Biocontrol of Citrus Canker Disease Caused by *Xanthomonas citri* subsp. *citri* Using an Endophytic *Bacillus thuringiensis*

Md. Nurul Islam1†, Md. Sarafat Ali1†, Seong-Jin Choi2, Jae-Wook Hyun3, and Kwang-Hyun Baek1*

1Department of Biotechnology, Yeungnam University, Gyeongsan 38541, Korea
2Department of Biotechnology, Catholic University of Daegu, Gyeongsan 38430, Korea
3Citrus Research Station, National Institute of Horticultural and Herbal Science, Rural Development Administration, Seogwipo 63607, Korea

(Received on March 20, 2019; Revised on July 5, 2019; Accepted on August 12, 2019)

Citrus canker is a devastating disease of citrus caused by *Xanthomonas citri* subsp. *citri* (*Xcc*). A total of 134 endophytic bacteria were isolated from various gymnospermic and angiospermic plants. They were screened for their antagonistic activities against three wild-type and six streptomycin-resistant *Xcc* strains. TbL-22 and TbL-26, both later identified as *Bacillus thuringiensis*, inhibited all the wild and resistant *Xcc* strains. TbL-22 exerted the highest antagonistic activity against *Xcc*W3 and *Xcc*M6 with inhibition zones of 20.64 ± 0.69 and 19.91 ± 0.87 mm, respectively. Similarly ethyl acetate extract of TbL-22 showed highest inhibition zones 15.31 ± 2.08 and 19.37 ± 3.17 mm against *Xcc*W3 and *Xcc*M6, respectively. TbL-22 reduced canker incidence on infected leaves by 64.05% relative to positive controls. Scanning electron microscopy revealed that the cell membranes of *Xcc* treated with ethyl acetate extract of TbL-22 were ruptured, lysed, and swollen. *B. thuringiensis* TbL-22 can effectively and sustainably controls streptomycin-resistant citrus canker.

**Keywords:** *Bacillus thuringiensis*, biocontrol, citrus canker, endophytic bacteria

**Handling Editor:** Sang, Mee Kyung

†These authors contributed equally to this work.
*Corresponding author.
Phone) +82-53-810-3029, FAX) +82-53-810-4769
E-mail) khbaek@ynu.ac.kr

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0) which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Articles can be freely viewed online at www.ppjonline.org.
Biocontrol of Citrus Canker Disease by *Bacillus thuringiensis*

developed due to its prolonged and excessive use (Carter et al., 2000; Hyun et al., 2012). In a recent study, 72% of the *Xcc* strains isolated from Jeju Island, Republic of Korea were found to be resistant to streptomycin (Hyun et al., 2012).

The cultivation of citrus varieties resistant to citrus canker is an effective and eco-friendly means of controlling the disease. The development of disease-resistant varieties, however, is time-consuming, laborious, and not widely available. Therefore, it is neither practical nor convenient as a citrus canker control method. For sustainable citrus production, more effective and sustainable techniques are required to control citrus canker.

Endophytes are important components in plant-microbe ecosystems (Li et al., 2012) and may serve as environmentally sustainable resources for citrus canker control. Endophytic microorganisms are bacteria or fungi that colonize the internal host tissues in high numbers but do not harm the host or induce plant disease symptoms (Quispel, 1992). Endophytic bacteria (EB) are endosymbiotic microorganisms that colonize the same ecological niches as phytopathogens, so they can function as biocontrol agents and coexist safely with plant tissues (Berg et al., 2005; Kloeper and Beauchamp, 1992). The application of EB as biocontrol agents enhances disease resistance (Van Loon et al., 1998) and provides a safe alternative to chemical control products (Backman and Sikora, 2008). Several EB used as biocontrol agents have been isolated from various plants, e.g., *Burkholderia cepacia* from citrus (Tan et al., 2007), *Aureobacterium saperdae*, *Bacillus pumilus*, *Phyllobacterium rubiacearum*, and *Pseudomonas putida* from cotton (Chen et al., 1995), and *Enterobacter cloacae* from rice (Yang et al., 2001).

In this study, EB isolated from different gymnosperm and angiosperm tissues were evaluated for their efficacy against citrus canker disease. Antibacterial efficacy was tested using three wild-type and six streptomycin-resistant mutant *Xcc* strains. The objectives of this research were to (1) screen for effective EB against *Xcc* strains and (2) determine the efficacies of the best EB able to control citrus canker through both *in vitro* and *in vivo* assays.

### Materials and Methods

**Collection of plant materials and isolation of EB.** Various gymnosperms and angiosperms, including *Metasequoia glyptostroboides*, *Ginkgo biloba*, *Taxus brevifolia*, *Pinus densiflora*, *Salix babylonica*, and *Salix chaenomeloides* were sampled at Yeungnam University, Gyeongsan, Republic of Korea in October 2014. Leaves, fruit, seeds, and cones (Table 1) were collected, placed in plastic bags, and brought to the laboratory for EB isolation.

EB were isolated according to standard procedures (De Oliveira Costa et al., 2012) with minor modifications. Plant parts were washed under running tap water. Visibly injured organs were discarded. The samples were dried and weighed on a digital balance. Tissues were sterilized by exposure to 70% v/v alcohol for 60 s, 2% sodium hypochlorite for 180 s, and 100% ethanol for 30 s. The tissues were then rinsed several times with sterile distilled water (SDW) and dried with sterile filter papers. The plant parts were then ground with sterile 3 ml/g of aqueous NaCl solution (0.9% w/v) using a sterile mortar and pestle. The tissue extracts were incubated at 28°C for 3 h to release all of the EB.

