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Endophyte *Bacillus velezensis* Isolated from *Citrus* spp. Controls Streptomycin-Resistant *Xanthomonas citri* subsp. *citri* That Causes Citrus Bacterial Canker

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Abstract: Citrus bacterial canker (CBC), caused by the plant pathogenic bacterium *Xanthomonas citri* subsp. *citri* (*Xcc*), is a devastating disease in many commercial citrus cultivars. Every year, CBC causes a substantial reduction in fruit quality and quantity that corresponds to significant economic losses worldwide. Endophytic microorganisms produce numerous bioactive secondary metabolites that can control plant pathogens. We investigated the antagonistic activities of 66 endophytic bacteria isolated from nine citrus cultivars to control streptomycin-resistant *Xcc*. The suspension of Endophytic Bacteria-39 (EB-39), identified as *Bacillus velezensis*, exhibited the highest antibacterial activity against three wild-type and six streptomycin-resistant *Xcc* strains, with the inhibition zones between 39.47 ± 1.6 and 45.31 ± 1.6 mm. The ethyl acetate extract of EB-39 also controlled both wild-type and streptomycin-resistant *Xcc* strains, with the inhibition zones between 29.28 ± 0.6 and 33.88 ± 1.3 mm. Scanning electron microscopy indicated the ethyl acetate extract of EB-39-induced membrane damage and lysis. The experiments using the detached leaves of a susceptible *Citrus* species showed that EB-39 significantly reduced the incidence of canker on the infected leaves by 38%. These results strongly suggest that our newly isolated EB-39 is a novel biocontrol agent against CBC caused by wild-type and streptomycin-resistant *Xcc* strains.

Keywords: *Bacillus velezensis*; citrus bacterial canker; endophyte; streptomycin-resistance

1. Introduction

Citrus species are fruit crops that can be found worldwide, and they have high value in both fresh and processed fruit industries [1]. Because of the increasing demand for citrus fruits and juices, the global production of citrus fruit for 2018–2019 was forecast to expand from 4.2 million metric tons in the previous year to 51.8 million metric tons as favorable weather leads to larger crops in Brazil and the United States [2]. Citrus fruits are very popular worldwide for their flavor, and they are the source of secondary metabolites, such as flavonoids with anti-inflammatory and antimicrobial activities. Flavonoids, such as hesperetin, naringenin, eriodictyol, and isosakuranetin, have been investigated for their possible roles in the prevention of life-threatening cardiovascular diseases and cancer [3].

Citrus bacterial canker (CBC) is the most severe and contagious disease of *Citrus* spp. and cultivars [4–6]. The severity of this disease was first reported around 1912 in Florida, and it spread throughout the U.S. as a result of imported seedlings from Japan [4]. To date, three distinct types of CBC have been reported—types A, B, and C—and they are caused by different pathovars and variants of the strain *Xanthomonas* [4,7,8]. CBC type A (Asiatic type of canker) caused by *X. citri* subsp. *citri* (*Xcc*), previously named *X. axonopodis* pv. *citri*, is the most destructive and widespread form of the disease in *C. paradisi* (grapefruit) and *C. aurantifolia* (Mexican lime) [8]. CBC types B and C are caused by *X. fuscans*

subsp. *aurantifolii* strain B and *X. fuscans* subsp. *aurantifolii* strain C, respectively [6]. CBC type B was first identified in Argentina in 1923, and the disease symptoms were observed in lemon and orange; CBC type C is restricted to the São Paulo state in Brazil, and it infects Mexican limes [7]. Because the symptoms of the three CBC types are almost similar, the disease type is only distinguishable by examining the phenotypic and genotypic characteristics of the *Xanthomonas* strains [4].

The genus *Xanthomonas* (family Xanthomonadaceae) is composed of 27 species, which collectively cause serious diseases in ≈400 plant species, including citrus, rice, tomato, cabbage, and bean [9]. During the infection process, *Xanthomonas* enters the host through the hydathodes or wounds, and it colonizes and multiplies in the mesophyll parenchyma and apoplastic regions of the host [9]. The virulence factors encoded by *Xanthomonas* include secretion systems (i.e., type III secretion system genes), effectors, cell-wall degrading enzymes (i.e., pectate lyase, polygalacturonase), toxins, and bacterial adhesins, which collectively cause tissue maceration, electrolyte loss, and cell death in the host plant [10].

CBC is characterized by the development of small necrotic lesions on the lower surface of the leaf that expand over time because of hyperplasia (an increase in the number of cells) and hypertrophy (enlargement of an organ) [11]. With time, the lesions become brown with oily water-soaked margins usually surrounded by a yellow chlorotic halo that become visible on the upper surface of the leaf. Lesions on young stems and fruits are generally raised and corky and sometimes open like a blister or volcano (erumpent) [5,12]. In a susceptible citrus host, the disease caused by *Xanthomonas* results in defoliation, premature fruit abscission, and shoot dieback, triggering a significant decrease in the crop yield and fruit marketability [4]. CBC is mostly transmitted through contaminated seeds, soils, insects, and possibly agricultural practices [9]. In addition, an orchard in Florida produced the first documented evidence of CBC spreading over longer distances because of high winds and heavy rainfall during a thunderstorm in 1990 [4,13].

For many years, the most common approaches to control CBC were the eradication of symptomatic citrus trees to prevent the spread of the pathogens [4]. However, some citrus producers rely on less susceptible cultivars planted in *Xanthomonas*-free nurseries. Spraying of copper-based bactericides (i.e., copper hydroxide, copper oxychloride, and copper oxide) has been used for more than two decades to control CBC [11]. Unfortunately, frequent use of these bactericides to control the pathogenic strains of *Xanthomonas* spp. has led to the development of copper-resistant strains [14,15]. Besides, copper-based bactericides leave copper residuals on the plants and cultivated soils, with potential phytotoxic and adverse environmental effects that eventually lead to an increase in the cost of production [16]. Streptomycin sulfate is most widely used to control CBC caused by *Xanthomonas* [17]. In addition to the advent of streptomycin-resistant strains due to the frequent spraying of streptomycin, streptomycin has been banned by European authorities because of the risks associated with the development of antibiotic resistance in non-target microbes [18]. CBC continues to spread, and approximately 12 million USD is spent on the control of CBC every year [12]; there is a pressing need to find alternative methods to control CBC.

Recently, endophytic microorganisms as biocontrol agents are gaining attention over chemical bactericides regarding the control of plant diseases [19,20]. Endophytes are preferred for controlling pathogenic microbes because they are environment-friendly and versatile in their mode of action [21,22]. Nearly 300,000 plant species exist on Earth, and each of them is the host of one or more endophytes [23]. The term "endophyte" was first coined by De Bary in 1866, and he defined endophytes as a group of microorganisms, often bacteria or fungi, that colonize intracellular locations of healthy plants without displaying any external sign of infection in the host [24,25]. However, some bacteria classified as endophytes seem to act as latent pathogens that participate in host plant infection under favorable conditions [26]. Endophytic microorganisms can promote plant growth, defense, and disease resistance by synthesizing antimicrobial molecules and phytohormones or activating host plant immunity through induced systemic resistance (ISR) and systemic acquired resistance (SAR) [20,27].

Several *Bacillus* spp., in particular, *B. subtilis*, *B. amyloliquefaciens* FZB42, *B. amyloliquefaciens* subsp. *plantarum*, *B. methylotrophicus*, *B. oryzicola*, and *B. velezensis*, have been reported for their biocontrol ability against a wide range of phytopathogenic microorganisms, for example, *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* [28], *Erwinia amylovora* [18], *Botrytis cinerea* [29], *Acidovorax oryzae* [30], *Rhizoctonia solani* [31], *Ralstonia solanacearum* [27], and *Fusarium graminearum* [32]. *Bacillus*-based products are now commercially available, for example, RhizoVital® (*B. amyloliquefaciens* FZB42; ABiTEP, GmbH, Berlin, Germany), Amylo-X® WG (*B. amyloliquefaciens* subsp. *plantarum* D747; Certis Europe BV, Netherlands), RhizoPlus® (*B. subtilis* FZB24; ABiTEP), Sonata® (*B. pumilus* QST2808; AgraQuest, Inc., Davis, CA, USA), and Taegro® (*B. subtilis* var. *amyloliquefaciens* FZB24; Novozymes Biologicals, Inc., Salem, VA, USA) [33].

