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# Biological control of the shot-hole disease in flowering cherry tree using antimicrobial compounds produced by *Bacillus velezensis* 8–2

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## Abstract

**Background** Effective control of shot-hole disease in flowering cherries is challenging because of multiple causative pathogens (bacteria and fungi). *Bacillus* species are well-known for their ability to control plant pathogens; therefore, biological control potential of a *Bacillus* isolate, *B. velezensis* 8–2, against SH disease on flowering cherry trees was investigated.

**Results** This study revealed strong antimicrobial activity of *Bacillus velezensis* 8–2 against various plant pathogenic bacteria and fungi, particularly focusing on *Xanthomonas arboricola* pv. *pruni* (Xap) and *Mycosphaerella cerasella* (Mc), which cause shot-hole (SH) disease in flowering cherry trees. In vitro assays showed that the fermentation filtrate of *B. velezensis* 8–2 inhibited bacterial and fungal growth with minimum inhibitory concentrations of 1.25–10% and 2.5–10%, respectively. UPLC-Q–Orbitrap–MS analysis revealed that *B. velezensis* 8–2 produced antagonistic compounds, including polyketides (difficidin and oxydifficidin) and cyclic lipopeptides (iturin A, fengycin, and surfatin). To enhance antimicrobial activity, fermentation parameters for optimal production of two antibacterial and three antifungal compounds were investigated in a 5 L jar fermenter. By regulating the agitation speed to sustain the state of vegetative cells, the production period was extended by 20 h at 400 rpm, resulting in maximum yields of 86.6 µg/mL for difficidin and 150.0 µg/mL for oxydifficidin within a 72 h fermentation period. In a field trial, a 500-fold diluted 10% suspension concentrate formulation of *B. velezensis* 8–2 effectively inhibited the development of SH disease, demonstrating 66.6% disease control and a 90.2% disease symptoms reduction.

**Conclusions** This is the first report to assess the disease control efficacy of *B. velezensis* for the biocontrol of SH disease in the field. These results suggest that the application of *B. velezensis* 8–2 could serve as a practical alternative for managing various bacterial and fungal diseases, including the management of SH disease in flowering cherry trees.

**Keywords** *Bacillus velezensis*, Biological control, Polyketides, Cyclic lipopeptide, Oxygen supply

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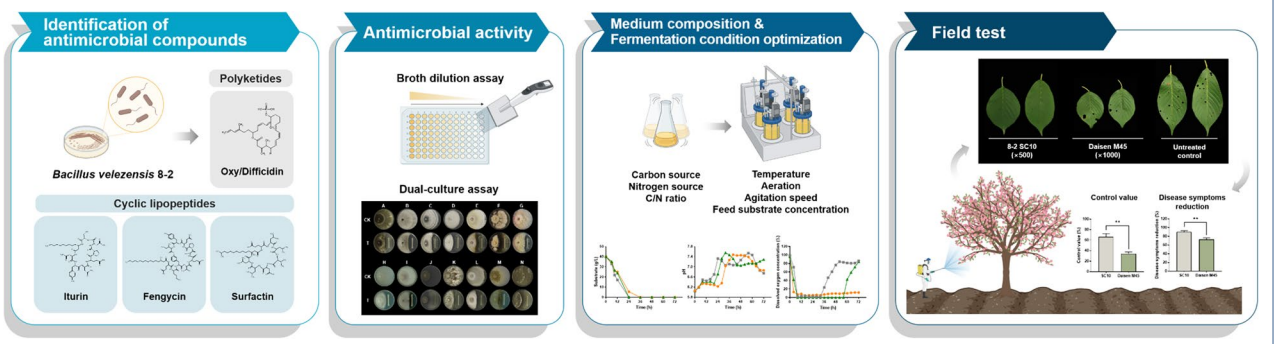
**Graphical Abstract**




## Background

Flowering cherry (*Prunus x yedoensis* Matsumura; Somei-yoshino) is an ornamental tree commonly planted along the road in the Republic of Korea [1]. This tree produces beautiful flowers in spring and is celebrated with annual cherry blossom festivals held in more than 20 countries around the world. Like all cherry trees, this tree is susceptible to shot-hole (SH) disease, resulting in decreased photosynthetic rates, premature defoliation, and reduced blooming of cherry blossoms in the following year [2]. Lesions appear as water-soaked brown spots that are observed sporadically along infected leaf midribs or margins [3]. Several pathogens, i.e., *Epicoccum tobaicum* (Et), *Burkholderia contaminans* (Bc), *Mycosphaerella cerasella* (Mc), *Pseudomonas syringae* pv. *syringae* (Pss), and *Xanthomonas arboricola* pv. *pruni* (Xap), are reported to cause SH disease in flowering cherry trees [4]. Controlling SH disease on flowering cherry trees is challenging because of the simultaneous involvement of two or three pathogens (bacterium and bacterium/bacterium and fungus/bacteria and fungus).