The tissue extracts were diluted to $10^{-1}$ and $10^{-2}$ with sterile NaCl solution. They were then applied with a stainless steel spreader onto Petri dishes containing 25% yeast extract, nutrient broth and agar (YNA), 25% nutrient agar, de Man, Rogosa and Sharpe agar, or Luria-Bertani agar media to obtain single bacterial colonies. The plates were incubated for up to 15 days at 28°C. All colonies were counted and reported as the number of colony-forming units per gram fresh tissue (cfu/g fresh weight (FW)). Single colony isolation was repeated at least three times to obtain pure EB isolates. Colony form, margin, color, and elevation were recorded. The selected EB were cultured in various media broths and stored in 50% glycerol at –80°C until use.

The isolated EB were identified with 16S rRNA gene sequencing. The sequencing data, alignment, and phylogenetic trees were analyzed by Molecular Evolutionary Genetics Analysis (MEGA) 6 software version 6.0. The phylogenetic trees were also analyzed by the neighbor-joining method (Susilowati et al., 2015; Tamura et al., 2013).

**Antagonistic assay of EB against citrus canker pathogen Xcc.** Nine *Xcc* strains, including three wild-type and six streptomycin-resistant mutants (Hyun et al., 2012) were used in this study. The wild-type (*Xcc*W1 [*Xcc* 19-18], *Xcc*W2 [*Xcc* 10-5], and *Xcc*W3 [*Xcc* 53-4]) and streptomycin-resistant mutant (*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]) strains were kindly provided by Dr. Hyun of the Citrus Research Station, National Institute of Horticultural and Herbal Science, Rural Development Administration, Jeju, Korea. The wild-type *Xcc* were grown in yeast extract-nutrient broth (YNB) medium. The streptomycin-resistant *Xcc* strains were incubated in YNB supplemented with 50 mg/ml streptomycin. All *Xcc* were stored in 50% glycerol at –80°C until use.
The EB isolates were screened for antagonistic activity against the nine strains of \textit{Xcc} following standard procedures (Roh et al., 2009) with minor modifications. Screening was performed by culturing 134 EB isolates in 96-well plates with 10 µl individual bacterial strain grown in YNB and the plates were incubated for 36 h at 28°C. Then the cultured bacteria were again grown on square plates (size as 127.94 × 85.50 × 16.25 mm, SPL Life Sciences Co., Ltd., Pocheon, Korea) using 25% YNA (1.5% agar) media following incubation for 36 h at 28°C. The EB were then killed by placing 1 ml chloroform on the square plate lids and inverting the plates for 15 min. The lids were then removed and the plates were left to stand for 20 min to vent any chloroform residue, then the plates were treated with UV light for 15 min to kill the bacteria completely. Simultaneously, 30 µl aliquots of \textit{Xcc} cultures that had been grown overnight in YNB media at 28°C were suspended in 10-ml fresh YNB media (0.75% agar) at 55°C, mixed gently, then poured slowly and uniformly on the square plates. After the agar solidified, all the plates were sealed and incubated at 28°C for 24 h. Inhibition zone diameters were measured using an electronic digital caliper (M500-182M, Hando, Cheonan, Korea) with a 1/100 mm precision.

Further analysis for the antagonistic activity was done using Tbl-22 (\textit{B. thuringiensis}) and Tbl-26 (\textit{B. thuringiensis}) as the isolates with the highest antagonistic activity along with TbS-8 (\textit{B. altitudinis}) and Tbl-19 (\textit{B. altitudinis}) as the isolates of another \textit{Bacillus} species against all of \textit{Xcc} strains. The above-described method was used the same way using all \textit{Xcc} strains and using Petri dish (90 mm diameter) instead of square plates.

**Antagonistic activity of the extracted EB against Xcc.**

After being sub-cultured on YNA plates for 24 h, single colonies of the antagonistic EB were added to 200 ml YNB in 1-liter Erlenmeyer flasks with continuous shaking at 180 rpm at 28°C for 48 h. The culture broths were centrifuged at 3,500 rpm for 15 min at 4°C. The supernatant was collected, mixed with equal volumes of ethyl acetate and incubated overnight. Ethyl acetate was selected for the metabolite extraction process for its low boiling point and moderate polarity. Simultaneously, the bacterial pellets were suspended in 20 ml methanol, placed in a sonicating bath (WUC-A03H, Daihan Scientific Co., Wonju, Korea) for 15 min to break the cells and incubated overnight. The ethyl acetate layer was then collected in a separating funnel. The methanol extract was separated from the bacterial debris by centrifugation at 3,500 rpm for 15 min at 4°C. Both extracts were dried at 50°C in a rotary evaporator (A-1000S, EYELA, Tokyo, Japan). The dried extracts were then removed from the evaporation flask, dissolved in 2 ml methanol, and air-dried in tubular glass vials. The ethyl acetate extract was measured and dissolved in 5% dimethyl sulfoxide (DMSO) in methanol to make 0.1 g/ml. Total 50-µl aliquots of this dilution were used to the well diffusion assay. The antagonistic activities of the EB against the \textit{Xcc} strains were determined by measuring the inhibition zone diameters.

**Determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC).**
The MIC and MBC of the ethyl acetate extract of Tbl-22 against \textit{Xcc} were determined by the twofold dilution method (Patra et al., 2015) with minor modifications. Five percent DMSO was used both as a control and a solvent to homogenize the ethyl acetate extract for the MIC and MBC tests. Various dilutions of the ethyl acetate extract of Tbl-22 in YNB media were prepared. The lowest concentration of Tbl-22 at which there was zero visible \textit{Xcc} growth was taken to be the MIC and was recorded in mg/ml. The MIC and the next higher concentration were selected, spread on YNA plates and incubated at 28°C for 24 h. The lowest concentration of Tbl-22 at which no pathogenic bacteria grew on the 25% YNA plates was considered the MBC and was expressed in mg/ml.