Recent phylogenomic studies based on RNA polymerase beta-subunit (*rpoB*) gene sequences and core genome sequences of bacterial strains suggested that *B. velezensis* is synonymous with *B. amyloliquefaciens* subsp. *plantarum*, *B. amyloliquefaciens* FZB42, *B. methylotrophicus*, and *B. oryzicola* [34–36]. *B. velezensis* is considered a microbial factory for the production of diverse biologically active secondary metabolites, such as cyclic lipopeptides (i.e., surfactin, bacillomycin-D, fengycin, and bacillibactin) and polyketide molecules (i.e., macrolactin, bacillaene, and difficidin) synthesized by modularly organized mega-enzymes, known as non-ribosomal peptide synthetases and polyketide synthases, respectively [21,32]. Besides, this bacterium is capable of synthesizing bacilysin, a non-ribosomally synthesized dipeptide antibiotic, and ribosomally synthesized modified peptide antibiotics plantazolicin (bactericidal and nematicidal molecules) [37] and amylocyclicin (bactericidal) [38].

In this study, we searched for effective endophytic bacteria from different *Citrus* spp. to control *Xcc*. We isolated an endophytic *B. velezensis* that can control the wild type and even streptomycin-resistant *Xcc* strains. To our knowledge, this is the first report of *B. velezensis* displaying an antibacterial effect against *Xcc* pathogens that cause CBC.

2. Materials and Methods

2.1. Plant Materials

Nine 3-year-old *Citrus* spp. (Table 1) were purchased from the Jeju Hanla Farm (Jeju, Republic of Korea), planted in pots (18 cm × 20 cm), and grown under greenhouse conditions. Fully expanded fresh leaves were collected for the isolation of endophytic bacteria. The leaves of Hwanggeum hyang, which is susceptible to CBC, were used for pathogen infection experiments in a detached leaf assay.

2.2. Bacterial Strains and Growth Conditions

Endophytic bacteria isolated from *Citrus* spp. were routinely grown on yeast-extract nutrient agar (YNA; 5 g yeast extract, 8 g nutrient broth, and 15 g agar per liter). These isolates were used for the antibacterial assay against nine *Xcc* pathogens: three were wild type (*Xcc*W1 (*Xcc* 19-18), *Xcc*W2 (*Xcc* 10-5), and *Xcc*W3 (*Xcc* 53-4)) and six were streptomycin-resistant mutants (*Xcc*M4 (*Xcc* 27-9), *Xcc*M5 (*Xcc* 8-4), *Xcc*M6 (*Xcc* 57-2), *Xcc*M7 (*Xcc* 57-5), *Xcc*M8 (*Xcc* 27-13), and *Xcc*M9 (*Xcc* 27-12)) [39]. All these strains were kindly provided by Dr. Hyun of the Citrus Research Station (NIHH, RDA, Jeju, Republic of Korea). The wild-type *Xcc* strains were grown in YNA, and the mutant streptomycin-resistant *Xcc* strains were grown in YNA with 50 mg mL^{-1} streptomycin for selective growth. For long-term storage, the bacterial cultures were maintained at −80 °C in YNB (5 g yeast extract and 8 g nutrient broth per liter) with 35% glycerol.

2.3. Isolation of Endophytic Bacteria

The endophytic bacteria were isolated according to previously described standard procedures [40]. Briefly, healthy and disease-free leaves were collected from the plants and washed thoroughly with sterile distilled water (SDW) to remove adherent soil particles and microbes. The samples were then

dried with tissue paper, weighed with a digital balance, and surface-sterilized with 70% ethanol for 1 min, 2% sodium hypochlorite (NaOCl) solution for 3 min, and 100% ethanol for 30 s. The samples were washed three times with SDW and dried using sterilized filter paper. The surface-sterilized samples were ground to a slurry with a sterilized mortar and pestle in the ratio of 1 gm fresh weight per 3 mL NaCl solution (0.9%). The tissue extracts were then incubated for 3 h at 28 °C for the complete release of the endophytic bacteria, homogenized, and serially diluted up to 10^{-2} with sterilized NaCl solution (0.9%). For each dilution, 50 µL suspensions were spread on 25% YNA (1.25 g yeast extract, 2 g nutrient broth, and 15 g agar per liter) with a stainless-steel spreader and incubated at 28 °C for 15 days. The colonies with a different morphology and color were selected and purified by sub-culture under aseptic conditions. The morphological characteristics of the isolated strains, such as form, margin, color, and elevation of the colonies, were recorded. A total of 66 endophytic bacteria were obtained from the nine *Citrus* spp. (Table 1).

2.4. Primary Screening of the Antagonistic Bacterial Isolates Against Xcc

All the endophytic bacterial isolates were screened for their antibacterial activity against the *Xcc*W1 strain, according to a previously described method [41] with minor modifications. Briefly, all 66 isolates were cultured in 96-well microtiter plates containing 190 µL YNB and 10 µL individual bacterial strains and incubated in a rotary shaker for 48 h at 28 °C and 100 rpm. Subsequently, 10 µL individual isolates were transferred onto square plates containing YNA and incubated for 36 h at 28 °C. The patches of endophytic bacteria grown on the square plates were killed with chloroform; subsequently; the plates were uncovered for 20 min to remove any traces of the chloroform residue. The petri plates were then exposed to UV radiation for 15 min. Finally, the plates were overlaid with 10 mL soft agar (0.75% agar in YNA) pre-inoculated with a 25 µL *Xcc*W1 strain grown overnight. After further incubation at 28 °C for 24 h, the overlaid plates were checked for any zones of inhibition against the test pathogen. The diameters of the zones of inhibition were measured using an electronic digital caliper. Based on the activity against *Xcc*W1, the isolates were subjected to further analysis against the three wild-type and six streptomycin-resistant strains of *Xcc*.

2.5. Phylogenetic Analyses of 16S rRNA Gene Sequences

Out of the 66 isolates, three endophytic bacterial isolates (EB-35, EB-39, and EB-44) exhibited antibacterial activity against *Xcc* strains. These isolates were identified using 16S rRNA sequence analysis. The 16S rRNA sequences were compared using Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database. The phylogenetic analysis was performed using the neighbor-joining method in the MEGA program (https://www.megasoftware.net/). The phylogenetic tree was inferred from the type strains of the species related to *Bacillus* spp. (Figure 1 and Figure S1). Bootstrap replication (1000) was used to statistically analyze the nodes in the phylogenetic tree.

2.6. Antagonistic Activities of Ethyl Acetate Extracts of the Endophytic Bacteria Against Xcc

Metabolites were extracted according to a previously described procedure [42]. Ethyl acetate was selected for the metabolite extraction process because of its low boiling point and moderate polarity. The seed cultures (5 mL YNB) of EB-35, EB-39, and EB-44 were transferred to 200 mL YNB in 0.5 L Erlenmeyer flasks, cultured in a rotary shaker at 180 rpm and 28 °C, and allowed to grow for 36–48 h until the OD_{600nm} reached 1. An equal volume of ethyl acetate was added to the bacterial cultures, and the flasks were sonicated for 5 min and maintained overnight with vigorous shaking. The culture broth was centrifuged at 3500 rpm for 15 min at 4 °C, and the supernatant was collected and dried in a rotary evaporator (A-1000S; Eyela, Tokyo, Japan) at 50 °C. The residues were dissolved in 0.5 mL High-Performance Liquid Chromatography (HPLC) grade methanol, collected in tubular glass vials, and air-dried under a chemical hood. The antibiotic assay was performed by dissolving the extracts in methanol to a concentration of 100 mg mL^{-1}, and 30 µL of the solution was used for the agar well

diffusion assay. The antagonistic activities of the isolates against the *Xcc* strains were determined using the diameter of the inhibition zone.

