Saudi Journal of Biological Sciences 29 (2022) 2363–2371

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Biocontrol of citrus bacterial canker caused by *Xanthomonas citri* subsp. *citri* by *Bacillus velezensis*

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ARTICLE INFO

Article history:
Received 30 June 2021
Revised 3 December 2021
Accepted 4 December 2021
Available online 10 December 2021

Keywords:
Bacillus velezensis
B. amyloliquefaciens
Biocontrol
Citrus bacterial canker
Xanthomonas citri subsp. *citri*

ABSTRACT

Microorganisms with biocontrol capabilities against plant pathogens are considered as one of the most promising approaches for healthy crop management. In this study, ethyl acetate extracts of 25 *Bacillus* strains were investigated for their antagonistic effect on *Xanthomonas citri* subsp. *citri* (*Xcc*), which causes the citrus bacterial canker (CBC) disease. Among them, 21 strains exerted antibacterial activity against wild-type *Xcc* strains. Based on the strength of the antibacterial activity, nine *Bacillus* strains were selected for 16S rRNA analysis. 16S rRNA sequence homology revealed that several strains were closely related to *B. velezensis*, where strains with no antibacterial activity grouped as the soil-associated community of *B. amyloliquefaciens*. *B. velezensis* Bv-21 exhibited the highest antibacterial activity against wild type and streptomycin resistant *Xcc* with inhibition zones of 22.91 ± 0.45 and 20.28 ± 0.53, respectively. Furthermore, *B. velezensis* Bv-21 strain was tested for biocontrol activity against a streptomycin-resistant *Xcc*M4 in detached susceptible citrus leaves. The strain reduced the incidence of CBC by 26.30% and pathogen density of *Xcc*M4 by 81.68% over control. The results of the study strongly suggest that *B. velezensis* can be used as an effective and eco-friendly biocontrol agent either by itself or as an active compound, against both, the wild-type and streptomycin-resistant *Xcc*.

1. Introduction

Citrus bacterial canker (CBC) is a severe, economically harmful, and highly contagious disease caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) in most commercial citrus cultivars (Hu et al., 2014). The initial outbreak of the disease reported in Florida, USA in the early 1910s and the disease was caused by imported citrus seedlings from Japan (Li et al., 2007). The symptoms of CBC include elevated hyperplastic lesions on the leaves, stems, and fruits leading to defoliation and premature fruit drop (Gottwald et al., 2002). The most common bactericides used to manage citrus canker are copper-based compounds such as copper hydroxide, cuprous oxide, and copper oxychloride (Marin et al., 2019). However, copper-based compounds posed considerable risks to the environment and long-term usage of these bactericides may result in the development of copper-resistant strains of *Xcc* (Behlau et al., 2011; Marin et al., 2019). Copper-resistant strains against *Xcc* and *X. alfalfae* subsp. *citrumelonis* were first discovered in Argentina in the mid-1990s in citrus nurseries exposed to copper-based bactericides on a regular basis (Behlau et al., 2011). Furthermore, overuse of these copper-based sprays results in phytotoxicity in the fruit skin and accumulates to toxic levels in the citrus root (Graham et al., 2016). An alternative to copper is zinc, which is generally recognized as safe, and zinc oxide based nanoformulations (Zinkicide) reduced the canker lesions by 38–42% in Pineapple leaves under greenhouse condition. However, the level of protection of zinc-based formulations against *Xcc* was not satisfactory in the field trials (Graham et al., 2016). Several other potential alternatives are availabe such as antibiotics (e.g., streptomycin). There is also concern of resistant bacteria selection from the overuse of these antibiotics. Moreover, streptomycin is prohibited in Brazil and Europe for agricultural use (Martins et al., 2020). Hence, there is compelling need to develop environmentally acceptable alternative to control the pathogenic strains of *Xcc*. During last decades, the potential use of *Bacillus* spp. as a

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2021.12.005
1319-562X/

biocontrol agent has been highlighted as a promising alternative to chemical pesticides (Chen et al., 2020; Leal et al., 2021). *Bacillus* spp. (including more than 300 species) are a wide group of saprophytic, gram-positive, aerobic, spore-forming ubiquitous organisms that can be recovered from almost any niche in the world (Cao et al., 2018).

Bacillus species are considered to be one of the most abundant sources of bioactive secondary metabolites with antipathogenic activity, that largely remain unexplored for plant disease control (Chen et al., 2007; Ongena and Jacques, 2007). *Bacillus* spp., including *B. amyloliquefaciens*, *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, and *B. pumilus* are known as a natural factory of biologically active compounds such as cyclic lipopeptides and polyketides. Many of these compounds are potentially useful against deleterious microbes that cause various plant diseases (Huang et al., 2012; Nigris et al., 2018; Wu et al., 2015; Xiong et al., 2015; Zhang and Sun, 2018). In addition to the *Bacillus* spp., the *Pseudomonas* spp. has also gained considerable attention as biocontrol agents. Unfortunately, owing to the challenges associated with the preparation of stable and long-lived bioformulations with the *Pseudomonas* spp., the commercial application of this biocontrol agent is limited in agriculture (Borriss, 2011). Therefore, *Bacillus* spp. are commonly recommended in agricultural practices as a result of their ability to form endospores that can withstand exposure to heat and desiccation. In addition, *Bacillus* spp. based products can be formulated into stable dry powders with longer shelf life (Ongena and Jacques, 2007). Furthermore, *Bacillus* spp. based natural antagonistic organisms are ecofriendly and can be combined with traditional chemical control systems to reduce the possibility of the development of microbial resistance (Ongena and Jacques, 2007; Wu et al., 2015).

