 cell suspension (T1) started to exhibit yellowing and wilting. At 30 days after treatment (DAT), all the plants were completely wilted, whereas the negative control (T2), S37 crude extract only (T3) and S37 cell suspension only (T4) remained alive and healthy (Fig. 3 - 1B to 4B). Three out of five plants inoculated with M1 combined with the S37 crude extract (T5) stayed alive 30 DAT (Fig. 3 – 5B). The two plants exhibited wilting only on the 4th week. Two out of five plants inoculated with M1 and S37 whole cell suspension (T6) remained unwilted at 30 DAT though the plants did not remain as healthy (Fig.3 – 6B). The three plants wilted only on the 4th week. This preliminary study showed the potential of S37 as a possible biocontrol agent against moko disease of banana as shown in the results from treatments 5 and 6.

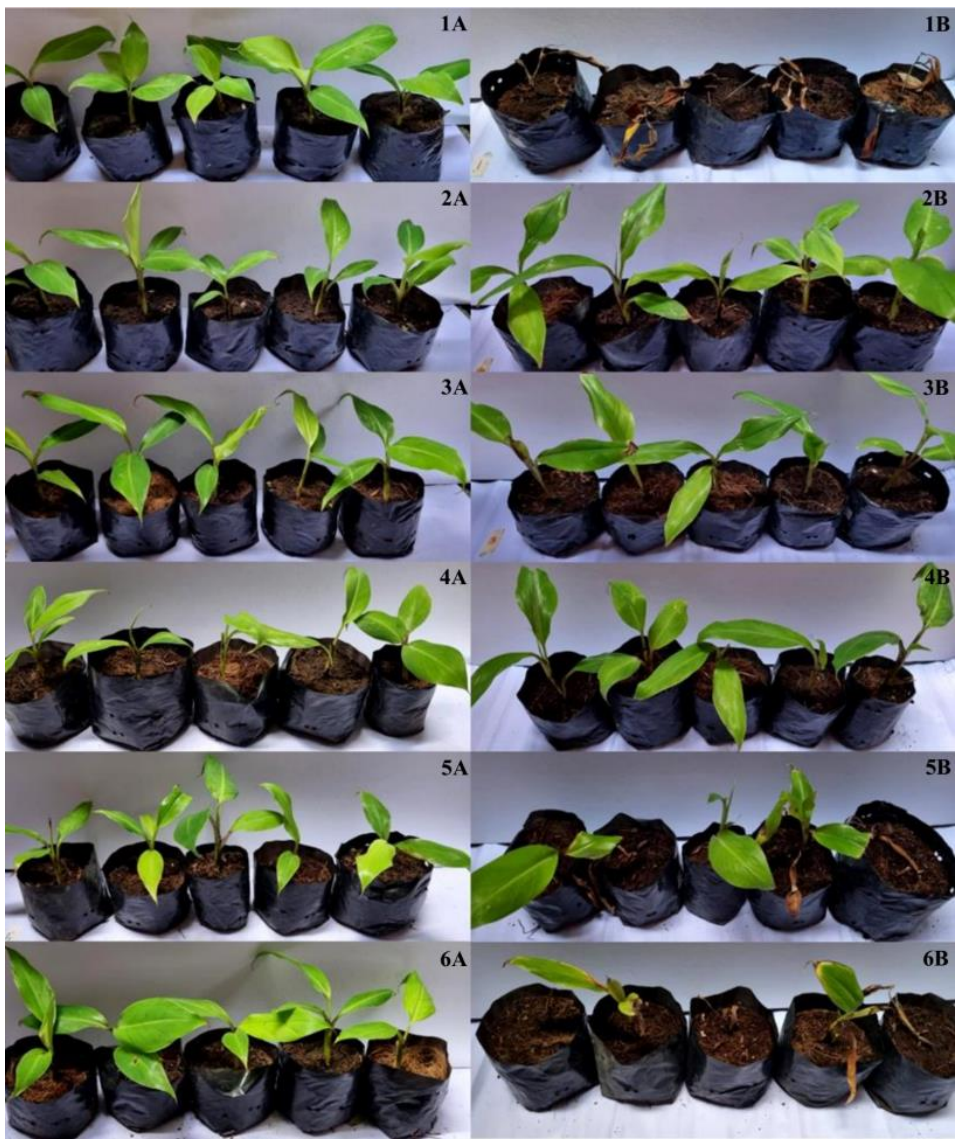

Fig. 3. Effect of the six screenhouse treatments on the Cavendish banana test plants: (1A to 6A) appearance of plants at Day 0 and (1B to 6B) condition of the same plants 30 DAT.