2.7. Determination of the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the streptomycin and ethyl acetate extracts of EB-39 were determined using the broth micro-dilution method [43]. Various dilutions of the ethyl acetate extract of EB-39, from 23.4–1500 μg mL^{-1}, were prepared for the MIC analysis. The streptomycin concentrations ranged from 1.95–125 μg mL^{-1} to 46.87–3000 μg mL^{-1} against the wild-type and streptomycin-resistant *Xcc* strains, respectively. In 96-well microtiter plates, 100 μL of the ethyl acetate extract was added to a mixture of 90 μL YNB and 10 μL *Xcc* (standardized to ≈2.2×10^4 CFU/well). The positive control consisted of 190 μL YNB and 10 μL *Xcc*, and the negative control had 200 μL YNB broth without the addition of *Xcc*. The plates were incubated at 28 °C for 24 h. MIC was recorded as the lowest concentration of the ethyl acetate extract or streptomycin that prevented visible growth of the *Xcc*. For MBC determination, 10 μL of the MIC cultures from the microtiter plates were transferred onto YNA plates and incubated at 28 °C for 24 h. The lowest concentrations of the ethyl acetate extract and streptomycin that prevented visible growth of the bacteria on the YNA plates were indicated as the MBCs. Both MIC and MBC were expressed in μg mL^{-1}.

2.8. Scanning Electron Microscopy Analysis

Scanning electron microscopy (SEM) analysis was performed to determine the effects of the ethyl acetate extract of EB-39, as well as streptomycin on *Xcc*W1, *Xcc*M4, and *Xcc*M5 cells. *Xcc* strains treated with methanol were used as the control. For the SEM observation, both wild-type and streptomycin-resistant *Xcc* strains (≈3.2×10^4 CFU mL^{-1}) were treated with the MIC of the ethyl acetate extract of EB-39 and streptomycin for 12 h at 28 °C. The suspension was centrifuged at 6000 rpm for 5 min, and the supernatant was discarded. The pellet was further washed five times with 1 mL phosphate buffer (0.1 M) at pH 7.4. Subsequently, 10 μL of the bacterial pellet was placed in poly-L-lysine-coated glass slides and pre-fixed overnight with 2.5% glutaraldehyde solution at 4 °C. The fixed cells were rinsed five times for 10 min with a 0.1 M phosphate buffer, post-fixed for 3 h in 2% osmium tetroxide, and washed three times with SDW to remove traces of salt crystals from the phosphate buffer. Finally, the cells were dehydrated in a graded ethanol series (50%, 70%, 80%, 85%, 90%, and 100%) for 15 min. The samples were mounted on copper grids, sputter-coated with platinum, and examined with a Hitachi S-4800 scanning electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 15.0 kV under standard operating conditions [28,44].

2.9. Testing the Pathogenicity and Disease Suppression Ability of EB-39

Pathogenic wild-type *Xcc*W2 were grown overnight at 28 °C in YNB and centrifuged at 3500 rpm for 15 min. The pellet was resuspended in SDW and adjusted to a concentration of 0.4 OD_{600nm} that was determined with a nanodrop (ASP-3700; ACTGene, Piscataway, NJ, USA). For the detached leaf assay, fully expanded and immature (four-week-old) leaves were excised from the Hwanggeum hyang cultivar (highly canker-susceptible), generated from a cross between *C. unshiu* × *C. sinensis* and *C. unshiu*. The leaves were washed with SDW and subjected to infiltration by pushing a needleless syringe against the surface of the citrus leaf supported by a finger on the opposite side of the leaf. For each treatment, a 0.1 mL suspension was used to infiltrate the citrus leaves. The freshly detached leaves of Hwanggeum hyang were inoculated with SDW (negative control), *Xcc*W2 at 0.4 OD_{600nm} (positive control), and a mixture of *Xcc*W2:EB-39 at 0.4 OD_{600nm} and EB-39 at 0.4 OD_{600nm}. The mixture was prepared by mixing equal volumes of *Xcc*W2 at 0.8 OD_{600nm} and EB-39 at 0.8 OD_{600nm}. The inoculated plant leaves were maintained in a humid box with three layers of sterile tissue paper moistened with SDW and incubated in a growth chamber at 28 °C. The severity of the disease symptoms on the Hwanggeum hyang leaves were scored in triplicate 7 days post-infection [12,45].

3. Results

3.1. Primary Screening of the Endophytic Bacteria Isolated from Citrus spp.

A total of 66 endophytic bacterial strains were isolated from nine different *Citrus* spp. (Table 1). The morphological characteristics of the isolated EB were documented on the basis of colony color, form, elevation, and margin.

Table 1. List of endophytic bacteria isolated from *Citrus* spp.

Serial No.	*Citrus* Species	Tissue	Number of Isolates
1	Summer mandarin	Leaves	5
2	Yakeum mandarin	Leaves	2
3	Navel orange	Leaves	6
4	Leeyegam mandarin	Leaves	4
5	Manbeckyu mandarin	Leaves	4
6	Early season citrus	Leaves	6
7	Palsak mandarin	Leaves	11
8	Dangyuja mandarin	Leaves	4
9	Hwangkeum hyang	Leaves	24
	Number of total isolates		66

All the isolates were screened for their antagonistic effects against *Xcc*W1 by using the agar overlay method [41]. The top three bacterial strains with antibacterial activity were EB-35, EB-39, and EB-44, isolated from Palsak mandarin, Dangyuja mandarin, and Hwangkeum hyang *Citrus* spp., respectively. The largest inhibition zone against *Xcc*W1 was formed by EB-39, followed by EB-44 and EB-35 (Table 2). The BLAST homology analysis revealed that the 16S rRNA gene sequences of the three bacteria belong to the genus *Bacillus*. In addition, the phylogenetic tree inferred from the 16S rRNA gene sequences indicated that the isolates EB-35, EB-39, and EB-44 share a high degree of identity with *B. wiedmannii*, *B. velezensis*, and *B. toyonensis*, respectively (Figure 1 and Figure S1).

Table 2. Primary screening of endophytic bacteria by *Xcc*W1.

Isolates	Species	Max Score	E. Value	Max Identity (%)	Zone of Inhibition (mm) *
EB-35	*B. wiedmannii*	2074	0.0	99.74	18.2
EB-39	*B. velezensis*	2368	0.0	99.92	36.7
EB-44	*B. toyonensis*	2636	0.0	99.59	18.5

* Diameter of the inhibition zones.

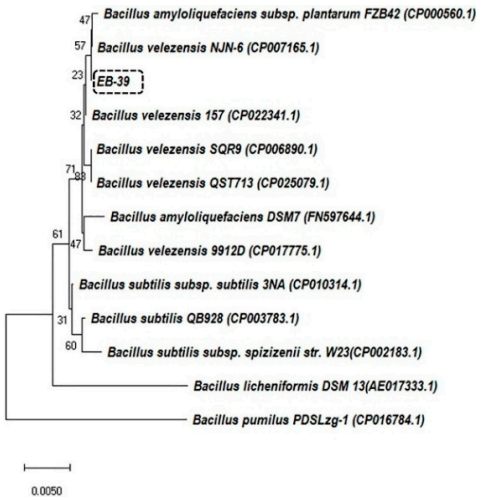

Figure 1. Phylogenetic tree based on the 16S rRNA gene sequences that highlights the position of EB-39 relative to the other strain types within the genus *Bacillus*. Accession numbers retrieved from the NCBI

database are provided within parentheses. Bootstrap values (%) are mentioned at the nodes and obtained by repeating the analysis 1000 times. The scale bar indicates 0.005 nucleotide substitutions per nucleotide position.

3.2. Antibacterial Activity of the Live Endophytic Bacteria Against Xcc

The three identified strains were further challenged with all three wild-type and six mutant strains of *Xcc* for a comparison of the antibacterial activities (Figure 2A,B). EB-39 exhibited the highest antibacterial effect against all the *Xcc* strains, with the inhibition zones ranging from 39.47 ± 1.6 to 45.31 ± 1.6 mm. EB-35 and EB-44 also exhibited antagonistic effects against all wild-type and mutant strains; however, their antibacterial activities were lower than that of EB-39. The inhibition zones of EB-35 and EB-44 ranged from 20.13 ± 0.1 to 23.5 ± 0.5 mm and 20.28 ± 0.2 to 22.78 ± 0.8 mm, respectively (Figure 2A,B).