Chemical pesticides have been reported to prevent or control various plant diseases. Because there is no chemical agent specific for SH disease, Mancozeb (MCZ), an ethylene–bis-dithiocarbamate fungicide, was primarily applied to infected leaves of flowering cherry trees to control SH disease [5, 6]. However, MCZ's registration as a pesticide has not been renewed due to undesirable side effects, such as neurotoxicity, endocrine disruption, and reproductive toxicity [7–10]. Moreover, MCZ has been phased out in the European Union since January 2021 [11]. Furthermore, fungicide treatment alone is not effective when the pathogens are bacterial species or bacterium–fungus combinations. Since flowering cherry trees are planted along sidewalks, eco-friendly alternatives such as microbial antagonists have attracted attention in view of public safety.

Several antagonistic *Bacillus* strains have been described as effective biocontrol agents against plant pathogens owing to their capability to produce both antibacterial and antifungal metabolites [12, 13]. Among *Bacillus* species, *B. velezensis* is a promising candidate exhibiting strong biocontrol potential towards various plant pathogens. It interferes with the growth of pathogenic microorganisms, producing various metabolites, polyketides (PKs), such as bacillaene, difficidin, and macrolactin [14], cyclic lipopeptides (CLPs), such as iturins, fengycins, and surfactins [15], and volatile organic compounds (VOCs), such as sulfur- and nitrogen-containing VOCs, fatty acids, and terpenoids [16]. However, despite its immense potential, comprehensive research is required to develop this species as a biocontrol agent. The lower control efficiency of this species compared to synthetic chemical pesticides hinders its industrialization. For commercial viability, it is necessary to make it cost-effective by increasing the yield of antimicrobial metabolites.

We isolated an antagonistic bacterium exhibiting biological control potential against SH disease on flowering cherry trees and identified it as *B. velezensis* 8–2. In this study, we investigated the in vitro antimicrobial activity of *B. velezensis* 8–2 against various phytopathogenic bacteria and fungi and identified antimicrobial compounds. Based on the production profiles of antimicrobial compounds, fermentation strategy on regulating the agitation speed to sustain the state of vegetative cells were studied to improve antagonistic activity. In addition, biocontrol efficacy against SH disease on flowering cherry trees was evaluated in the field.

## Materials and methods

### Isolation and identification of bacterial communities and in vitro screening for biocontrol potential

Bacterial strains were isolated from soil samples in several sites, viz., Chonnam National University (35.176°N, 126.906°E) and Daejeon (36.350°N, 127.384°E) in South Korea. Each soil sample of 1 g was suspended in 9 mL of

sterile distilled water and subsequently vortexed for 10 min. Resulting solution was serially diluted to $1\times10^{-7}$. A 100 µL aliquot of the diluted solution was spread on tryptic soy agar plates (TSA; Difco, USA) inoculated with *Xanthomonas arboricola* pv. *pruni* (Xap) at $1\times10^{6}$ CFU/mL. Plates were placed at 30 °C for 3–5 days. Seventy-two antagonistic bacteria showing the inhibition zone were isolated from 100 soil samples. Their antibacterial activity against Xap was compared using the broth dilution method, and strain 8–2 was finally selected due to the strongest antibacterial activity. Strain 8–2 was identified as *Bacillus velezensis* 8–2 by complete genome sequencing, and it has been deposited in the Korea Collection for Type Cultures (KCTC, Jeongeup, South Korea) as KCTC 13418BP with accession number CP028439.1. For the maintenance of *B. velezensis* 8–2, bacterial cultures in 1.2 mL Cryovial tubes (Simport, Canada) were stored in a freezer at − 80 °C to avoid genetic mutation due to successive culture. For seed cultures, a bacteria stock was thawed at 25 °C and cultured in a 500 mL Erlenmeyer flask containing 100 mL of tryptic soy broth (TSB; Difco, USA). The flasks were incubated on a rotary shaker (IS-971RF; Jeiotech, South Korea) at 30°C and 150 rpm for 3 days.