Among the *Bacillus* spp., *B. velezensis* has been gaining attention for its eco-friendly nature and versatile mode of action (Kim et al., 2021). *B. velezensis* is a plant-associated *Bacillus* that colonizes plant roots, promotes plant growth, and suppresses pathogens (Cao et al., 2018). *B. amyloliquefaciens* FZB42^T (reclassifed as *B. velezensis* FZB42^T) is a gram-positive plant growth promoting rhizobacterium and was first isolated from sugar beet in Brandenburg, Germany (Borriss et al., 2011). This bacterium was originally described in 2005 by Ruiz-Garcia, when it was isolated from the Vélez River in the province of Málaga, Spain (Ruiz-García et al., 2005). Further research has shown that *B. velezensis* FZB42^T is genetically equipped with nine giant gene-clusters that encode secondary metabolites that make up approximately 10% of the total genome (Chen et al., 2007), which have been documented to be useful tools for plant disease control (Ongena and Jacques, 2007). Five of these nine gene clusters were involved in the synthesis of cyclic lipopeptides (i.e., surfactin, fengycin, and bacillomycin D), an unknown peptide, and siderophore producing compound bacillibactin (Chen et al., 2009). Meanwhile, three other polyketide synthase gene clusters were described for the biosynthesis of macrolactin, bacillaene, and difficidin (Chen et al., 2006, 2009). The last gene cluster was found to be responsible for the synthesis and export of the dipeptide antibiotic bacilysin (Chen et al., 2009). Besides, two other ribosomally synthesized bacteriocins (i.e., plantazolicin and amylocyclicin) were identified in *B. velezensis* and displayed high antibacterial activity against closely related Gram-positive bacteria (Scholz et al., 2014, 2011).

Besides the direct antagonism of *B. velezensis* against pathogenic microbes, this bacterium has also been reported to contribute to the plant defense by competing with deleterious microbes for essential nutrients (i.e., iron) through the secretion of siderophore bacillibactin (Xiong et al., 2015) or by expressing the global defense response in the plant by induced systemic resistance (Li et al., 2017). In fact, *Bacillus* spp. based products account for about half of all commercially available biological control agents (Ongena and Jacques, 2007). To date, commercially available *B. velezensis* based products include RhizoVital® (*B. velezensis* FZB42; ABiTEP, GmbH, Berlin, Germany), BioYield™ (*B. amyloliquefaciens* GB99 + *B. subtilis* GB122; Bayer Crop Science, USA), Amylo-X® WG (*B. amyloliquefaciens* subsp. *plantarum* D747; Certis Europe BV, Netherlands), Taegro® (*B. subtilis* var. *amyloliquefaciens* FZB24; Novozymes Biologicals, Virginia, USA etc. (Borriss et al., 2011). The fungicide Serifel® formulated from the *B. amyloliquefaciens* strain MBI600 has also been shown to have antiviral action against tomato spotted wilt virus (TSWV) and potato virus Y (PVY). A drench application of this biocontrol agent reduced the incidence of tomato spotted wilt virus by 80% and delayed the development of potato virus Y by accumulating the defense-related genes for jasmonic acid and salicylic acid (Beris et al., 2018). Botrybel (Agricaldes, Spain) based on *B. velezensis* has been found to be effective against *Botrytis cinerea*, the leading cause of gray mold disease infecting around 200 plant species worldwide (Romanazzi and Feliziani, 2020).

In our previous study, we reported the isolation and identification of *B. velezensis* EB-39 (an endophytic bacterium) from Dangyuja mandarin citrus and the bacterium was found to have antibacterial activity against the wild-type and streptomycin-resistant mutants of *Xcc* (Rabbee et al., 2019b). In this study, we perfomred a comparative study of antibacterial activity of various strains of *B. velezensis* and *B. amyloliquefaciens* to control both the wild-type and streptomycin-resistant *Xcc* and found *B. velezensis* Bv-21 exhibited the highest antibacterial activity against wild type and streptomycin resistant *Xcc*. The biocontrol efficacy of *B. velezensis* Bv-21 was also examined against streptomycin-resistant *Xcc*M4 in detached susceptible citrus leaves. The application of this strain could be benificial for long-term agricultual management and disease control.

2. Materials and methods

2.1. Plant materials

A three-year-old Hwanggeum hyang *Citrus* trees were purchased from the Jeju Hanla Farm (Jeju province, Republic of Korea), planted in pots (18 cm × 20 cm), and grown in a temperature-controlled glasshouse. The leaves of Hwanggeum hyang citrus, which are susceptible to CBC, were used in detached leaf assays for the *Xcc* infection experiments.

2.2. Culture conditions of microorganisms

A total of 25 *Bacillus* spp. (Table 1) were screened for their biocontrol activity against *Xcc*. The Korean Agricultural Culture Collection (KACC, RDA, Republic of Korea) kindly supplied 24 strains. Bv-25, an endophytic *B. velezensis* strain was isolated from the leaves of Hwanggeum hyang citrus in our laboratory. All the *Bacillus* spp. were cultured on Yeast-Nutrient-Agar (YNA) media (5 g yeast extract, 8 g nutrient broth, 15 g agar in 1L distilled water). Pathogenic *Xcc* strains included two wild-type *Xcc* [*Xcc*W1 (*Xcc* 19-18), *Xcc*W2 (*Xcc* 10-5)] and five 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)], provided by Dr. Hyun at the Citrus Research Station, RDA, Jeju, Korea. These streptomycin resistant *Xcc* strains were isolated from citrus orchard which was previously sprayed heavily with streptomycin. All *Xcc* strains were grown in YNA medium.

Table 1
List of *B. velezensis* and *B. amyloliquefaciens* used in this study.