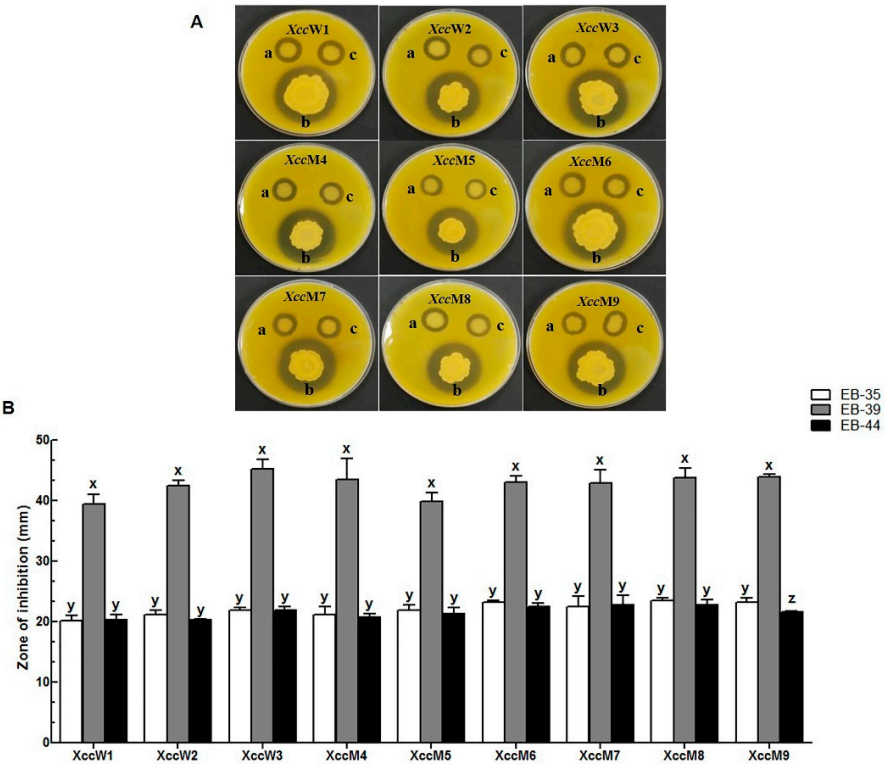

Figure 2. In vitro antagonistic activity of endophytic bacteria against *X. citri* subsp. *citri.* (**A**) The test was conducted using the pathogenic strains *Xcc*W1–*Xcc*W3 (wild-type strains) and *Xcc*M4–*Xcc*M9 (streptomycin-resistant mutant strains). a: EB-35 (*B. wiedmannii*); b: EB-39 (*B. velezensis*); c: EB-44 (*B. toyonensis*). (**B**) Antagonistic activity of the endophytic bacteria was measured using the wild-type and streptomycin-resistant *X. citri* subsp. *citri* strains. Different letters on bars indicate significant differences at $p < 0.05$ using Duncan's test.

3.3. Antagonistic Effects of the Ethyl Acetate Extracts of the Endophytic Bacteria on Xcc

The three endophytic bacteria were further tested for the antibacterial effects of their ethyl acetate extracts on all *Xcc* strains. EB-39 was found to have active secondary metabolites that showed strong antibacterial activity against both wild-type and mutant *Xcc* strains, with the zone of inhibition ranging from 29.28 ± 0.6 to 33.88 ± 1.3 mm (Figure S2). The ethyl acetate extracts of EB-35 and EB-44 did not display any antagonistic effects against the *Xcc* strains.

The antibacterial activity of the ethyl acetate extract of EB-39 (100 mg mL^{-1}) was further compared with those of two streptomycin concentrations (S1: streptomycin (50 mg mL^{-1}) and S2: streptomycin

(5 mg mL^{-1})). The ethyl acetate extract of EB-39 had an antibacterial effect on both wild-type and streptomycin-resistant *Xcc* strains, with the zone of inhibition ranging from 29.82 ± 1.1 to 32.20 ± 2.9 mm (Figure 3A,B). S1 and S2 had antagonistic effects on the wild-type *Xcc* strains, with the zone of inhibition ranging from 40.96 ± 1.38 to 43.79 ± 1.05 mm and 32.11 ± 0.35 to 34.60 ± 0.0 mm, respectively. However, S1 exhibited a significantly reduced antibacterial effect on the streptomycin-resistant *Xcc* strains, with the zone of inhibition ranging from 10.5 ± 0.6 to 12.9 ± 2.3 mm; S2 did not have any antibacterial effect on the streptomycin-resistant *Xcc* strains (Figure 3A,B). We compared the antibacterial activity of EB-39 with a standard *B. velezensis* (KACC no. 17177) obtained from the Rural Development Agriculture (RDA), Jeonju-si, Republic of Korea. Compared with the extract of the standard *B. velezensis*, that of our newly isolated *B. velezensis* EB-39 had 21.1% and 18.0% higher antibacterial activity against *Xcc*W1 and *Xcc*M4 using 1 mg of ethyl acetate extract mL^{-1}, respectively (Figure 3C).

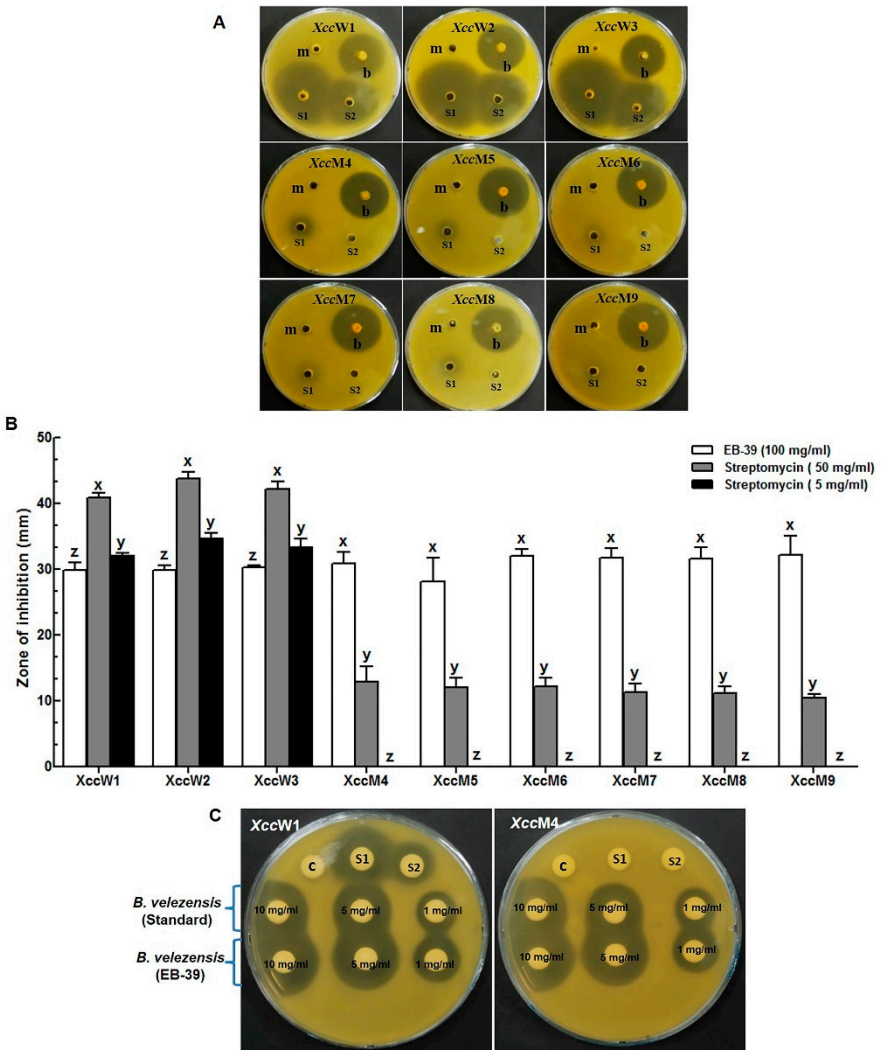

Figure 3. Comparison of the antibacterial effects of the ethyl acetate extract of *B. velezensis* (EB-39) and streptomycin on the wild-type and streptomycin-resistant *X. citri* subsp. *citri* strains. (**A**) The antibacterial activity was measured using the agar well diffusion assay. *Xcc*W1–*Xcc*W3 (wild-type strains) and *Xcc*M4–*Xcc*M9 (streptomycin-resistant mutants). m: methanol control; b: ethyl acetate extract of EB-39 (100 mg mL^{-1}); S1: streptomycin (50 mg mL^{-1}); S2: streptomycin (5 mg mL^{-1}). (**B**) Antagonistic activity of the ethyl acetate extracts of the endophytic bacteria was measured against the wild-type and streptomycin-resistant *X. citri* subsp. *citri* strains. Different letters on bars indicate significant differences at $p < 0.05$ using Duncan's test. (**C**) Comparison of antibacterial effects of the ethyl acetate extract of the standard *B. velezensis* (KACC no. 17,177) and our newly isolated *B. velezensis*

(EB-39). **c**: methanol control; S1: streptomycin (0.5 mg mL^{-1}); S2: streptomycin (0.1 mg mL^{-1}); Ethyl acetate extract of the standard and EB-39 *B. velezensis* (10, 5, and 1 mg mL^{-1}).