### Phytopathogenic microorganisms

In this study, 15 plant pathogenic bacteria were used, including *Acidovorax avenae* subsp. *cattleyae* (Aac), *Agrobacterium tumefaciens* (At), *Burkholderia contaminans* (Bc), *Bur. glumae* (Bg), *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), *Erwinia amylovora* (Ea), *E. pyrifoliae* (Ep), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), *Pec. chrysanthemi* (Pc), *Pseudomonas syringae* pv. *actinidiae* (Psa), *Pse. syringae* pv. *syringae* (Pss), *Ralstonia solanacearum* (Rs), Xap, *X. campetris* pv. *citri* (Xcc), and *X. euvesicatoria* (Xe). Bc and Pss were isolated from flowering cherry tree leaves infected SH disease in CNU [2]. Psa was kindly obtained from Department of Plant Medicine, Suncheon National University, while Rs was given from Department of Applied Bioscience, Dong-A University. The other bacterial strains were obtained from Korean Agricultural Culture Collection (KACC), South Korea [17]. These phytopathogens were isolated from infected plants in South Korea. The pathogenic bacteria were grown in TSB at 30 ± 2 °C and stored in 15% glycerol at − 80 °C during the study period. Fifteen fungal strains, plant pathogenic fungi, were used in this study, including *Aspergillus niger* (An), *Botryosphaeria dothidea* (Bd), *Botrytis cinerea* (Bc,), *Colletotrichum acutatum* (Ca), *Cryphonectria parasitica* (Cp), *Epicoccum tobaicum* (Et), *Fusarium graminearum* (Fg), *F. oxysporum* (Fo), *F. verticillioides* (Fv), *Mycosphaerella cerasella* (Mc), *Ophiostoma ulmi* (Ou), *Penicillium italicum* (Pi), *Raffaelea quercus–mongolicae* (Rq), *Sclerotinia homoeocarpa* (Sh), and *Valsa cerasperma* (Vc). Et was obtained from flowering cherry tree leaves infected SH disease in Hadong, South Korea (35°07′48.9″N, 127°46′53.8″E) [18], while the rest of fungal strains were obtained from Korean Agricultural Culture Collection (KACC) [19]. The fungal strains were grown in potato dextrose broth (PDB; Difco, USA) at 25 °C and stored in 25% glycerol at − 80 °C.

### In vitro antimicrobial assay

#### *Dual-culture assay*

The inhibitory activity of *B. velezensis* 8–2 against the mycelial growth of fourteen fungal strains was evaluated using a dual-culture assay following a previously described method [20] with slight modifications. A 5-mm fungal disk was placed on one side of the potato dextrose agar (PDA; Difco, USA) plate. The bacterial suspension was streaked on the opposite side of the plate at a distance of 4 cm from the disks. Negative control included fungal disk only. After incubation at 25 °C for 5–7 days, the mycelial growth inhibition rate was calculated when the control plate was completely covered with mycelia. The inhibition rate was calculated by the following formula:

$$Inhibition\ rate(\%) = \frac{r_1 - r_2}{r_1} \times 100 \quad (1)$$

where $r_1$ is the radius (mm) of the mycelial growth on the control plate and $r_2$ is the radius (mm) of the mycelial growth towards the bacterial colonies. The experiment was repeated twice in triplicate.

#### *Preparation of fermentation filtrate and cyclic lipopeptides*

*B. velezensis* 8–2 was grown in TSB and a modified medium, respectively, at 30 °C and 150 rpm for 3 days. The bacterial suspension was centrifuged at 12,000 g for 15 min to separate the pellet and supernatant. The culture supernatant was filtered using a 0.2 µm membrane filter and stored at − 20 °C until further experiment. The standard samples of CLPs iturin A, fengycin, and surfactin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and a 1:1:1 (by volume) mixture of the three standards was prepared from a 5000 µg/mL solution of each CLP in 100% methanol (MeOH).