CONCLUSION

The mangrove bacterial isolates, designated as S31 (*Bacillus altitudinis*), S37 (*B. subtilis*) and S38 (*B. thuringiensis*), may be considered potential biological agents to control moko disease-causing *R. solanacearum* due to their non-pathogenicity, short generation times, non-fastidious nature, spore-forming ability thus more hardy compared to non-sporeformers, and capacity to produce antimicrobial metabolites. The bacterium with the strongest activity against the pathogenic *R. solanacearum* strains, S37, may be harnessed to develop a new microbial-based product that can help suppress moko disease in banana. Its biocontrol potential, however, will have to be tested in the field. The proper concentration/formulation and time of application will have to be studied. Having six antimicrobial secondary metabolites, the wider application of isolate S37 against other important plant disease-causing microorganisms, especially *Fusarium oxysporum* TR 4 which is also a big problem in the banana industry not only in the Philippines, will be explored in future studies. These isolates will be available for stakeholders in the country to acquire as they have been deposited at the PNCM culture collection.

ACKNOWLEDGEMENT

The study is a portion of the Ph.D. dissertation of A.R. Creencia. The authors would like to extend their gratitude to the Department of Science and Technology (DOST) Accelerated Science and Technology Human Resource Development Program – National Science Consortium (ASTHRDP-NSC) Scholarship for funding the research; the Palawan Council for Sustainable Development (PCSD) and the Protected Area Management Board (PAMB) for granting the sampling permit; Lapanday Banana Plantation in Davao, Philippines for providing the *R. solanacearum* test strains; the National Plant Genetic Resources Laboratory and the Plant Pathology Laboratory of the Institute of Plant Breeding (IPB), UPLB for providing the tissue-cultured banana plants and assisting in the pathogenicity testing, respectively; the Antibiotics Laboratory of BIOTECH, UPLB for providing the rotary evaporator; and the PNCM of BIOTECH, UPLB for providing the laboratory chemicals, supplies and other equipment.

REFERENCES CITED

Alvarez, E., A. Pantoja, L. Gañan and G. Ceballos 2015. Current status of moko disease and black sigatoka in Latin America and the Caribbean, and options for managing them. Colombia: Centro Internacional de Agricultura Tropical (CIAT). 50 pp.

Babasaki, K., T. Takao, Y. Shimonishi and K. Kurahashi. 1985. Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: Isolation, structural analysis, and biogenesis. J. Biochem. 98(3):585-603. DOI: 10.1093/oxfordjournals.jbchem.a135315.

Blin K., S. Shaw, K. Steinke, R. Villebro, N. Ziemert, S.Y. Lee, M.H. Medema and T. Weber. 2019. antiSMASH 5.0: Updates to the secondary metabolite genome mining pipeline. Nucleic Acids Res. 47(W1):W81-W87.

Blomme, G., M. Dita, K. Jacobsen, L. Perez Vicente, A. Molina, W. Ocimati, S. Poussier and P. Prior. 2017. Bacterial diseases of bananas and enset: Current state of knowledge and integrated approaches toward sustainable management. Front. Plant Sci. 8:1290. DOI: 10.3389/fpls.2017.01290.

Bouhdid, S., J. Abrini, M. Amensour, A. Zhiri, M.J. Espuny and A. Manresa. 2010. Functional and ultrastructural changes in *Pseudomonas aeruginosa* and *Staphylococcus aureus* cells induced

by *Cinnamomum verum* essential oil. J. Appl. Microbiol. 109(4):1139-1149. DOI: 10.1111/j.1365-2672.2010.04740.x.

Buddenhagen, I. and A. Kelman. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopath. 2:203-230.

CAB International. 2014. Invasive species compendium: *Ralstonia solanacearum* Race 2. https://www.cabi.org/isc/datasheet/44999/aqb/. Accessed 10 February 2022.

Chakraborty, K., V.K. Kizhakkekalam, M. Joy and R.D. Chakraborty. 2022. Bacillibactin class of siderophore antibiotics from a marine symbiotic *Bacillus* as promising antibacterial agents. Appl. Microbiol. Biotechnol. 106(1):329-340. DOI: 10.1007/s00253-021-11632-0.

Chen, X.H., R. Scholz, M. Borriss, H. Junge, G. Mogel, S. Kunz and S. Borriss. 2009. Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* are efficient in controlling blight disease. J. Biotechnol. 140(1-2):38-44. DOI: 10.1016/j.jbiotec.2008.10.015.