3.4. Determination of MIC and MBC

Among the antagonistic endophytic bacterial strains, EB-39 had the highest antibacterial activity against all *Xcc* strains and was therefore selected for the determination of MIC and MBC. The MIC and MBC values of EB-39 were 46.8–93.7 μg mL^{-1} and 93.7–187.5 μg mL^{-1}, respectively (Table 3). In contrast, the MIC of streptomycin against the wild-type *Xcc* strains was considerably lower (1.95 μg mL^{-1}) than that against the mutant *Xcc* strains (370–755 μg mL^{-1}). The MBC of streptomycin against the *Xcc* strains was 3.9–750 μg mL^{-1} (Figures S3 and S4, Table 3).

Table 3. MIC and MBC values of the ethyl acetate extract of *B. velezensis* and streptomycin against *X. citri* subsp. *citri* (*Xcc*) on YNA plates.

***Xcc* Strains**	**Ethyl Acetate Extracts of *B. velezensis***		**Streptomycin**	
	MIC (μg mL^{-1})	**MBC (μg mL^{-1})**	**MIC (μg mL^{-1})**	**MBC (μg mL^{-1})**
*Xcc*W1	93.7	187.5	1.95	3.90
*Xcc*W2	93.7	187.5	1.95	3.90
*Xcc*W3	46.8	93.7	1.95	3.90
*Xcc*M4	46.8	93.7	375.0	750.0
*Xcc*M5	93.7	187.5	375.0	750.0
*Xcc*M6	93.7	187.5	375.0	750.0
*Xcc*M7	46.8	93.7	375.0	750.0
*Xcc*M8	93.7	187.5	750.0	1500.0
*Xcc*M9	46.8	93.7	375.0	750.0

3.5. SEM Analysis of the B. velezensis Extract (EB-39)

The SEM analysis was performed to visualize the cellular damage caused by the ethyl acetate extract of EB-39 and streptomycin on the *Xcc* strains (*Xcc*W1, *Xcc*M4, and *Xcc*M5; Figure 4). *Xcc*W1 was selected to represent the wild type, and *Xcc*M4 and *Xcc*M5 were selected to represent the streptomycin-resistant mutants on the basis of their genotypic classification. The control *Xcc* strains, as well as *Xcc*M4 and *Xcc*M5 treated with streptomycin, did not show any changes in morphology, and they had an intact rod shape with a smooth exterior. In contrast, all the *Xcc* strains (both the wild-type and streptomycin-resistant mutants), upon exposure to the ethyl acetate extract of EB-39, showed distortion of their normal rod structure, and cells eventually lysed. The same morphological changes were observed in the streptomycin-treated wild-type *Xcc*W1; however, the changes were not found in the mutant *Xcc* strains after the streptomycin treatment (Figure 4).

3.6. Biocontrol Efficacy of the Antagonistic Endophytic Bacteria

EB-39 was tested for biocontrol efficacy against *Xcc*W2. In the detached leaf assay, all citrus leaves were infiltrated with 0.1 mL distilled water, *Xcc*W2, and a mixture of *Xcc*W2:EB-39 and EB-39 (Figure 5A,B). Seven days post-infiltration (dpi), both *Xcc*W2 and the *Xcc*W2:EB-39 mixture generated canker lesions. However, the leaves infiltrated with the mixture of XccW2:EB-39 developed smaller infection lesions than those infiltrated with *Xcc*W2 alone (Figure 5A,B). The sizes of the necrotic lesions of *Xcc*W2 were significantly smaller than those of the mixture of *Xcc*W2:EB-39 (14.35 ± 0.45 mm vs. 8.27 ± 0.47 mm; Figure 5C). The lesions developed on the leaves infiltrated with *Xcc*W2 were visible on both surfaces and appeared in the form of water-soaked margins surrounded by yellow rings. The leaves infiltrated with EB-39, however, did not display any visible disease symptoms (Figure 5A,B).

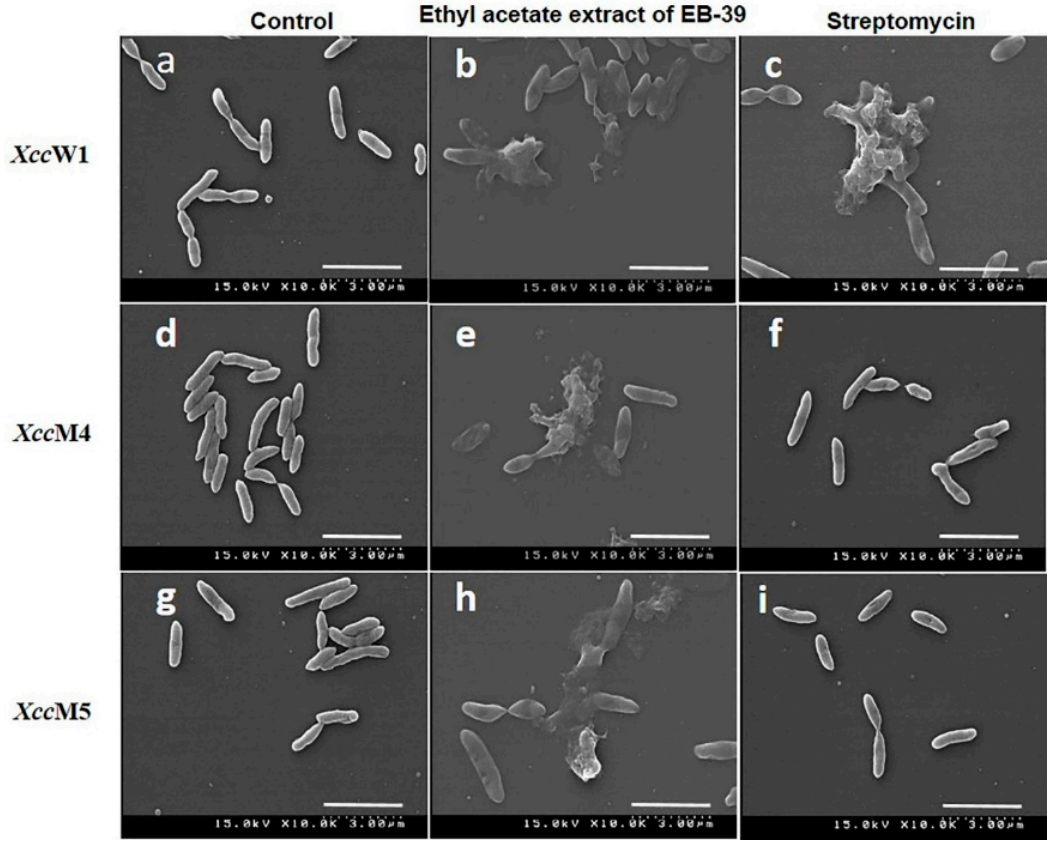

Figure 4. Scanning electron micrographs of *Xcc* strains treated with methanol as the negative control (**a**,**d**,**g**); ethyl acetate extract of EB-39 (**b**,**e**,**h**); and streptomycin as the positive control (**c**,**f**,**i**). The methanol-treated *Xcc* strains had a regular, uniform, and smooth surface. The EB-39-treated strains showed disruption, collapse, elongation, and cell lysis. The streptomycin-treated *Xcc*W1 cells were distorted and eventually lysed; however, the resistant *Xcc* strains (*Xcc*M4 and *Xcc*M5) had an intact, rod shape with a smooth surface. a–c: *Xcc*W1 treated with methanol (control), ethyl acetate extract of EB-39, and streptomycin; d–f: *Xcc*M4 treated with methanol (control), ethyl acetate extract of EB-39, and streptomycin; g–i: *Xcc*M5 treated with methanol (control), ethyl acetate extract of EB-39, and streptomycin. Scale bar: 3 µm.