Strain no.	KACC* no.	Strain Name	Source of isolation
Bv-1	**15814**	***B. velezensis***	**Red chili paste (Gochujang)**
Ba-2	**15815**	***B. amyloliquefaciens***	**Soybean paste (Doenjang)**
B-3	15816	*B. amyloliquefaciens*	Soybean paste (Doenjang)
B-4	15817	*B. amyloliquefaciens*	Soy sauce (Ganjang)
B-5	15818	*B. amyloliquefaciens*	Soy sauce (Ganjang)
Ba-6	**15819**	***B. amyloliquefaciens***	**Soy sauce (Ganjang)**
B-7	15848	*B. amyloliquefaciens*	Red chili paste (Gochujang)
B-8	15865	*B. amyloliquefaciens*	Soybean paste (Doenjang)
B-9	15866	*B. amyloliquefaciens*	Soybean paste (Doenjang)
B-10	15868	*B. amyloliquefaciens*	Soybean paste (Doenjang)
Bv-11	**15875**	***B. velezensis***	**Soybean paste (Doenjang)**
B-12	15877	*B. amyloliquefaciens*	Soybean paste (Doenjang)
B-13	16017	*B. amyloliquefaciens*	Dried fermented soybeans (Meju)
B-14	16023	*B. amyloliquefaciens*	Dried fermented soybeans (Meju)
B-15	16024	*B. amyloliquefaciens*	Dried fermented soybeans (Meju)
B-16	16032	*B. amyloliquefaciens*	Dried fermented soybeans (Meju)
Bv-17	**17029**	***B. velezensis***	**Soil**
B-18	17030	*B. amyloliquefaciens*	Soil
Bv-19	**17031**	***B. velezensis***	**Soil**
B-20	17032	*B. amyloliquefaciens*	Soil
Bv-21	**17073**	***B. velezensis***	**Onion**
Ba-22	**18611**	***B. amyloliquefaciens***	***Agaricus bisporus* media**
B-23	18650	*B. amyloliquefaciens*	Soil
B-24	19163	*B. amyloliquefaciens*	Pig fecal
Bv-25	**Lab strain**	***B. velezensis* EB-39**	**Endophyte isolated from Dangyuja mandarin citrus**

* KACC: Korean Agricultural Culture Collection; the strains identified by 16S sequence analysis are highlighted.

2.3. Sequencing of the 16s rRNA gene and phylogenetic analysis

To identify the bacterial species, 16S rRNA sequences were compared with the corresponding whole gene sequences available in the GenBank nucleotide database http://www.ncbi.nlm.nih.gov/BLAST. The phylogenetic analysis was carried out in the MEGA-7 program using the Maximum Likelihood method (Kumar et al., 2016). The phylogenetic tree was constructed using the type strains of *Bacillus*-related species. To statistically evaluate the nodes in the phylogenetic tree, bootstrap replication (1000) was used.

2.4. Morphology of Bacillus strains

Bacillus spp. were grown overnight in YNB or Landy broth (L-glutamic acid, 5.0 g; glucose, 20.0 g; yeast extract, 1.0 g; phenylalanine, 2.0 mg; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 g; $MnSO_4$, 5.0 mg; $CuSO_4 \cdot 5H_2O$, 0.16 mg; $FeSO_4 \cdot 7H_2O$, 0.15 mg; KH_2PO_4, 1.0 g; distilled water, 1L; pH was adjusted to 7.0 with 5 N NaOH), then 5 µl cultures were plated on YNA or Landy agar plates. The morphological features were observed after a 24 h and 48 h incubation period at 28 °C.

2.5. Selection of antagonistic Bacillus strains against Xcc

Ethyl acetate extracts of 25 *Bacillus* isolates were prepared according to Goodson et al. (2017) and tested for their antibacterial activity against *Xcc*W1 by the disk diffusion method. Briefly, the *Xcc*W1 strain grown overnight was mixed with 5 mL of 0.7% YNA soft agar and poured directly onto the YNA plates (1.5% agar). After air-drying, the ehtyl acetate extracts were dissolved in HPLC grade methanol to a concentration of 1.0 mg mL^{-1} for the antibiotic assay. Ethyl acetate was selected in the metabolite extraction process due to its low boiling point and polarity. Consequently, 30 µl of extracts were placed on a sterile filter paper disk. The antibacterial activity of the ethyl acetate extract was compared to those of the two positive controls composed of streptomycin S1 (streptomycin 1.0 mg mL^{-1}) and S2 (streptomycin 0.1 mg mL^{-1}) and a negative control where the filter paper discs were impregnated with 30 µl of methanol. After 24 h incubation of the plates at 28 °C, diameter of inhibition zone was measured around the paper disk (8 mm in size). Based on the activity against *Xcc*W1, the metabolite extracts of nine *Bacillus* isolates (Bv-1, Ba-2, Ba-6, Bv-11, Bv-17, Bv-19, Bv-21, Ba-22, and Bv-25) were further selected for evaluating their *in vitro* antagonistic effect against the two wild-type (*Xcc*W1 and *Xcc*W2) and the two streptomycin-resistant *Xcc* strains (*Xcc*M4 and *Xcc*M6). The experiment was performed twice with three replicates. The error bars in a two-tailed *t*-test represented the standard deviation, and the result was found to be statistically significant at $p < 0.05$.