**Analysis using scanning electron microscopy (SEM).**
The effects of the ethyl acetate extract of Tbl-22 on \textit{Xcc} cell morphology were determined by SEM analysis. Both the 5% DMSO control and the samples treated with the ethyl acetate extract of Tbl-22 were applied to the four \textit{Xcc} strains and observed under SEM using a slightly modified version of the procedure described by Bajpai et al. (2009). The four different \textit{Xcc} strains used for SEM analysis were \textit{Xcc}W1, \textit{Xcc}M4, \textit{Xcc}M6, and \textit{Xcc}M8, which were selected based on their genotypic classification. Overnight bacterial culture samples were washed gently with 0.05 M phosphate buffer solution (pH 7.4) and fixed with 2.5% v/v glutaraldehyde in SDW. The specimens were dehydrated with sequential exposures to ethanol ranging from 50 to 100%. The ethanol was then replaced with \textit{tert}-butyl alcohol. The specimens were sputter-coated with platinum in an ion coater for 120 s then examined under an SEM (S-4100, Hitachi, Tokyo, Japan).

**Biocontrol efficacy of the antagonistic EB.** Pathogenic wild-type \textit{Xcc}W2 were grown at 28°C in YNB media. Overnight bacterial cell cultures were harvested at 4°C, centrifuged at 3,500 rpm for 15 min, and suspended in SDW to make a concentration of 0.4 OD$_{600nm}$ determined.
by spectrophotometer (ASP-3700, ACTGene, Piscataway, NJ, USA). For the detached leaf assay (Brown and Soepena, 1994), fully expanded immature (4-week-old) leaves were excised from Hwanggeum hyang, a highly canker-susceptible cultivar generated from the cross between Citrus unshiu × C. sinensis and C. unshius. The leaves were infiltrated with a 0.1 ml bacterial suspension using a 1-ml needleless syringe. The bacterial suspensions were prepared by mixing 0.4 OD 600nm XccW2 either with 0.4 OD 600nm EB suspension or with SDW. The infiltrated leaves were placed on three layers of sterile tissue paper moistened with SDW in a humid box and incubated in a growth chamber at 28°C. The infiltrated leaves were photographed, and any canker lesions that developed were measured 7 days post-infiltration (dpi) (Martínez-Medina et al., 2013).

**Pathogenicity test of the isolated EB.** To determine whether TbL-22 and TbL-26 could cause disease in citrus plants, bacterial suspensions at 0.4 OD 600nm concentration were infiltrated into Hwanggeum hyang leaves as described by Brown and Soepena (1994). The pathogenic bacterium XccW2 and SDW were used as a positive and negative control, respectively. Citrus canker disease symptoms were assessed for up to 7 dpi.

### Statistical analysis
All data were expressed as the mean ± standard deviation by measuring four independent replicates per experiment. Statistical analysis of the results was conducted by one-way ANOVA followed by the Duncan’s multiple range test at $P < 0.05$ using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

**Table 1.** Isolation of endophytic bacteria from different gymnosperms and angiosperms

| Plant species          | Tissues          | No. of isolates | cfu/g of fresh tissue |
|------------------------|------------------|-----------------|-----------------------|
| Metasequoia glyptostroboides | Leaves        | 4               | $3.3 \times 10^4$     |
| Ginkgo biloba          | Leaves          | 11              | $1.8 \times 10^3$     |
| Taxus brevifolia       | Leaves, Seeds   | 23, 8           | $6.0 \times 10^4$, $7.2 \times 10^3$ |
| Pinus densiflora       | Leaves          | 2               | $9.6 \times 10^3$     |
| Salix babylonica       | Leaves          | 24              | $1.5 \times 10^4$     |
| Salix chaenomeloides   | Leaves          | 9               | $5.9 \times 10^4$     |
| Total                  |                 | 134             |                       |

**Table 2.** Screening and identification of antibacterial activity of endophytic bacteria isolated from gymnosperms and angiosperms against XccW1

| Isolate | Species                  | Coverage (%) | Max. identity (%) | Antibacterial activity a |
|---------|--------------------------|--------------|-------------------|--------------------------|
| TbL-6   | Kocuria palustris        | 97           | 99                | 18.05                    |
| TbL-7   | Moraxella osloensis      | 99           | 99                | 18.03                    |
| TbS-8   | Bacillus altitudinis     | 98           | 99                | 18.15                    |
| TbL-9   | Moraxella osloensis      | 99           | 99                | 16.79                    |
| TbL-11  | Staphylococcus epidermidis | 98         | 99                | 17.30                    |
| TbF-18  | Staphylococcus saprophyticus | 99       | 99                | 12.59                    |
| TbL-19  | Bacillus altitudinis     | 97           | 99                | 17.65                    |
| TbF-20  | Dermacoccus profundi    | 97           | 99                | 17.39                    |
| TbF-21  | Staphylococcus warneri   | 97           | 99                | 16.05                    |
| TbL-22  | Bacillus thuringiensis   | 97           | 99                | 18.68                    |
| TbS-23  | Bacillus stratosphericus | 98           | 99                | 18.18                    |
| TbL-24  | Bacillus stratosphericus | 83           | 99                | 18.15                    |
| TbL-26  | Bacillus thuringiensis   | 98           | 100               | 18.52                    |
| TbL-31  | Staphylococcus epidermidis | 98       | 99                | 12.73                    |
| TbL-34  | Bacillus altitudinis     | 97           | 99                | 18.06                    |
| SbL-44  | Pseudomonas tremae       | 97           | 99                | 18.12                    |
| SbL-46  | Pantoea vagans          | 94           | 97                | 18.17                    |
| SbL-50  | Xanthomonas gardneri     | 83           | 99                | 12.38                    |

TbL, Taxus brevifolia leaf; TbS, T. brevifolia seed; TbF, T. brevifolia fleshy part of fruit; SbL, Salix babylonica leaf.
aDiameter of inhibition zones of antibacterial activity are expressed in millimeters.
Isolation and identification of EB. A total of 134 morphologically distinct EB were isolated from *M. glyptostrobodies*, *G. biloba*, *T. brevifolia*, *P. densiflora*, *S. babylonica*, and *S. chaenomeloides*. They were obtained from the leaves (73 isolates, 54.48%), fruit (49 isolates, 36.57%), seeds (8 isolates, 5.97%), and cones (4 isolates, 2.98%) (Table 1). The tissue-specific cell counts (CFU) per gram FW were in the range of $1.8 \times 10^2$-$6.0 \times 10^4$ cfu/g in the fruits, $1.8 \times 10^3$-$1.5 \times 10^5$ cfu/g in the leaves, $7.2 \times 10^3$ cfu/g in the seeds, and $1.2 \times 10^3$ cfu/g in the cones (Table 1).