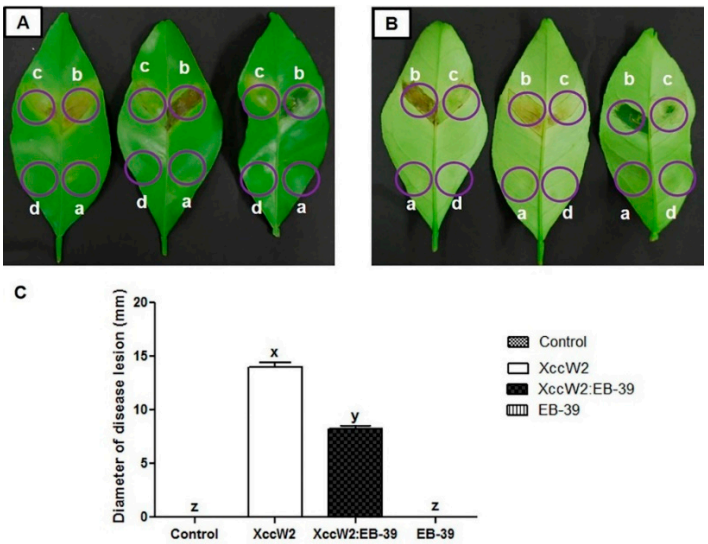

Figure 5. Testing the pathogenicity and disease suppression ability of EB-39 against *X. citri* subsp. *citri* (*Xcc*W2) on citrus leaves. (**A**) adaxial surface and (**B**) abaxial surface of the same leaves. In all cases, the

leaves were infiltrated with 0.1 mL of (a) sterile distilled water, (b) *Xcc*W2, (c) *Xcc*W2 mixed with EB-39, and (d) EB-39. (**C**) Disease lesion diameter was measured at 7 dpi. The mean ± standard deviation values from three independent replicates were calculated for each treatment. Different letters on bars indicate significant differences at $p < 0.05$ using Duncan's test.

4. Discussion

CBC is one of the most destructive diseases in citrus plants in many production areas worldwide [5]. In the last few decades, citrus growers in many countries have been forced to eradicate millions of citrus plants because of the severity of CBC [5,16]. Treatment of the disease with copper-based chemical sprays and streptomycin has led to an increase in the number of resistant *Xanthomonas* strains [14,39]. The development of alternative biocontrol agents has now become a major challenge for scientists. Endophytic bacteria may play a significant role in controlling the disease [23].

In this study, a total of 66 endophytic bacteria were isolated from different varieties of *Citrus* spp. Among the isolated strains, three isolates exhibited in vitro inhibition of *Xcc* by forming inhibition zones (Figure 2A,B). The isolates were identified as *Bacillus* spp., namely, *B. wiedmannii* (EB-35), *B. velezensis* (EB-39), and *B. toyonensis* (EB-44) using 16S rRNA sequence analysis. To date, many strains of endophytic bacteria have been isolated from *Citrus* spp., for example, *B. pumilus*, *B. megaterium*, *Enterobacter cloacae*, *Pantoea agglomerans*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Curtobacterium flaccumfaciens* [40,46].

The solvent extracts of the selected strains were evaluated for their antibacterial activity against all the *Xcc* strains. The ethyl acetate extract of *B. velezensis* EB-39 exhibited the highest antibacterial activity against the wild-type and mutant strains of *Xcc*, and those of EB-35 and EB-44 did not show any antibacterial activity against the *Xcc* strains (Figure S2). In the comparative study of EB-39 metabolites and two different streptomycin concentrations, S1 (50 mg mL^{-1}) and S2 (5 mg mL^{-1}), S1 and S2 displayed antagonistic effects against the wild-type *Xcc* strains. However, S1 had a significantly lower antibacterial effect against the mutant *Xcc* strains, whereas S2 had no effect on the mutant *Xcc* strains. These data strongly indicate that the ethyl acetate extract of EB-39 contained biologically active secondary metabolites that could suppress both wild-type and streptomycin-resistant mutant *Xcc* strains (Figure 3A,B).

SEM was used to detect the morphological changes caused by exposure to the ethyl acetate extracts in the *Xcc* strains. The SEM analysis indicated that the *Xcc* cells became distorted from their normal rod-shaped structure and eventually lysed because of treatment with the ethyl acetate extract of *B. velezensis* (Figure 4). The antimicrobial compounds possibly caused the *Xcc* cell membranes to destabilize, leading to leaking of the cell contents, leaving cell debris. Similar morphological deformities were observed in *X. oryzae* pv. *oryzae* cells exposed to 50 µg mL^{-1} difficidin or bacilysin isolated from *B. amyloliquefaciens* FZB42 (reclassified as a strain of *B. velezensis*) [28]. Although further research would be required to identify the antibacterial compounds, the ethyl acetate extract of *B. velezensis* should contain antibacterial compounds that induce lysis of *Xcc*.

The detached citrus leaves infiltrated with a bacterial mixture of *Xcc*W2:EB-39 significantly reduced the disease symptoms when compared with those infiltrated with *Xcc*W2; however, those infiltrated with EB-39 did not show any disease symptoms, indicating EB-39 is not a pathogen of citrus (Figure 5). Reductions in CBC severity and incidence have been observed in a *Citrus* spp. (Mexican lime) treated with the culture suspensions of *B. amyloliquefaciens* strains WG6-14 and *B. subtilis* TKS1-1 [47]. The disease severity of black pod rot of cacao caused by *Phytophthora capsici* was significantly reduced when detached cacao leaf disks were challenged with the bacterial endophyte *B. cereus* [48]. Several *Bacillus* spp., including *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, *B. thuringiensis*, *B. cereus*, and *B. sphaericus*, reduced disease severity via the elicitation of ISR in diverse host plants such as tomato, sugar beet, watermelon, bell pepper, and cucumber [49]. Surfactin and other non-ribosomally synthesized

secondary metabolites produced by *B. amyloliquefaciens* FZB42 enhanced the plant defense levels toward the bottom rot pathogen *R. solani* via the higher expression of plant defensin factor 1.2 (*PDF 1.2*) [50].

Overall, to the best of our knowledge, this is the first report of the control of CBC by the endophyte *B. velezensis* isolated from *Citrus* spp. In a similar study, the methanolic extract of *B. velezensis* exhibited strong antagonistic activity against *R. solanacearum*, which infects many plant species such as potato (*Solanum tuberosum*), eggplant (*Solanum melongena*), and tomato (*Solanum lycopersicum*) [27]. *B. velezensis* is also known as a plant-associated bacterium with the ability to promote plant growth and produce different types of secondary metabolites that suppress phytopathogens [21,28]. The antibacterial activity of *B. velezensis* is mainly due to non-ribosomal synthesis of polyketides such as bacillaene, difficidin, and macrolactin [21,38]. *B. velezensis* showed the ability to suppress the growth of plant pathogenic bacteria, such as *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, the causative agents of bacterial blight and bacterial leaf streak of rice, respectively [28]. All the data strongly suggest that our newly isolated EB-39 is a novel antibacterial agent that can be used to control both wild-type and streptomycin-resistant mutant *Xcc* strains efficiently.

5. Conclusions

The isolated *B. velezensis* EB-39 exhibited antibacterial activity against wild-type and streptomycin-resistant *Xcc* pathogens. Therefore, EB-39 could be a practical and powerful biocontrol agent (either bacterial spray or extract) against CBC. Further studies are required for the identification of antibacterial compounds that control the streptomycin-resistant *Xcc* strains. Elucidation of the genes responsible for bioactive secondary metabolites and the expression control of the genes are additional important steps required for increasing the production of metabolites by *B. velezensis*.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/8/470/s1, Figure S1: Phylogenetic tree based on the 16S rRNA gene sequences. (A). Highlighted position of EB-35 and (B). Highlighted position EB-44 relative to the other type strains within the genus *Bacillus*, Figure S2: (A). The ethyl acetate extracts of the *Bacillus* strains against the *X. citri* subsp. *citri* (*Xcc*) strains; m: methanol control a: EB-35 (*B. wiedmannii*); b: EB-39 (*B. velezensis*); c: EB-44 (*B. toyonensis*). (B). Antagonistic activity of the ethyl acetate extracts was measured against the wild-type and streptomycin-resistant *X. citri* subsp. *citri* strains, Figure S3: MIC and MBC values of the ethyl acetate extract of *B. velezensis* EB-39 against *X. citri* subsp. *citri* (*Xcc*) on YNA plates; *Xcc*W1–*Xcc*W3 (wild-type strains) and *Xcc*M4–*Xcc*M9 (streptomycin-resistant mutant strains), Figure S4: MIC and MBC values of the streptomycin against *X. citri* subsp. *citri* (*Xcc*) on YNA plates; *Xcc*W1–*Xcc*W3 (wild-type strains) and *Xcc*M4–*Xcc*M9 (streptomycin-resistant mutant strains).