2.6. Determination of the MIC and MBC of Bv-17 and Bv-21

To perform MIC and MBC experiments, we selected the most effective strains of Bv-17 and Bv-21. The MIC and MBC of ethyl acetate extracts of Bv-17 and Bv-21 and streptomycin were determined by the broth microdilution method (Adimpong et al., 2012). For the MIC analysis, various concentration of ethyl acetate extracts of Bv-17 and Bv-25 were prepared ranging from 15.625 to 1000 µg mL^{-1} as two-fold serial dilution in 96-well microtiter plates. Each well contained 100 µl of the ethyl acetate extract from Bv-17 or Bv-21, 90 µl of YNB broth, and 10 µl of *Xcc* strains at around 2.2×10^4 colony-forming units/ mL. The positive control was composed of YNB broth inoculated with *Xcc* whereas the negative control was only the YNB broth. After incubation at 28 °C for 24 h, MIC was recorded as the lowest metabolite extract concentration that prevented the visible growth of the indicator bacterial strain. However, MBC was determined by plating 10 µl of each well's cultures onto YNA plates and incubating the plates at 28 °C for 24 h. MBC was defined as the lowest concentration of metabolite extract that, after an incubation period of 24 h at 28 °C, did not show any bacterial growth in the YNA plates. MIC and MBC were both expressed in µg mL^{-1}.

2.7. In vivo CBC disease suppression by B. velezensis Bv-21

In vivo biological control tests were performed on detached Hwanggeum hyang citrus leaves (Randhawa, 1985). For the assay, fully expanded immature (four-week-old) leaves were excised from Hwanggeum hyang (highly susceptible) cultivar, surface-sterilized with 70% ethanol for 1 min, 2% NaOCl (sodium hypochlorite) solution for 3 min, and 100% ethanol for 30 s. The leaves were washed three times with sterile distilled water (SDW) and then dried with sterilized filter paper. *Xcc*W4, a streptomycin-resistant mutant, and *B. velezensis* Bv-21 were used for inoculation. Both the strains were grown on liquid YNB media at 28 °C in a rotary

shaker incubator at 180 rpm for two days and harvested by centrifugation at 4 °C at 6000 rpm for 15 min. The pellet was then re-suspended in SDW and adjusted to an approximate density of OD_{600} = 0.4 [approximately 10^8 colony-forming units (cfu) mL^{-1}]. For each treatment of the freshly detached leaves of Hwanggeum hyang, approximately 0.1 mL of bacterial suspension, were infiltrated into the intercellular spaces of the leaves by applying steady and gentle pressure and holding the open end of the syringe to the leaf. For each concentration, three separate leaves were infiltrated and around 6–8 mm area of the leaves were water-soaked. The bacterial suspensions were prepared by mixing 0.8 OD_{600nm} *Xcc*W4 with 0.8 OD_{600nm} Bv-21. The freshly detached leaves of Hwanggeum hyang were infiltrated with SDW (negative control), *Xcc*M4 at 0.4 OD_{600nm} (positive control), and a mixture of *Xcc*M4:Bv-21 at 0.4 OD_{600nm} and Bv-21 at 0.4 OD_{600nm}.

To avoid splashing of inoculum, the leaves were lightly blotted with sterile tissue to remove excess inoculum. Following inoculation, the leaves were placed in a humid box, upward (abaxial side), on two layers of sterile tissue paper moistened with SDW and later sealed with a parafilm to maintain high humidity. The boxes containing the inoculated leaves were kept at 24 °C in a plant growth chamber for the development of disease symptoms. The lesion area was measured using a Vernier digital electronic caliper and photographed at 7 days post-infiltration (dpi). All experiments were carried out three times and yielded the same results each time. Data were analyzed by one way ANOVA (analysis of variance) test and result was found significant ($p < 0.0001$) among the groups.

2.8. Quantification of Xcc populations in leaf tissues

Following the onset of CBC symptoms on the leaves of Hwanggeum-hyang citrus citrus at 7 dpi, the populations of *Xcc*W4 in the inoculated sites were counted on the YNA plates. In brief, the leaves were rinsed five times with SDW and dried under a laminar-flow hood to remove the moisture. The lesions (10 mm in diameter) were removed individually with a sterilized cork borer. The leaf disks were homogenized using a grinder in 1.0 mL of SDW and the resulting suspension was serially diluted from 10^{-2} to 10^{-8} in a microtiter plate. By plating10 μl of each suspension onto YNA supplemented with streptomycin, the number of viable cells was calculated. The YNA plates were incubated for 3 days at 28 °C for colony formation and counted to calculate the cfu mL^{-1}. Data were analyzed by one way ANOVA test and result was found significant ($p < 0.0001$) among the groups.

3. Results

3.1. Identification of bacteria based on 16S rRNA gene sequencing

A total of 25 strains of *B. velezensis* or *B. amyloliquefaciens* (Table 1) were used in this experiment. After 16S rRNA sequence homology analysis with corresponding gene sequences for which complete genomes are available, we found that the 24 *B. amyloliquefaciens* strains provided by KACC were actually two groups of *Bacillus* spp., namely *B. velezensis* and *B. amyloliquefaciens*. Furthermore, the phylogenetic tree constructed from the 16S rRNA gene sequences revealed that all the isolates (except Bv-11) that suppressed the growth of *Xcc* belong to *B. velezensis* clade (Fig. 1). Both the species of *B. velezensis* and *B. amyloliquefaciens* differed in morphological features obtained on two different growth media namely the Landy and YNA media after an incubation period of 2 days at 28 °C (Fig. S1).

3.2. Antagonistic activity of ethyl acetate extracts of B. velezensis against Xcc

In the disc diffusion assay, ethyl acetate extracts of 25 *Bacillus* strains were initially screened for potential antibacterial activity against the wild-type *Xcc*W1 (Fig. 2). Among these, Bv-21 and Bv-17 showed the strongest activity against *Xcc*W1 with an inhibition zone of 22.16 ± 1.58 and 18.29 ± 0.57 mm, respectively. Four isolates (Ba-2, Ba-6, Bv-11, and Ba-22) had no antibacterial effect against *Xcc*W1.