### Table 3. Antagonistic activity of isolated endophytic bacteria against *Xanthomonas citri* subsp. *citri* (*Xcc*)

| Isolate | Species | *Xcc*W1 | *Xcc*W2 | *Xcc*W3 | *Xcc*M4 | *Xcc*M5 | *Xcc*M6 | *Xcc*M7 | *Xcc*M8 | *Xcc*M9 |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Tbl-22  | *Bacillus thuringiensis* | 15.33 ± 0.42 a | 20.64 ± a | 19.72 ± a | 19.90 ± a | 19.91 ± a | 19.84 ± a | 19.78 ± a | 18.44 ± a |
| Tbl-26  | *Bacillus thuringiensis* | 11.65 ± 0.22 b | 16.54 ± b | 16.96 ± b | 15.16 ± b | 17.34 ± b | 15.89 ± b | 16.64 ± b | 15.78 ± b |

*a* Diameter of inhibition zone (mm) of three independent replicates expressed as the mean ± standard deviation.<br>

*b* Values with different lower case letters in the same column are significantly different at $P < 0.05$ by Duncan’s test.

**Results**

### Isolation and identification of EB. A total of 134 morphologically distinct EB were isolated from *M. glyptostrobodies*, *G. biloba*, *T. brevifolia*, *P. densiflora*, *S. babylonica*, and *S. chaenomeloides*. They were obtained from the leaves (73 isolates, 54.48%), fruit (49 isolates, 36.57%), seeds (8 isolates, 5.97%), and cones (4 isolates, 2.98%) (Table 1). The tissue-specific cell counts (CFU) per gram FW were in the range of $1.8 \times 10^2$-$6.0 \times 10^4$ cfu/g in the fruits, $1.8 \times 10^3$-$1.5 \times 10^5$ cfu/g in the leaves, $7.2 \times 10^3$ cfu/g in the seeds, and $1.2 \times 10^3$ cfu/g in the cones (Table 1).

### Antagonistic activity of EB against *Xcc.* In the primary screening, all EB isolates were tested for antagonistic activity against *Xcc*W1. Eighteen of the EB efficacious against *Xcc*W1 were identified by 16S rRNA gene sequencing analysis (Table 2). Among them, Tbl-22 and Tbl-26 exerted the highest antagonistic activity against all *Xcc*, so
they were selected for further study (Table 3). Phylogenetic trees were constructed using the 16S rRNA sequences of TbL-22 and TbL-26 and those of their closest relatives. The phylogenetic tree analysis revealed that TbL-22 and TbL-26 had 99% and 100% identity with the 16S rRNA gene sequences of *B. thuringiensis* Bt 407 and *B. thuringiensis* IAM12077, respectively (Fig. 1A and B).

In *Xcc* cultures, TbL-22 and TbL-26 exhibited the highest antagonistic activity with inhibition zones of 20.64-15.33 and 17.34-11.65 mm, respectively (Table 3, Fig. 2). TbL-22 had greater activity than TbL-26 against all *Xcc*. Only the ethyl acetate extract of TbL-22 was antagonistic against all *Xcc* strains (Table 4, Fig. 3). The extract of TbL-22 exerted the highest antagonistic activity of all, and produced inhibition zones of 15.31 ± 2.08 mm and 19.37 ± 3.17 mm against *Xcc* W3 and *Xcc* M6, respectively. The extract of TbL-26 produced inhibition zones of 13.81 ± 1.05 mm and 18.08 ± 4.69 mm against *Xcc* W3 and *Xcc* M6, respectively, but had no antagonistic activity against *Xcc* W2 (Table 4, Fig. 3).

**Table 4.** Antagonistic activity of ethyl acetate extract of endophytic bacteria TbL-22 and TbL-26 against *Xcc*

| Isolate | Species       | *Xcc* (inhibition zone in mm)$^a$ |
|---------|---------------|----------------------------------|
|         | *Xcc* W1      | *Xcc* W2 | *Xcc* W3 | *Xcc* M4 | *Xcc* M5 | *Xcc* M6 | *Xcc* M7 | *Xcc* M8 | *Xcc* M9 |
| TbL-22  | *Bacillus*    | 15.24    | 15.07    | 15.31    | 16.31    | 17.16    | 19.37    | 14.32    | 14.26    | 16.81    |
|         | *thuringiensis*| ± 1.14 a$^b$ | ± 0.08 a | ± 3.95 a | ± 1.30 a | ± 3.17 a | ± 1.08 a | ± 0.46 a | ± 3.04 a |
| TbL-26  | *Bacillus*    | 15.27    | –        | 13.81    | 13.96    | 15.54    | 18.08    | 15.64    | 13.44    | 12.56    |
|         | *thuringiensis*| ± 0.09 a | ± 1.05 a | ± 1.39 a | ± 3.97 a | ± 4.69 a | ± 1.92 a | ± 0.88 a | ± 0.20 a |

*$Xcc*, *Xanthomonas citri* subsp. *citri*; –, no inhibition zone.

$^a$Diameter of inhibition zone in (mm) of three independent replicates expressed as the mean ± standard deviation.

$^b$Values with different lower case letters in the same column are significantly different at $P < 0.05$ by Duncan’s test.
**Determination of MIC and MBC.** Since the extract of TbL-22 had the highest antagonistic activity against all Xcc, it was selected for the determination of MIC and MBC. The values of MIC and MBC for the ethyl acetate extract of TbL-22 ranged from 0.625 to 5.00 mg/ml for MIC and from 1.25 to >5.00 mg/ml for MBC (Table 5). The lowest MIC and MBC values were found for XccW2 (0.625 mg/ml and 1.25 mg/ml, respectively) (Table 5).