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References

1. Shi, Q.; Febres, V.J.; Jones, J.B.; Moore, G.A. A survey of *FLS2* genes from multiple citrus species identifies candidates for enhancing disease resistance to *Xanthomonas citri* ssp. *citri*. *Hortic. Res.* **2016**, *3*, 1–11. [CrossRef] [PubMed]
2. USDA. *Citrus: World Markets and Trade*; United States Department of Agriculture Foreign Agriculture Service: Washington, DC, USA, 2019; pp. 1–11.
3. Khan, M.K.; Huma, Z.E.; Dangles, O. A comprehensive review on flavanones, the major citrus polyphenols. *J. Food Compos. Anal.* **2014**, *33*, 85–104. [CrossRef]
4. Graham, J.H.; Gottwald, D.T.R.; Cubero, J.; Achor, D.S. *Xanthomonas axonopodis* pv. *citri*: Factors affecting successful eradication of citrus canker. *Mol. Plant Pathol.* **2004**, *5*, 1–15. [CrossRef] [PubMed]
5. Gottwald, T.R.; Graham, J.H.; Schubert, T.S. Citrus canker: The pathogen and its impact. *Plant Manag. Netw.* **2002**. [CrossRef]

6. Rigano, L.A.; Marano, M.R.; Castagnaro, A.P.; Do Amaral, A.M.; Vojnov, A.A. Rapid and sensitive detection of citrus bacterial canker by loop-mediated isothermal amplification combined with simple visual evaluation methods. *BMC Microbiol.* **2010**, *10*, 176. [CrossRef]
7. Moreira, L.M.; Almeida, N.F.; Potnis, N.; Digiampietri, L.A.; Adi, S.S.; Bortolossi, J.C.; da Silva, A.C.; da Silva, A.M.; de Moraes, F.E.; de Oliveira, J.C.; et al. Novel insights into the genomic basis of citrus canker based on the genome sequences of two strains of *Xanthomonas fuscans* subsp. *aurantifolii*. *BMC Genom.* **2010**, *11*, 238. [CrossRef]
8. Cubero, J.; Graham, J.H. Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. *Appl. Environ. Microbiol.* **2002**, *68*, 1257–1264. [CrossRef]
9. Ryan, R.P.; Vorhölter, F.J.; Potnis, N.; Jones, J.B.; Van Sluys, M.-A.; Bogdanove, A.J.; Dow, J.M. Pathogenomics of *Xanthomonas*: Understanding bacterium-plant interactions. *Nat. Rev. Microbiol.* **2011**, *9*, 344–355. [CrossRef]
10. Silva, A.C.R.; Ferro, J.A.; Reinach, F.C.; Farah, C.S.; Furlan, L.R. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* **2002**, *417*, 459–463. [CrossRef]
11. Ference, C.M.; Gochez, A.M.; Behlau, F.; Wang, N.; Graham, J.H.; Jones, J.B. Recent advances in the understanding of *Xanthomonas citri* ssp. *citri* pathogenesis and citrus canker disease management. *Mol. Plant Pathol.* **2018**, *19*, 1302–1318. [CrossRef]
12. Pitino, M.; Armstrong, C.M.; Duan, Y. Rapid screening for citrus canker resistance employing pathogen-associated molecular pattern-triggered immunity responses. *Hortic. Res.* **2015**, *2*, 1–8. [CrossRef] [PubMed]
13. Gottwald, T.R.; Hughes, G.; Graham, J.H.; Sun, X.; Riley, T. The citrus canker epidemic in Florida: The scientific basis of regulatory eradication policy for an invasive species. *Phytopathology* **2000**, *91*, 30–34. [CrossRef] [PubMed]
14. Behlau, F.; Canteros, B.I.; Minsavage, G.V.; Jones, J.B.; Graham, J.H. Molecular characterization of copper resistance genes from *Xanthomonas citri* subsp. *citri* and *Xanthomonas alfalfae* subsp. *citrumelonis*. *Appl. Environ. Microbiol.* **2011**, *77*, 4089–4096. [CrossRef] [PubMed]
15. Cooksey, D.A.; Azad, H.R.; Cha, J.S.; Lim, C.K. Copper resistance gene homologs in pathogenic and saprophytic bacterial species from Tomato. *Appl. Environ. Microbiol.* **1990**, *56*, 431–435. [PubMed]
16. Silva, I.C.; Regasini, L.O.; Petrãnio, M.S.; Silva, D.H.S.; Bolzani, V.S.; Belasque, J.; Sacramento, L.V.S.; Ferreira, H. Antibacterial activity of alkyl gallates against *Xanthomonas citri* subsp. *citri*. *J. Bacteriol.* **2013**, *195*, 85–94. [CrossRef] [PubMed]
17. Graham, J.H.; Leite, R.P.; Yonce, H.D.; Myers, A.M. Streptomycin controls citrus canker on sweet orange in Brazil and reduces risk of copper burn on grapefruit in Florida. *Proc. Fla. State Hort. Soc.* **2008**, *121*, 118–123.
18. Chen, X.H.; Scholz, R.; Borriss, M.; Junge, H.; Mogel, G.; Kunz, S.; Borriss, R. Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* are efficient in controlling fire blight disease. *J. Biotechnol.* **2009**, *140*, 38–44. [CrossRef]
19. Liu, B.; Qiao, H.; Huang, L.; Buchenauer, H.; Han, Q.; Kang, Z.; Gong, Y. Biological control of take-all in wheat by endophytic *Bacillus subtilis* E1R-j and potential mode of action. *Biol. Control* **2009**, *49*, 277–285. [CrossRef]
20. Gouda, S.; Das, G.; Sen, S.K.; Shin, H.S.; Patra, J.K. Endophytes: A treasure house of bioactive compounds of medicinal importance. *Front. Microbiol.* **2016**, *7*, 1–8. [CrossRef]
21. Chen, X.H.; Koumoutsi, A.; Scholz, R.; Eisenreich, A.; Schneider, K.; Heinemeyer, I.; Morgenstern, B.; Voss, B.; Hess, W.R.; Reva, O.; et al. Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nat. Biotechnol.* **2007**, *25*, 1007–1014. [CrossRef]
22. Paulsen, I.T.; Press, C.M.; Ravel, J.; Kobayashi, D.Y.; Myers, G.S.A.; Mavrodi, D.V.; DeBoy, R.T.; Seshadri, R.; Ren, Q.; Madupu, R.; et al. Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nat. Biotechnol.* **2005**, *23*, 873–878. [CrossRef] [PubMed]
23. Strobel, G.; Daisy, B. Bioprospecting for microbial endophytes and their natural products. *Microbiol. Mol. Biol. Rev.* **2003**, *67*, 491–502. [CrossRef] [PubMed]
24. Hallmann, J.; Quadt-Hallmann, A.; Mahaffee, W.F.; Kloepper, J.W. Bacterial endophytes in agricultural crops. *Can. J. Microbiol.* **1997**, *43*, 895–914. [CrossRef]