Nine *Bacillus* spp. (Bv-1, Ba-2, Ba-6, Bv-11, Bv-17, Bv-19, Bv-21, Ba-22, and Bv-25) were further selected from the initial screening data to check the antibacterial activity against two wild-type (*Xcc*W1 and *Xcc*W2) and two streptomycin-resistant strains of *Xcc* (*Xcc*M4 and *Xcc*M6). The ethyl acetate extract of Bv-1, Bv-17, Bv-19, Bv-21, and Bv-25 inhibited the growth of both wild-type and streptomycin-resistant *Xcc* strains (Fig. 3a, 3c). However, streptomycin S1 and S2 exerted no antibacterial effect on the streptomycin-resistant *Xcc* strains (Fig. 3c, 3d). S1 and S2 had antagonistic effects on the wild-type *Xcc* strains, with inhibition zones ranging from 28.17 ± 0.69 to 28.88 ± 0.61 mm and 15.10 ± 0.81 to 15.54 ± 0.58 mm, respectively. Bv-17 showed antibacterial activity with inhibition zones ranging from 20.0 ± 1.21 to 21.58 ± 0.81 and 19.70 ± 0.81 to 19.81 ± 1.49 for both wild-type and mutant *Xcc* strains, respectively. However, Bv-21 exhibited the highest antibacterial activity against *Xcc*W2 and *Xcc*M4 with inhibition zones of 22.91 ± 0.45 and 20.28 ± 0.53, respectively (Fig. 3b, 3d).

3.3. Determination of MIC and MBC of the ethyl acetate extracts against Xcc

The MIC and MBC of crude ethyl acetate extracts of strain Bv-17 and Bv-21 were determined against all *Xcc* strains by using the broth micro dilution method. Among the antagonistic strains of *B. velezensis*, Bv-17 and Bv-21 showed the highest antibacterial activity during the *in vitro* study against *Xcc*. Therefore, the metabolites of these two isolates were further selected for the determination of MIC and MBC against the wild-type *Xcc* strain (*Xcc*W1) and the streptomycin-resistant mutant *Xcc* strains (*Xcc*M4, *Xcc*M5, *Xcc*M6, *Xcc*M7, and *Xcc*M8). For the Bv-17 isolate, the MIC and MBC values of the ethyl acetate extract ranged from 62.5 to 125.0 μg mL^{-1} and for *B. velezensis* Bv-21 the MIC and MBC ranged from 31.25 to 125.0 μg mL^{-1}. However, against *Xcc*M5, the MIC and MBC of ethyl acetate extract of Bv-21 were determined to be 31.25 μg mL^{-1} and 62.5 μg mL^{-1}, respectively. The MIC of streptomycin against wild-type *Xcc*W1 strain ranged from 78 to 156 μg mL^{-1}. However, the MBC of streptomycin against the mutant *Xcc* strains was relatively high and ranged from 375 to 1500 μg mL^{-1} (Fig. S2; Table 2).

3.4. In vivo biocontrol efficacy of B. velezensis Bv-21

Among the metabolites of *Bacillus* spp. tested, *B. velezensis* Bv-17 and *B. velezensis* Bv-21 held the highest antibiotic activity. Furthermore, *B. velezensis* Bv-21 was tested for biocontrol efficacy against a streptomycin-resistant mutant *Xcc*M4. In the detached leaf assay, all the citrus leaves were infiltrated with 0.1 mL of SDW, *Xcc*M4, and a mixture of *Xcc*M4:Bv-21. After a period of 7 dpi, both *Xcc*M4 and the mixture of *Xcc*M4:Bv-21 generated lesions of CBC (Fig. 4a). Lesions developed on *Xcc*M4-infiltrated leaves were visible on both faces and appeared in the form of water-soaked margins that were surrounded by yellow rings. However, the leaves infiltrated with Bv-21 exhibited no visible toxic effects or disease symptoms on the leaves of Hwanggeum hyang citrus (Fig. 4a). The leaves infiltrated with the mixture of *Xcc*M4:Bv-21

Fig. 1. Phylogenetic analysis of 16S rRNA gene sequences that highlight the position of selected *Bacillus* species closely related to the representatives of the *Bacillus* genus, such as, *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis, B. tequilensis*, *B. cereus,* and *B. pumilus.* Phylogenetic tree was constructed using Maximum Likelihood method. Parentheses indicate the accession numbers obtained from the National Center for Biotechnology Information (NCBI) database. The bootstrap values (%) are mentioned at the nodes and are obtained by repeating the analysis 1000 times. The scale bar represents a nucleotide substitution rate of 0.005 per nucleotide position.