**SEM analysis with the extract of TbL-22.** The effects of the ethyl acetate extract of TbL-22 on cell morphology were observed for XccW1, XccW2, XccW3, XccM4, XccW9 (Fig. 4). XccW1 was selected to represent the wild-type, and XccM4, XccM6, and XccM8 were selected as representatives of each mutant on the basis of their genotypic classification. All Xcc treated with the ethyl acetate extract presented with ruptured cell surfaces. They were elongated, shrunken, lysed, and swollen. In contrast, the control Xcc cell surfaces were regular, uniform, and smooth (Fig. 4).

**In vivo biocontrol efficacy of the antagonistic endophytes.** TbL-22 and TbL-26 were tested for in vivo biocontrol efficacy against Xcc. In the detached leaf assay, all citrus leaves infiltrated with XccW2, TbL-22, and TbL-26 showed disease symptoms. Nevertheless, the infected areas of the leaves infiltrated with TbL-22 and TbL-26 were

---

Table 5. Evaluation of MIC and MBC of ethyl acetate extract of endophytic bacterium *Bacillus thuringiensis* TbL-22 against *Xanthomonas citri* subsp. *citri* (Xcc) (XccW1–XccW3 [wild-type strains] and XccM4–XccM9 [streptomycin-resistant mutant type strains]) determined by well diffusion assay. 

| Xcc strains | MIC (mg/ml) | MBC (mg/ml) |
|-------------|-------------|-------------|
| XccW1       | 5.000       | >5.00       |
| XccW2       | 0.625       | 1.25        |
| XccW3       | 5.000       | >5.00       |
| XccM4       | 2.500       | 5.00        |
| XccM5       | 5.000       | >5.00       |
| XccM6       | 5.000       | >5.00       |
| XccM7       | 5.000       | >5.00       |
| XccM8       | 5.000       | >5.00       |
| XccM9       | 1.250       | 2.50        |

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.
Biocontrol of Citrus Canker Disease by Bacillus thuringiensis

Fig. 4. Photograph of scanning electron microscopy images of Xanthomonas citri subsp. citri (Xcc) strains treated with 5% dimethyl sulfoxide as control (A, C, E, G) and those treated with the ethyl acetate extract of TbL-22 as treatment (B, D, F, H). (A, B) XccW1. (C, D) XccM4. (E, F) XccM6. (G, H) XccM8. The collapsed and lysed cells are indicated by red arrows.

Discussion

Citrus trees are cultivated throughout the tropical and subtropical regions of the world (Webber, 1967). Citrus canker, which is caused by the bacterial pathogen Xcc, is particularly destructive to citrus. Copper-based bactericides and streptomycin antibiotics have been used in the attempt to control this disease (Behlau et al., 2011; Graham et al., 2008). Nevertheless, they are relatively ineffective on certain Xcc strains (Voloudakis et al., 2005) which have developed resistance to these disease control products (Hyun et al., 2012). Therefore, our aim was to find efficacious alternative citrus canker control measures. EB may serve as powerful canker biocontrol agents.

A total of 134 EB were isolated from the leaves, fruit, seeds, and cones of six different plant species (Table 1). The EB population densities (in cfu/g FW) varied depending on the plant species and tissue (Table 1). Similar findings were published elsewhere (Hallmann et al., 2002; Quadt-Hallman et al., 1997; Sturz and Nowak, 2000). In the present study, the highest EB densities were found in the leaves of all species tested except for G. biloba. The EB density in its fruit was 33 times higher than that in its leaves (Table 1).

Based on the relative diameters of the inhibition zones they formed on Xcc plates, 18 EB isolates exhibited varying degrees of positive antibacterial activity (Table 2). In addition, solvent extracts of these isolates were evaluated for their antagonistic activity against XccW1 (data not shown). Based on the screening data using the EB and the extracts, the endophytes TbL-22 and TbL-26 were selected for their antibacterial activities against all Xcc strains (Table 3, Fig. 2). Both the endophytes and their ethyl acetate extracts created inhibition zones on Xcc culture media. Therefore, it may be the metabolites of TbL-22 and TbL-26 that are antagonistic to Xcc. Nevertheless, the inhibition zones produced by these two EB differed in size. This discrepancy may be explained by differences in the amount, stability, and diffusion rate of the metabolites secreted by TbL-22 and TbL-26 (Kim et al., 1999). Previous studies reported on the antimicrobial activity of Bacillus spp. like B. subtilis, B. pumilus, B. amyloliquefaciens, B. pasteurii, B. cereus, B. mycoides, and B. sphaericus. These endophytes were isolated from various plants and suppressed the growth of microbial plant pathogens including X. oryzae pv. oryzae (Chithrashree et al., 2011; Lin et al., 2001), P. syringae (Bais et al., 2004; Wei et al., 1996), Phytophthora medicaginis (Silo-Suh et al., 1994), Pythium torulosum (Shang et al., 1999), and Botrytis cinerea (Touré et al., 2004). Endophytic B. thuringiensis was used as a...
biocontrol agent against the phytopathogens *Ciboria shiraiana*, *Urocystis agropyri*, and *Sclerotinia sclerotiorum*, and against insects such as silkworms (*Bombyx mori*) (De Goes et al., 2012; Roh et al., 2007; Schnepf et al., 1998; Sultana and Kim, 2016; Tao et al., 2014; Zhou et al., 2008). *B. thuringiensis* is a Gram-positive, soil-borne bacterium. It has been widely used as a biocontrol agent and bioinsecticide (Chattopadhyay et al., 2004; Jouzani et al., 2017).