25. Nisa, H.; Kamili, A.N.; Nawchoo, I.A.; Shafi, S.; Shameem, N.; Bandh, S.A. Fungal endophytes as prolific source of phytochemicals and other bioactive natural products: A review. *Microb. Pathog.* **2015**, *82*, 50–59. [CrossRef] [PubMed]
26. Hentschel, U.; Steinert, M.; Hacker, J. Common molecular mechanisms of symbiosis and pathogenesis. *Trends Microbiol.* **2000**, *8*, 226–231. [CrossRef]
27. Cao, Y.; Pi, H.; Chandrangsu, P.; Li, Y.; Wang, Y.; Zhou, H.; Xiong, H.; Helmann, J.D.; Cai, Y. Antagonism of two plant-growth promoting *Bacillus velezensis* isolates against *Ralstonia solanacearum* and *Fusarium oxysporum*. *Sci. Rep.* **2018**, *8*, 1–14. [CrossRef] [PubMed]
28. Wu, L.; Wu, H.; Chen, L.; Yu, X.; Borriss, R.; Gao, X. Difficidin and bacilysin from *Bacillus amyloliquefaciens* FZB42 have antibacterial activity against *Xanthomonas oryzae* rice pathogens. *Sci. Rep.* **2015**, *5*, 1–9. [CrossRef] [PubMed]
29. Ge, B.; Liu, B.; Nwet, T.T.; Zhao, W.; Shi, L.; Zhang, K. *Bacillus methylotrophicus* strain NKG-1, isolated from changbai mountain, China, has potential applications as a biofertilizer or biocontrol agent. *PLoS ONE* **2016**, *11*, e0166079. [CrossRef] [PubMed]
30. Masum, M.M.I.; Liu, L.; Yang, M.; Hossain, M.M.; Siddiqa, M.M.; Supty, M.E.; Ogunyemi, S.O.; Hossain, A.; An, Q.; Li, B. Halotolerant bacteria belonging to operational group *Bacillus amyloliquefaciens* in biocontrol of the rice brown stripe pathogen *Acidovorax oryzae*. *J. Appl. Microbiol.* **2018**, *11*, 1–13. [CrossRef] [PubMed]
31. Asaka, O.; Shoda, M. Biocontrol of *Rizhoctonia solani* damping-off of Tomato with *Bacillus subtilis* RB14. *Appl. Environ. Microbiol.* **1996**, *62*, 4081–4085. [PubMed]
32. Chen, L.; Heng, J.; Qin, S.; Bian, K. A comprehensive understanding of the biocontrol potential of *Bacillus velezensis* LM2303 against *Fusarium* head blight. *PLoS ONE* **2018**, *13*, e0198560. [CrossRef] [PubMed]
33. Cawoy, H.; Bettiol, W.; Fickers, P.; Ongena, M. *Bacillus*-based biological control of plant diseases. *Pestic. Mordern World-Pestic. Use Manag.* **2009**, *1849*, 273–298.
34. Rabbee, M.F.; Ali, M.S.; Choi, J.; Hwang, B.S.; Jeong, S.C.; Baek, K. *Bacillus velezensis*: A valuable member of bioactive molecules within plant microbiomes. *Molecules* **2019**, *24*, 1046. [CrossRef] [PubMed]
35. Dunlap, C.A.; Kim, S.J.; Kwon, S.W.; Rooney, A.P. *Bacillus velezensis* is not a later heterotypic synonym of *Bacillus amyloliquefaciens*; *Bacillus methylotrophicus*, *Bacillus amyloliquefaciens* subsp. *plantarum* and '*Bacillus oryzicola*' are later heterotypic synonyms of *Bacillus velezensis* based on phylogenomics. *Int. J. Syst. Evol. Microbiol.* **2016**, *66*, 1212–1217. [PubMed]
36. Fan, B.; Blom, J.; Klenk, H.P.; Borriss, R. *Bacillus amyloliquefaciens*, *Bacillus velezensis*, and *Bacillus siamensis* form an "Operational Group, *B. amyloliquefaciens*" within the *B. subtilis* species complex. *Front. Microbiol.* **2017**, *8*, 1–15. [CrossRef] [PubMed]
37. Scholz, R.; Molohon, K.J.; Nachtigall, J.; Vater, J.; Markley, A.L.; Süssmuth, R.D.; Mitchell, D.A.; Borriss, R. Plantazolicin, a novel microcin B17/streptolysin S-like natural product from *Bacillus amyloliquefaciens* FZB42. *J. Bacteriol.* **2011**, *193*, 215–224. [CrossRef]
38. Scholz, R.; Vater, J.; Budiharjo, A.; Wang, Z.; He, Y.; Dietel, K.; Schwecke, T.; Herfort, S.; Lasch, P.; Borriss, R. Amylocyclicin, a novel circular bacteriocin produced by *Bacillus amyloliquefaciens* FZB42. *J. Bacteriol.* **2014**, *196*, 1842–1852. [CrossRef]
39. Hyun, J.W.; Kim, H.J.; Yi, P.H.; Hwang, R.Y.; Park, E.W. Mode of action of streptomycin resistance in the citrus canker pathogen (*Xanthomonas smithii* subsp. *citri*) in Jeju island. *Plant Pathol. J.* **2012**, *28*, 207–211. [CrossRef]
40. Araújo, W.L.; Marcon, J.; Maccheroni, W.; Van Elsas, J.D.; Van Vuurde, J.W.L.; Azevedo, J.L. Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. *Appl. Environ. Microbiol.* **2002**, *68*, 4906–4914. [CrossRef]
41. Guo, Y.; Huang, E.; Yuan, C.; Zhang, L.; Yousef, A.E. Isolation of a *Paenibacillus* sp. strain and structural elucidation of its broad-spectrum lipopeptide antibiotic. *Appl. Environ. Microbiol.* **2012**, *78*, 3156–3165. [CrossRef]
42. Goodson, J.R.; Klupt, S.; Zhang, C.; Straight, P.; Winkler, W.C. LoaP is a broadly conserved antiterminator protein that regulates antibiotic gene clusters in *Bacillus amyloliquefaciens*. *Nat. Microbiol.* **2017**, *2*, 1–10. [CrossRef] [PubMed]
43. Adimpong, D.B.; Sorensen, K.I.; Thorsen, L.; Stuer-Lauridsen, B.; Abdelgadir, W.S.; Nielsen, D.S.; Derkx, P.M.F.; Jespersen, L. Antimicrobial susceptibility of *Bacillus* strains isolated from primary starters for african traditional bread production and characterization of the bacitracin operon and bacitracin biosynthesis. *Appl. Environ. Microbiol.* **2012**, *78*, 7903–7914. [CrossRef] [PubMed]

44. Nanda, S.S.; Yi, D.K.; Kim, K. Study of antibacterial mechanism of graphene oxide using Raman spectroscopy. *Sci. Rep.* **2016**, *6*, 1–12. [CrossRef] [PubMed]
45. Chang, S.C.; Deng, W.L.; Huang, H.C.; Chung, K.R.; Tzeng, K.C. Differential expression of pectolytic enzyme genes in *Xanthomonas citri* subsp. *citri* and demonstration that pectate lyase Pel3 is required for the formation of citrus canker. *Microbiol. Res.* **2016**, *192*, 1–10. [CrossRef] [PubMed]
46. Rosenblueth, M.; Martínez-Romero, E. Bacterial endophytes and their interactions with hosts. *Mol. Plant-Microbe Interact.* **2006**, *19*, 827–837. [CrossRef]
47. Huang, T.P.; Tzeng, D.D.S.; Wong, A.C.L.; Chen, C.H.; Lu, K.M.; Lee, Y.H.; Huang, W.D.; Hwang, B.F.; Tzeng, K.C. DNA polymorphisms and biocontrol of *Bacillus* antagonistic to citrus bacterial canker with indication of the interference of phyllosphere biofilms. *PLoS ONE* **2012**, *7*, e42124. [CrossRef]
48. Melnick, R.L.; Zidack, N.K.; Bailey, B.A.; Maximova, S.N.; Guiltinan, M.; Backman, P.A. Bacterial endophytes: *Bacillus* spp. from annual crops as potential biological control agents of black pod rot of cacao. *Biol. Control* **2008**, *46*, 46–56. [CrossRef]
49. Kloepper, J.W.; Ryu, C.-M.; Zhang, S. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology* **2004**, *94*, 1259–1266. [CrossRef]
50. Chowdhury, S.P.; Uhl, J.; Grosch, R.; Alquéres, S.; Pittroff, S.; Dietel, K.; Schmitt-Kopplin, P.; Borriss, R.; Hartmann, A. Cyclic lipopeptides of *Bacillus amyloliquefaciens* subsp. *plantarum* colonizing the Lettuce rhizosphere enhance plant defense responses toward the bottom rot pathogen *Rhizoctonia solani*. *Mol. Plant-Microbe Interact.* **2015**, *28*, 984–995. [CrossRef]