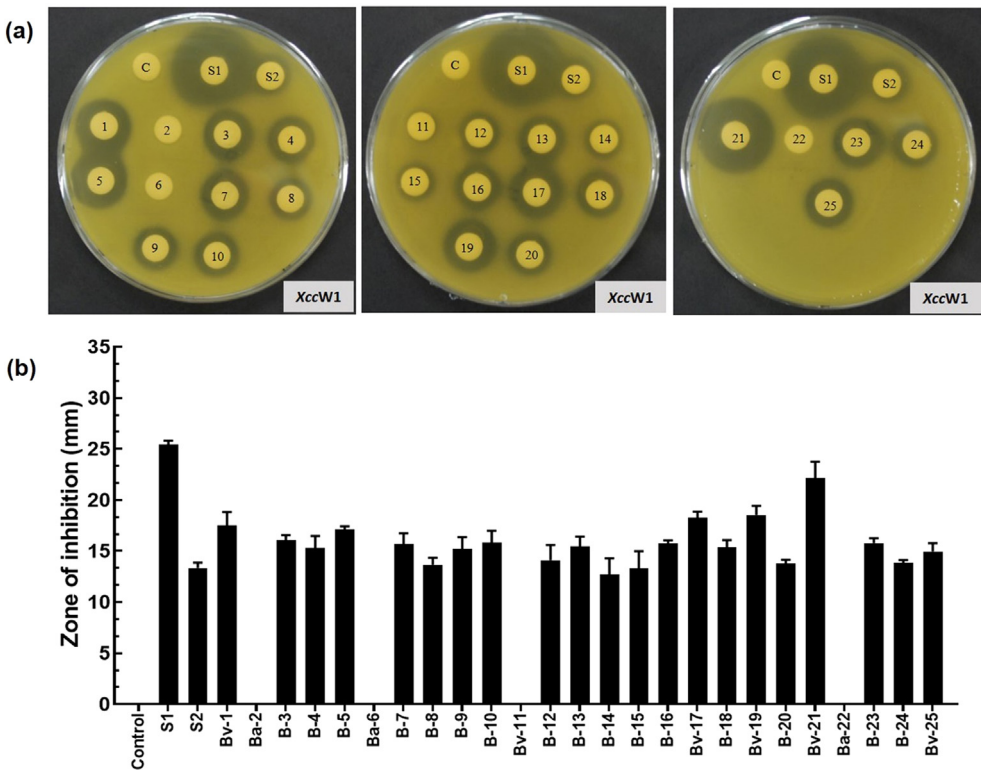

Fig. 2. *In vitro* antagonistic effect of the ethyl acetate extract of *Bacillus* spp. (1–25) and streptomycin on the virulence of *X. citri* subsp. *citri* (*Xcc*). (**a**) Bactericidal activity was conducted against the wild-type *Xcc*W1. 1–25: Ethyl acetate extract of 25 *Bacillus* strains (1.0 mg mL^{-1}); C: Control (methanol); S1: Streptomycin (1.0 mg mL^{-1}); S2: Streptomycin (0.1 mg mL^{-1}). (**b**) Measurement of inhibition zones (mm) of ethyl acetate extracts of *Bacillus* spp. against *Xcc*W1. The error bars in a two-tailed *t*-test represented the standard deviation, and the result was found to be statistically significant at $p < 0.05$.

developed smaller necrotic lesions than those infiltrated by *Xcc*M4 alone (15.67 ± 1.6 mm vs. 11.54 ± 3.7 mm; Fig. 4b). The artificial inoculation of *Xcc*M4:Bv-21 suspension reduced the *Xcc* populations (*Xcc*M4) in citrus leaves by approximately 81.68% compared to those in the *Xcc*M4 alone (Fig. 4c). On the other hand, leaves infiltrated with SDW and Bv-21 showed no colonies on the YNA plates containing streptomycin.

4. Discussion

The role of biological agents in the control of crop diseases is gradually gaining the attention of microbiologists and plant pathologists. Unlike conventional pesticides, biological agents are considered as sustainable, safer to the environment and enhance the soil bacterial diversity (Jaffuel et al., 2019; You et al., 2016).

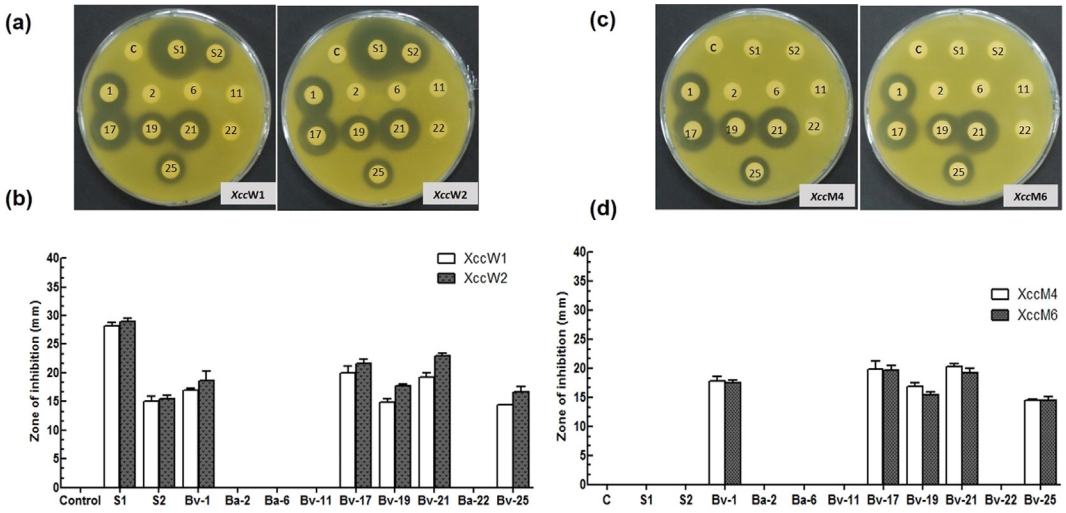

Fig. 3. *In vitro* antagonistic effect of the ethyl acetate extract of selected *Bacillus* spp. and streptomycin on wild-type and streptomycin-resistant *X. citri* subsp. *citri* (*Xcc*) strains. (**a**) Bactericidal activity was conducted using the pathogenic wild-type *Xcc*W1 and *Xcc*W2. (**b**) Measurement of inhibition zones (mm) of ethyl acetate extracts of *Bacillus* spp. against *Xcc*W1 and *Xcc*W2. (**c**) Bactericidal activity was conducted using the pathogenic strains *Xcc*M4 and *Xcc*M6 (streptomycin-resistant). (**d**) Measurement of inhibition zones (mm) of ethyl acetate extracts of *Bacillus* spp. against the streptomycin-resistant *Xcc*W4 and *Xcc*W6. C: control (methanol); 1, 2, 6, 11, 17, 19, 21, 22, and 25: Ethyl acetate extract (1.0 mg mL^{-1}) of selected *Bacillus* spp.; S1: streptomycin (1.0 mg mL^{-1}); S2: streptomycin (0.1 mg mL^{-1}). The error bars in a two-tailed *t*-test represented the standard deviation, and the result was found to be statistically significant at $p < 0.05$.