The ethyl acetate extract of *B. thuringiensis* TbL-22 exhibited the highest antagonistic activity against all *Xcc* (Table 4, Fig. 3). Therefore, it is a promising candidate as a biocontrol agent against the streptomycin-resistant strains *Xcc*M4-9 (Hyun et al., 2012). The ethyl acetate extract may have contained potent secondary metabolites which sup-
pressed the growth of Xcc. Many lipopeptides and fengycin-like substances isolated and purified from \textit{B. thuringiensis} CMNB26 were found to be fungicidal, bactericidal, and insecticidal (Kim et al., 2004). \textit{B. thuringiensis} suppresses plant diseases by producing antimicrobial compounds (Cherif et al., 2008; Dong et al., 2002; Raddadi et al., 2009; Reyes-Ramirez et al., 2004). The TBL-26 extract exerted no antibiotic activity against XccW2 but did suppress both XccW1 and XccW3. This observation suggests that the three pathogen strains may differ in terms of genotype and metabolite susceptibility (Fig. 3). When the Xcc strains were subjected to ethyl acetate extracts of TBL-22, SEM analysis indicated that the cells became irregular, rough, and deformed. They eventually collapsed and lysed (Fig. 4). Similar morphological deformities have been observed in \textit{Xanthomonas campestris pv. glycinea} and \textit{Pythium myriotylum} treated with the metabolites of endophytic \textit{Actinomycetes} spp. and \textit{Fusarium} spp. (Kerthi et al., 2016; Mingma et al., 2014).

EB isolates are known for their beneficial relationships with host plants and their antagonistic activity against plant pathogens (Rosenblueth and Martinez-Romero, 2006). The biocontrol approach is an eco-friendly alternative to chemical pest control products for the suppression of plant diseases. Both \textit{in vivo} and \textit{in vitro} tests with EB and/or their extracts can be used to screen for efficacy. Detached citrus leaves infiltrated with XccW2, TBL-22, and TBL-26 showed significantly reduced disease symptoms in comparison to XccW2 alone infected leaves (Fig. 5), but the two EB did not induce any disease symptoms themselves (Fig. 6). Reductions in citrus canker severity and incidence were also reported for citrus species treated with the \textit{Bacillus} strains WG6-14 and TKS1-1 and with \textit{B. subtilis} (S-12) (Das et al., 2014; Huang et al., 2012). \textit{B. subtilis}, \textit{B. amyloliquefaciens}, \textit{B. pumilus}, \textit{B. mycoides}, \textit{B. pasteurii}, and \textit{B. cereus} induce plant disease resistance and are commercially available as biocontrol agents (Kloepper et al., 2004; Nam et al., 2016).

In summary, the endophytic \textit{Bacillus thuringiensis} strains TBL-22 and TBL-26 were determined to be prospective antagonists against both wild-type and streptomycin-resistant Xcc. Both EB were highly active against Xcc, but TBL-22 was the more potent antibacterial and may serve either as a biocontrol agent or a source of biocontrol compounds. The purification and identification of the antimicrobial metabolites of TBL-22 will be necessary in order to formulate them for agricultural applications. Further \textit{in vitro} and \textit{in vivo} research with TBL-22 and/or its extracts may help develop sustainable control measures which could replace the synthetic agrochemicals currently being used to manage Xcc.

Acknowledgments

This work was supported by a grant from Yeungnam University, Gyeongsan, Korea.

References

Backman, P. A. and Sikora, R. A. 2008. Endophytes: an emerging tool for biological control. \textit{Biol. Control} 46:1-3.

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

Bajpai, V. K., Al-Reza, S. M., Choi, U. K., Lee, J. H. and Kang, S. C. 2009. Chemical composition, antibacterial and antioxidative activities of leaf essential oil and extracts of \textit{Metasequoia glyptostroboides} Miki ex Hu. \textit{Food Chem. Toxicol.} 47:1876-1883.

Behlau, F., Canteros, B. I., Minsavage, G. V., Jones, J. B. and Graham, J. H. 2011. Molecular characterization of copper resistance genes from \textit{Xanthomonas citri} subsp. \textit{citri} and \textit{Xanthomonas alfalfae} subsp. \textit{citrumelonis}. \textit{Appl. Environ. Microbiol.} 77:4089-4096.

Berg, G., Eberl, L. and Hartmann, A. 2005. The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. \textit{Environ. Microbiol.} 7:1673-1685.

Brown, A. E. and Soepena, H. 1994. Pathogenicity of \textit{Colletotrichum acutatum} and \textit{C. gloeosporioides} on \textit{Hevea} spp. \textit{Mycol. Res.} 98:264-266.

Carter, A. P., Clemons, W. M., Brodersen, D. E., Morgan-Warren, R. J., Wimberly, B. T. and Ramakrishnan, V. 2000. Functional insights from the structure of the 30S ribosomal subunit and its interaction with antibiotics. \textit{Nature} 407:340-348.

 Chattopadhyay, A., Bhatnagar, N. B. and Bhatnagar, R. 2004. Bacterial insecticidal toxins. \textit{Crit. Rev. Microbiol.} 30:33-54.

Chen, C., Bauske, E. M., Musson, G., Rodriguezkabana, R. and Kloepper, J. W. 1995. Biological control of \textit{Fusarium} wilt on cotton by use of endophytic bacteria. \textit{Biol. Control} 5:83-91.

Cherif, A., Rezgui, W., Raddadi, N., Daffonchio, D. and Bouldous, A. 2008. Characterization and partial purification of entomocin 110, a newly identified bacteriocin from \textit{Bacillus thuringiensis} subsp. \textit{entomocids} HD110. \textit{Microbiol. Res.} 163:684-692.

Chitrashree, Udayashankar, A. C., Chandra Nayaka, S., Reddy, M. S. and Srinivas, C. 2011. Plant growth-promoting rhizobacteria mediate induced systemic resistance in rice against bacterial leaf blight caused by \textit{Xanthomonas oryzae} pv. \textit{oryzae}. \textit{Biol. Control} 59:114-122.

Das, A. K. and Singh, S. 1999. Management of bacterial canker in acid lime. \textit{Intensive Agric.} 36:28-29.