Chun, J., A. Oren, A. Ventosa, H. Christensen, D. Arahal, M. da Costa, A. Rooney, H. Yi, X. Xu, S. De Meyer and M. Trujillo. 2018. Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. Int. J. Syst. Evol. Microbiol. 68:461-466. DOI: 10.1099/ijsem.0.002516.

Dadrasnia, A., M.M. Usman, R. Omar, S. Ismail and R. Abdullah. 2020. Potential use of *Bacillus* genus to control of banana diseases: Approaches toward high yield production and sustainable management. Science. 32:2336-2342. DOI:10.1016/j.jksus.2020.03.011.

Department of Agriculture (DA). 2018. Philippine banana industry roadmap 2019-2022. https://www.da.gov.ph/wp-content/uploads/2019/06/Philippine-Banana-Industry-Roadmap-2019-2022.pdf/. Accessed 10 May 2022.

Dimopoulou, A., I. Theologidis, D. Benaki, M. Koukounia, A. Zervakou, A. Tzima, G. Diallinas, D.G. Hatzinikolaou and N. Skandalis. 2021. Direct antibiotic activity of bacillibactin broadens the biocontrol range of *Bacillus amyloliquefaciens* MBI600. mSphere. DOI: 10.1128/mSphere.00376-21.

FAOSTAT. 2014. Banana and plantain surface and production in 2013. https://faostat.fao.org/site/339/default.aspx. Accessed 10 February 2022.

Goris, J., K.T. Konstantinidis, J.A. Klappenbach, T. Coenye, P. Vandamme and J.M. Tiedje. 2007. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int. J. Syst. Evol. Microbiol. 57(Pt 1):81-91. DOI: 10.1099/ijs.0.64483-0.

Hasinu, J.V., G.N.C. Tuhumury and H. Kesaulya. 2021. Potential of *Bacillus* spp. as a biocontrol agent against *Ralstonia* bacterial wilt in bananas. IOP Conf. Ser.: Earth Environ. Sci. 883:012039. DOI: 10.1088/1755-1315/883/1/012039.

Hayashida-Soiza, G., A. Uchida, N. Mori, Y. Kuwahara and Y. Ishida. 2008. Purification and characterization of antibacterial substances produced by a marine bacterium *Pseudoalteromonas haloplanktis* strain. J. Appl. Microbiol. 105(5):1672-1677. DOI: 10.1111/j.1365-2672.2008.03878.

Hayward, A.C. 1994. The hosts of *Pseudomonas solanacearum*. In: Hayward, A.C. and G.L. Hartman (eds). Bacterial wilt: the disease and its causative agent *Pseudomonas solanacearum*. Wallingford, UK: CAB International. p. 9-24.

Horng, Y.B., Y.H. Yu, A. Dybus, F.S. Hsiao and Y.H. Cheng. 2019. Antibacterial activity of *Bacillus* species-derived surfactin on *Brachyspira hyodysenteriae* and *Clostridium perfringens*. AMB Express. 9(1):188. DOI: 10.1186/s13568-019-0914-2.

Hudzicki, J. 2009. Kirby-Bauer disk diffusion susceptibility test protocol. https://asm.org. Accessed 10 February 2022.

Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology. 44:693-695.

Kim, P.I., J. Ryu, Y.H. Kim and Y.T. Chi. 2010. Production of biosurfactant lipopeptides iturin A, fengycin and surfactin A from *Bacillus subtilis* CMB32 for control of *Colletotrichum gloeosporioides*. J. Microbiol. Biotechnol. 20:138-145. DOI: 10.4014/jmb.0905.05007.

Lin, L.Z., Q.W. Zheng, T. Wei, Z.Q. Zhang, C.F. Zhao, H. Zhong, Q.Y. Xu, J.F. and L.Q. Guo. 2020. Isolation and characterization of fengycins produced by *Bacillus amyloliquefaciens* JFL21 and its broad-spectrum antimicrobial potential against multidrug-resistant foodborne pathogens. Front. Microbiol. 11:579621. DOI: 10.3389/fmicb.2020.579621.

Medeot, D.B., M. Fernandez, G.M. Morales and E. Jofre. 2020. Fengycins from *Bacillus amyloliquefaciens* MEP_218 exhibit antibacterial activity by producing alterations on the cell surface of the pathogens *Xanthomonas axonopodis* pv. *vesicatoria* and *Pseudomonas aeruginosa* PA01. Front. Microbiol. 10:3107. DOI: 10.3389/fmicb.2019.03107.