Table 2
MIC and MBC of the ethyl acetate extract of Bv-17 and Bv-21, and streptomycin against *Xcc* strains.

Xcc strains	Bv-17 ethyl acetate extract		Bv-21 ethyl acetate extract		Streptomycin	
	MIC (μg mL^{-1})	MBC (μg mL^{-1})	MIC (μg mL^{-1})	MBC (μg mL^{-1})	MIC (μg mL^{-1})	MBC (μg mL^{-1})
*Xcc*W1	62.5	125.0	62.5	125.0	78.0	156
*Xcc*M4	62.5	125.0	62.5	125.0	375.0	750.0
*Xcc*M5	62.5	125.0	31.25	62.5	375.0	750.0
*Xcc*M6	62.5	125.0	62.5	125.0	375.0	750.0
*Xcc*M7	62.5	125.0	62.5	125.0	750.0	1500.0
*Xcc*M8	62.5	125.0	62.5	125.0	375.0	750.0

In this study, we have investigated 25 *Bacillus* strains that were initially identified and stored as *B. amyloliquefaciens* (except Bv-25) in KACC (Table 1). However, the homology analysis of 16S rRNA gene sequences indicated that several KACC strains were indeed *B. velezensis*. According to previous studies, *B. amyloliquefaciens* is closely related to *B. velezensis* and is difficult to distinguish using traditional phenotypic approaches (Rabbee et al., 2019a). Furthermore, *B. amyloliquefaciens* strains are genetically related to *B. subtilis* species complex, which includes *B. subtilis*, *B. velezensis*, *B. pumilus*, and *B. licheniformis* (Fan et al., 2017). In 2017, Fan et al. analyzed the nucleotide sequence of 66 *B. velezensis* and related bacterial species based on its core genomes and *rpoB* (RNA polymerase beta-subunit) gene. Dendrograms based on sequence analysis revealed the presence of three tightly linked branches of (1) *B. siamensis* (2) soil-borne *B. amyloliquefaciens*, and (3) a conspecific group that included *B. methylotrophicus*, *B. amyloliquefaciens* subsp. *plantarum* and *B. velezensis* (Fan et al., 2017). Phylogenetic analysis and extended genome sequence data revealed that *B. amyloliquefaciens* subsp. *plantarum* and *B. methylotrophicus* is actually classified as *B. velezensis* (Dunlap et al., 2016; Rabbee et al., 2019a). *B. amyloliquefaciens* strains were well known for producing industrial enzymes such as amylase, protease, and glucanase (Fan et al., 2018) and demonstrated reduced potentiality in the synthesis of non-ribosomal secondary metabolites that act as antimicrobial compounds (Rückert et al., 2011). On the other hand, *B. velezensis* produces abundant secondary metabolites with antimicrobial activities against pathogenic microorganisms (Chen et al., 2018; Niazi et al., 2014).

To test the antibacterial efficacy of ethyl acetate extracts from all *Bacillus* spp. against *Xcc*, a disk diffusion assay was performed. The result showed that the ethyl acetate extracts of 21 *Bacillus* isolates exhibited different degrees of inhibition against *Xcc*W1. After initial screening against *Xcc*W1, we evaluated nine *Bacillus* strains for checking the antagonistic effect against two wild-type and two streptomycin-resistant *Xcc* strains. We found that ethyl acetate extracts of *B. velezensis* have antibacterial activity against *Xcc* pathogens. Moreover, the solvent extract of *B. velezensis* Bv-21 displayed the strongest antibacterial activity against both wild-type and streptomycin-resistant *Xcc* strains. Previously, the methanol extracts of *B. velezensis* displayed antimicrobial activity against *Ralstonia solanacearum* (causative agent of tomato wilt) and *Fusarium oxysporum* (causative agent of banana *Fusarium* wilt) under both laboratory and greenhouse conditions (Cao et al., 2018). In a another study, it was shown that ethyl acetate extract of *B. velezensis* Lle-9 exhibited antifungal activity against *F. oxysporum* with the growth inhibition percentage of 68.56 ± 2.35% (Khan et al., 2020). Inoculation of *B. velezensis* into the root of different plant seedlings also reported to increase the plant height as well as root and shoot biomass (Balderas-Ruíz et al., 2020).

Host plant colonization is a critical process for the survival of any plant pathogens. In our experiment, detached citrus leaves were infiltrated with a bacterial mixture of *Xcc*M4: Bv-21 and reduced the disease symptoms as compared to those infiltrated with *Xcc*M4 alone. Hwanggeum hyang leaves infiltrated with Bv-21, on the other hand, showed no signs of disease, indicating that Bv-21 is not pathogenic to citrus leaves. Furthermore, the number