Das, A. K. 2003. Citrus canker: a review. \textit{J. Appl. Hortic.} 5:52-60.

Das, A. K. and Singh, S. 2000. Management of acid lime canker by using chemicals with compatible cultural practices. In: \textit{Hitech Citrus Management: Proceedings of the International
Islam et al.  

Symposium Citiculture, eds. by S. P. Ghosh and S. Singh, pp. 1054-1056. Indian Society of Citiculture and National Research Centre for Citrus, Nagpur, India.

Das, A. K. and Singh, S. 2001. Managing citrus bacterial diseases in the state of Maharashtra. Indian Hortic. 46:11-13.

Das, R., Mondal, B., Mondal, P., Khatua, D. C. and Mukherjee, N. 2014. Biological management of citrus canker on acid lime through Bacillus subtilis (S-12) in West Bengal, India. J. Biopest. 7:38-41.

De Goes, K. C. G. P., de Castro Fisher, M. L., Cattelan, A. J., Nogueira, M. A., de Carvalho, C. G. P. and de Oliveira, A. L. M. 2012. Biochemical and molecular characterization of high population density bacteria isolated from sunflower. J. Microbiol. Biotechnol. 22:437-447.

De Oliveira Costa, L. E., de Queiroz, M. V., Borges, A. C., de Moraes, C. A. and de Araújo, E. F. 2012. Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (Phaseolus vulgaris). Braz. J. Microbiol. 43:1562-1575.

Dong, Y.-H., Gusti, A. R., Zhang, Q., Xu, J.-L. and Zhang, L.-H. 2002. Identification of quorum-quenching N-acyl homoserine lactonases from Bacillus species. Appl. Environ. Microbiol. 68:1754-1759.

Gottwald, T. R., Hughes, G., Graham, J. H., Sun, X. and Riley, T. 2001. The citrus canker epidemic in Florida: the scientific basis of regulatory eradication policy for an invasive species. Phytopathology 91:30-34.

Graham, J. H., Leite, R. P., Yonce, H. D. and Myers, M. 2008. Streptomycin controls citrus canker on sweet orange in Brazil and reduces risk of copper burn on grapefruit in Florida. Proc. Fla. State Hortic. Soc. 121:118-123.

Hallmann, J., Quadt-Hallmann, A., Miller, W. G., Sikora, R. A. and Lindow, S. E. 2002. Endophytic colonization of plants by the biocontrol agent Rhizobium etli G12 in relation to Meloidogyne incognita infection. Phytopathology 91:415-422.

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. and Tzeng, K.-C. 2012. DNA polymorphisms and biocontrol of Bacillus antagonistic to citrus bacterial canker with indication of the interference of phyllosphere biofilms. PLoS ONE 7:e42124.

Hyun, J.-W., Kim, H.-I., Yi, P.-H., Hwang, R.-Y. and Park, E.-W. 2012. Mode of action of streptomycin resistance in the citrus canker pathogen (Xanthomonas citri pv. citri) in Jeju Island. Plant Pathol. J. 28:207-211.

Jalan, N., Kumar, D., Yu, F., Jones, J. B., Graham, J. H. and Wang, N. 2013. Complete genome sequence of Xanthomonas citri subsp. citri strain Aw12879, a restricted-host-range citrus canker-causing bacterium. Genome Announc. 1:e00235-13.

Jouzani, G. S., Valijanian, E. and Sharafi, R. 2017. Bacillus thuringiensis: a successful insecticide with new environmental features and tidings. Appl. Microbiol. Biotechnol. 101:2691-2711.

Keerthi, D., Nair, R. A. and Prasath, D. 2016. Molecular phylogeneticts and anti-pythium activity of endophytes from rhizomes of wild ginger congener, Zingiber zerumbet Smith. World J. Microbiol. Biotechnol. 32:41.

Kim, B. S., Moon, S. S. and Hwang, B. K. 1999. Isolation, identification and antifungal activity of a macrolide antibiotic, oligomycin A, produced by Streptomyces libani. Can. J. Bot. 77:850-858.

Kim, P. I., Bai, H., Bai, D., Chae, H., Chung, S., Kim, Y., Park, R. and Chi, Y.-T. 2004. Purification and characterization of a lipopeptide produced by Bacillus thuringiensis CMB26. J. Appl. Microbiol. 97:942-949.

Kloepper, J. W. and Beauchamp, C. J. 1992. A review of issues related to measuring colonization of plant roots by bacteria. Can. J. Microbiol. 38:1219-1232.

Kloepper, J. W., Ryu, C. M. and Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by Bacillus spp. Phytopathology 94:1259-1266.

Li, H.-Y., Wei, D.-Q., Shen, M. and Zhou, Z.-P. 2012. Endophytes and their role in phytoremediation. Fungal Divers. 54:11-18.

Lin, D., Qu, L. J., Gu, H. and Chen, Z. 2001. A 3.1-kb genomic fragment of Bacillus subtilis encodes the protein inhibiting growth of Xanthomonas oryzae pv. oryzae. J. Appl. Microbiol. 91:1044-1050.

Liu, Y. Q., Heying, E. and Tanumihardjo, S. A. 2012. History, global distribution, and nutritional importance of citrus fruits. Compr. Rev. Food Sci. Food Saf. 11:530-545.

Martínez-Medina, A., Fernández, I., Sánchez-Guzmán, M. J., Jung, S. C., Pasqual, J. A. and Pozo, M. J. 2013. Deciphering the hormonal signaling network behind the systemic resistance induced by Trichoderma harzianum in tomato. Front. Plant Sci. 4:206.

McManus, P. S., Stockwell, V. O., Sundin, G. W. and Jones, A. L. 2002. Antibiotic use in plant agriculture. Annu. Rev. Phytopathol. 40:443-465.

Mingma, R., Pathom-aree, W., Trakulnaleamsai, S., Thamchaipenet, A. and Duangmal, K. 2014. Isolation of rhizospheric and roots endophytic actinomycetes from Leguminosae plant and their activities to inhibit soybean pathogen, Xanthomonas campestris pv. glycine. World J. Microbiol. Biotechnol. 30:271-280.