Meier-Kolthoff J.P. and M. Goker. 2019. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. Nat. Commun. 10:2182. DOI: 10.1038/s41467-019-10210-3.

Milewski, S., H. Chmara and E. Borowski. 1986. Antibiotic tetaine-a selective inhibitor of chitin and mannoprotein biosynthesis in *Candida albicans*. Arch. Microbiol. 145(3):234-240. DOI: 10.1007/BF00443651.

Molina, A.B. 1999. Fruit rot diseases of cooking banana in Southeast Asia. Infomusa. 8:29-30.

Molina, A.B. 2006. Managing bacterial wilt/fruit rot disease of banana in Southeast Asia. In: Karamura, E., M. Osiru, G. Blomme, C. Lusty and C. Picq (eds). Proceedings of the banana *Xanthomonas* wilt regional preparedness and strategy development workshop held in Kampala: Developing a regional strategy to address the outbreak of banana *Xanthomonas* wilt in East and Central Africa. p. 26-31.

Patel, P.S., S. Huang, S. Fisher, D. Pirnik, C. Aklonis, L. Dean, E. Meyers, P. Fernandes and F. Mayerl. 1995. Bacillaene, a novel inhibitor of procaryotic protein synthesis produced by *Bacillus subtilis*: production, taxonomy, isolation, physico-chemical characterization and biological activity. J. Antibiot (Tokyo). DOI: 10.7164/antibiotics.48.997.

Pava, H.M., N.F. Franje and T.J. Timario. 2003. Banana pilot demonstration studies for bukidnon: Table salt and early debudding to control "Bugtok" disease of cooking banana cultivars "Saba" and "Cardaba". Philipp. J. Crop Sci. 28:31-43.

Philippine Statistics Authority (PSA). 2016-2020. Crops Statistics of the Philippines. https://psa.gov.ph/sites/default/files/Crops%20Statistics%20of%20the20Philippines%202016-2020.pdf/. Accessed 08 May 2022.

Ramirez, J.G., M.A. Muñoz, L.H. Patiño and J.O. Morales. 2015. Banana moko disease management with resistance inducers and chlorine dioxide. Agron. Colomb. 33(2):194-202. DOI: 10.15446/agron/colomb.v33n2.48663.

Raymundo, A.K. 2001. The bacterial wilt organism: The crop nemesis with a new twist. Trans. Natl. Acad. Sci & Tech. 23:131-147.

Rodriguez, L.M. and K.T. Konstantinidis. 2016. The enveomics collection: A toolbox for specialized analyses of microbial genomes and metagenomes. PeerJ Preprints. 4:e1900v1. DOI: 10.7287/peerj.preprints.1900v1.

Santos, V.S.V., E. Silveira and B.B. Pereira. 2018. Toxicity and applications of surfactin for health and environmental biotechnology. J. Toxicol. Environ. Health B. Crit. Rev. 21(6-8):382-399. DOI: 10.1080/10937404.2018.1564721.

Seeman T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 30(14): 2068-9.

Thiribhuvanamala, G., S. Nakkeeran and K.E. Arutkani Aiyanathan. 2018. Major disease of horticultural crops and this management. https://agritech.tnau.ac.in. Accessed 10 February 2022.

Thwaites, R., S.J. Eden-Green and R. Black. 2000. Diseases caused by bacteria. In: Jones, D.R. (ed). Diseases of Banana, Abaca and Enset. Wallingford, UK: CAB International. p. 213-239.

Um, S., A. Fraimout, P. Sapountzis, D. Oh and M. Poulsen. 2013. The fungus-growing termite Macrotermes natalensis harbours bacillaene-producing *Bacillus* sp. that inhibit potentially antagonistic fungi. Sci. Rep. DOI: 10.1038/srep03250.

Wu, L. H. Wu, L. Chen, S. Xie, H. Zang, R. Borriss and X. Gao. 2014. Bacilysin from *Bacillus amyloliquefaciens* FZB42 has specific bactericidal activity against harmful algal bloom species. Appl. Environ. Microbiol. 80(24):7512-7520. DOI: 10.1128/AEM.02605-14.

Yuliar, Y.A. Nion and K. Toyota. 2015. Recent trends in control methods for bacterial wilt diseases caused by *Ralstonia solanacearum*. Microbes Environ. 30(1):1-11. DOI: 10.1264/jsme2.ME14144.