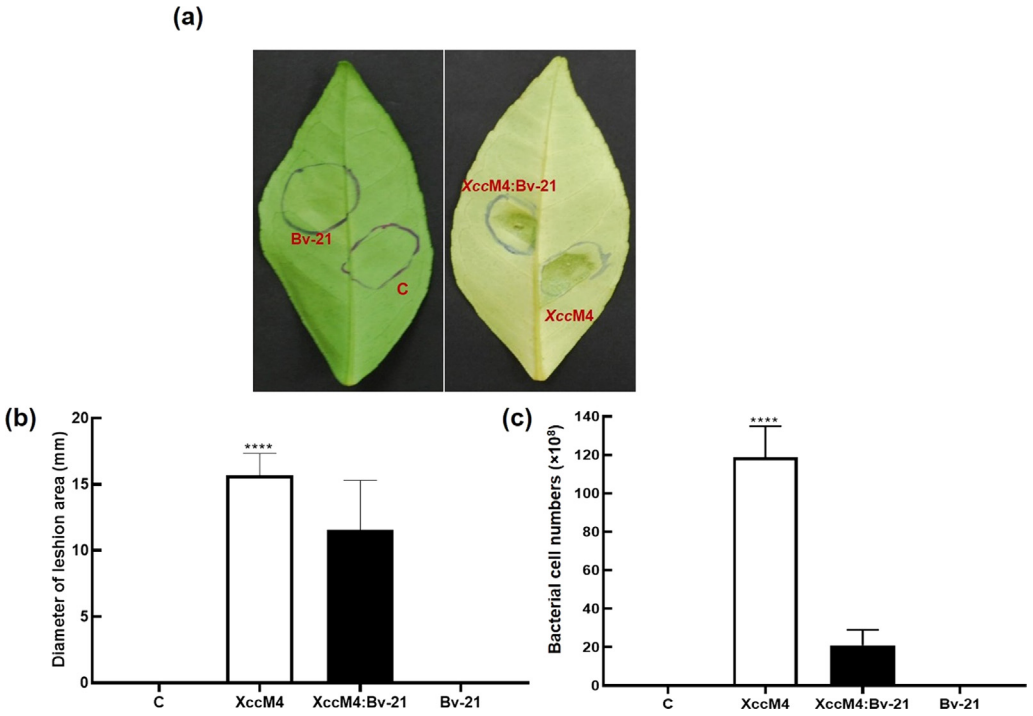

Fig. 4. Development of disease symptoms in the leaves of Hwanggeum hyang citrus at 7 dpi through the inoculation of *Xanthomonas citri* subsp. *citri* (*Xcc*M4) and suspension of *Xcc*M4:Bv-21 **(a)** The leaves were infiltrated with 0.1 mL aliquots of OD_{600nm} 0.4 *Xcc*M4 mixed with SDW (*Xcc*M4); OD_{600nm} 0.4 *Xcc*M4 mixed with OD_{600nm}0.4 Bv-21 (*Xcc*M4:Bv-21) and Bv-21. As a control, leaves were inoculated with SDW **(b)** Diameter of disease lesions measured at 7 dpi. **(c)** Quantification of *Xcc*M4 from the leaves treated with *Xcc*M4 and suspension of *Xcc*M4:Bv-21. Three independent replicates were tested for each treatment. One way ANOVA (analysis of variance) test was performed and result was found significant ($p < 0.0001$) among the groups.

of phytopathogenic bacteria was reduced in citrus leaves that had been artificially inoculated with *Xcc*M4:Bv-21. More specifically, the suspension of *Xcc*M4: Bv-21 reduced *Xcc* populations in citrus leaves by approximately 81.68% relative to *Xcc*M4 alone. In our previous work, co-infiltration of endophytic bacteria *B. thuringiensis* TbL-22 and *Xcc* reduced the lesions of CBC by 64.05% relative to positive controls (Islam et al., 2019). Biocontrol property of *B. velezensis* AP-3 was reported to control the severity of Fusarium wilt (caused by *F. oxysporum* f. sp. *lycopersici*) in tomato by 50% when compared with control (Medeiros and Bettiol, 2021).

Bacillus spp. are well known as biocontrol agents against pathogenic microbes due to their capability to produce a wide variety of antimicrobial substances, such as lipopeptides, polyketides and some other volatile organic compounds (Chen et al., 2020). *B. velezensis* and *B. amyloliquefaciens* are morphologically similar, however; genome analysis revealed that some of the *B. amyloliquefaciens* strains with biocontrol potential were *B. velezensis* (Magno-erez-Bryan et al., 2015). *B. velezensis* exhibits biocontrol activity against the rice blight and leaf streak pathogens *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola,* by producing the antibacterial compounds difficidin and bacilysin (Wu et al., 2015). Fengycins produced by *B. amyloliquefaciens* MEP_218 exhibited antibacterial potentiality against *X. axonopodis* pv. *vesicatoria*, a plant pathogen causing bacterial spot disease (Medeot et al., 2020). In a similar study, the *B. methylotrophicus* strain NKG-1 isolated from China exerted biocontrol activity against *B. cinerea* and thirteen phytopathogenic fungi. Strain NKG-1 reduced gray tomato mold infection by 60% in a detached leaf assay and enhanced tomato plant growth in both greenhouse and field trial experiments (Ge et al., 2016). In another research, it was shown that bacilysin synthesize by *B. velezensis* contributed biocontrol potentiality against Soybean root rot disease pathogen *Phytophthora sojae* (Han et al., 2021).

5. Conclusions

The ability of *B. velezensis* to synthesize antibacterial compounds has opened a new possibility in order to control plant pathogenic microbes. Our findings strongly suggest that metabolic extracts of *B. velezensis* can control the spread of *Xcc* under both *in vitro* and *in vivo* conditions. Therefore, identification of the antibacterial compounds of *B. velezensis*, and investigation of their biosynthetic pathways and regulatory genes under different culture conditions would be the additional steps in order to control *Xcc*. Furthermore, *B. velezensis* could be developed as a biofertilizer for more sustainable agriculture practices. All these findings point to *B. velezensis* can be the basis of biocontrol agent to combat *Xcc* in an efficient and eco-friendly manner.

Author contributions

M.F.R., Md. N. I., and K.-H.B. collected the data and wrote the manuscript. All authors have read and approved the final manuscript.

Funding

This research was funded by NRF-2019R1F1A1052625.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

Authors appreciate the research fund provided by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2019R1F1A1052625).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.12.005.

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