Nam, H.-S., Yang, H.-J., Oh, B. J., Anderson, A. J. and Kim, Y. C. 2016. Biological control potential of Bacillus amyloliquefaciens KB3 isolated from the feces of Alomyrina dichotoma larvae. Plant Pathol. J. 32:273-280.

Patra, J. K., Das, G. and Baek, K.-H. 2015. Antibacterial mechanism of the action of Enteromorpha linza L. essential oil against Escherichia coli and Salmonella typhimurium. Bot. Stud. 56:13.

Paul, D. K. and Shaha, R. K. 2004. Nutrients, vitamins and minerals content in common citrus fruits in the northern region of Bangladesh. Pak. J. Biol. Sci. 7:238-242.

Quadri-Hallman, A., Kloepper, J. W. and Benhamou, N. 1997. Bacterial endophytes in cotton: mechanisms of entering the plant. Can. J. Microbiol. 43:557-582.
Quispel, A. 1992. A search for signals in endophytic microorganisms. In: Molecular signals in plant-microbe communications, ed. by D. P. S. Verma, pp 471-490. CRC Press, Boca Raton, FL.

Raddadi, N., Belaouis, A., Tamagnini, I., Hansen, B. M., Hendriksen, N. B., Boudabous, A., Cherif, A. and Daffonchio, D. 2009. Characterization of polyvalent and safe Bacillus thuringiensis strains with potential use for biocontrol. J. Basic. Microbiol. 49:293-303.

Ramful, D., Bahorun, T., Bourdon, E., Tamus, E. and Aruoma, O. I. 2010. Bioactive phenolics and antioxidant propensity of flavedo extracts of Mauritian citrus fruits: potential prophylactic ingredients for functional foods application. Toxicology 278:75-87.

Reyes-Ramírez, A., Escudero-Abarca, B. I., Aguilar-Uscanga, G., Hayward-Jones, P. M. and Barboza-Corona, J. E. 2004. Antifungal activity of Bacillus thuringiensis chitinase and its potential for the biocontrol of phytopathogenic fungi in soybean seeds. J. Food Sci. 69:M131-M134.

Roh, E., Lee, S., Lee, Y., Ra, D., Choi, J., Moon, E. and Heu, S. 2009. Diverse antibacterial activity of Pectobacterium carotovorum subsp. carotovorum isolated in Korea. J. Microbiol. Biotechnol. 19:42-50.

Roh, J. Y., Choi, J. Y., Li, M. S., Jin, B. R. and Je, Y. H. 2007. Bacillus thuringiensis as a specific, safe, and effective tool for insect pest control. J. Microbiol. Biotechnol. 17:547-559.

Rosenblueth, M. and Martinez-Romero, E. 2006. Bacterial endophytes and their interactions with hosts. Mol. Plant Microbe Interact. 19:827-837.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R. and Dean, D. H. 1998. Bacillus thuringiensis and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62:775-806.

Shang, H., Chen, J., Handelsman, J. and Goodman, R. M. 1999. Behavior of Pythium torulosum zoospores during their interaction with tobacco roots and Bacillus cereus. Curr. Microbiol. 38:199-204.

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

Sturz, A. V. and Nowak, J. 2000. Endophytic communities of rhizobacteria and the strategies required creating yield enhancing associations with crops. Appl. Soil Ecol. 15:183-190.

Sultana, R. and Kim, K. 2016. Bacillus thuringiensis C25 suppresses popcorn disease caused by Ciboria shiraiana in mulberry (Morus australis L.). Biocontrol Sci. Technol. 26:145-162.

Susilowati, R., Sabdono, A. and Widowati, I. 2015. Isolation and characterization of bacteria associated with brown algae Sargassum spp. from Panjang Island and their antibacterial activities. Proc. Environ. Sci. 23:240-246.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: molecular evolutionary genetic analysis version 6. Mol. Biol. Evol. 30:2725-2729.

Tan, X., Huang, S., Ren, J., Yan, W. and Cen, Z. 2007. Characterization of an endophytic bacterium strain Be51 suppressing citrus canker. Acta Phytopathol. Sin. 37:9-17.

Tao, A., Pang, F., Huang, S., Yu, G., Li, B. and Wang, T. 2014. Characterization of endophytic Bacillus thuringiensis strains isolated from wheat plants as biocontrol agents against wheat flag smut. Biocontrol Sci. Technol. 24:901-924.

Touré, Y., Ongena, M., Jacques, P., Guiro, A. and Thonart, P. 2004. Role of lipopeptides produced by Bacillus subtilis GA1 in the reduction of grey mould disease caused by Botrytis cinerea on apple. J. Appl. Microbiol. 96:1151-1160.

Van Loon, L. C., Bakker, P. A. H. M. and Pieterse, C. M. J. 1998. Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. 36:453-483.

Voloudakis, A. E., Reignier, T. M. and Cooksey, D. A. 2005. Regulation of resistance to copper in Xanthomonas axonopodis pv. vesicatoria. Appl. Environ. Microbiol. 71:782-789.

Webber, H. J. 1967. History and development of the Citrus industry. In: The Citrus industry, Vol. I, eds. by W. Reuther, L. D. Batchelor and H. J. Webber, pp. 1-39. University of California Press, Berkely, CA, USA.

Wei, G., Kloeper, J. W. and Tuzun, S. 1996. Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. Phytopathology 86:221-224.

Yang, H. L., Sun, X. L., Song, W. and Wang, Y. S. 2001. Studies on the rice endophytic bacteria Entetobacter cloacae MR12’s identification and its effects of nitrogen fixation and biological control to plant disease. Acta Phytopathol. Sin. 31:92-93.

Zhou, Y., Choi, Y.-L., Sun, M. and Yu, Z. 2008. Novel roles of Bacillus thuringiensis to control plant diseases. Appl. Microbiol. Biotechnol. 80:563-572.