#### *Antibacterial activity*

The minimum inhibitory concentration (MIC) of the fermentation filtrates against 13 plant pathogenic bacteria was determined using the broth dilution method in a 96-well plate [21]. The first column of the plates was filled with 200 µL of bacterial suspension ($1\times10^{5}$ CFU/mL) containing 10% of the fermentation filtrate of *B.*

*velezensis* 8–2, and then, 100 µL of the suspension was added to the remaining columns. Twofold serial dilutions were carried out by transferring 100 µL of the suspension in the first column to the adjacent wells. The positive control was streptomycin sulfate dissolved in sterile distilled water, and TSB was used as the negative control. The plates were incubated at 30 ± 2 °C for 24–48 h. The bacterial growth was measured as optical density (OD) at 600 nm using a microplate reader (UV-1800, Shimadzu, Kyoto, Japan). The MIC value was the lowest concentration that inhibited the bacterial growth. The assay was repeated twice, in triplicate. The inhibition rate was calculated according to the following formula:

$$Inhibition\ rate(\%) = \frac{OD_{600}\ of\ negative\ control - OD_{600}\ of\ treated\ samples}{OD_{600}\ of\ negative\ control} \times 100 \quad (2)$$

#### *Antifungal activity of fermentation filtrate and cyclic lipopeptides*

The fermentation filtrates were used for MIC tests against seven fungal strains using antifungal assay described by Nguyen et al. [22]. The 96-well plates containing 100 µL of PDB were inoculated with 1% of mycelial suspensions (50 mg fresh mycelia in 1 mL of sterile distilled water). All treatments were added to each well in the same way as in the section *Antibacterial activity*. PDB without inoculation of mycelial suspensions was used as a negative control consisted of, while PDB inoculated with 1% of mycelial suspensions was used as a growth control. All test plates were incubated at 25 °C for 3–5 days. Mycelial growth was measured at 595 nm using a microplate reader. The inhibition rate was calculated according to the following formula:

$$Inhibition\ rate(\%) = 100 - \left(\frac{OD_{595}\ of\ treatment - AverageOD_{595}\ of\ negative\ control}{AverageOD_{595}\ of\ growth\ control - AverageOD_{595}\ of\ negative\ control}\right) \times 100 \quad (3)$$

The MIC value was defined as the lowest treatment concentration required for the inhibition rate to above 90%. The assay was repeated twice, in triplicate.

The synergistic interaction of three CLPs was identified using the diagonal measurement of *n*-way drug interactions (DiaMOND), as described previously [23]. The fractional inhibitory concentration (FIC) index was calculated as follows: $FIC_n = o_n/e_1 \times 100$, where $FIC_n$ is the FIC index for *n*-way combinations, $o_n$ is the observed MIC value in *n*-way combinations, and $e_1$ is the predicted MIC value calculated using a single response in *n*-way combinations. If the $FIC_n$ score is 1, then the *n*-way combination is additive; if the score is less than 1, then the combination is synergistic; if the score is greater than 1, then the combination is antagonistic.

### Identification of the active antimicrobial compounds

#### *Extraction of crude active compounds from Bacillus velezensis 8–2*

To extract PKs and CLPs, the supernatant of *B. velezensis* 8–2 was mixed with an equal volume of n-butanol (BuOH) and vigorously vortexed for 1 min at 25 °C according to a previously described method [24]. The BuOH layers were collected after centrifugation, diluted with 20% MeOH, and stored at − 80 °C until further analysis.

#### *Identification and quantification of active metabolites by UPLC-Q–Orbitrap MS analysis*

UPLC-quadrupole–Orbitrap mass spectrometry (UPLC-Q–Orbitrap–MS) analysis was performed to identify and quantify the active metabolites using a UPLC–HESI–MS system comprised of a Dionex Ultimate 3000 UHPLC module and a heated electrospray ionization (HESI) quadrupole Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Thermo Xcalibur 3.0 software (Thermo Scientific, Germany) was used to control the instruments. Samples were injected into a $C_{18}$ reversed phase column (BEH $C_{18}$, 1.7 µm, 2.1 × 100 mm; Waters, Milford, MA, USA), which was maintained at 40 °C. The mobile phase consisted of the following 20 min sequence of linear gradients and isocratic flows of 0.1% formic acid in acetonitrile (solvent B, *v*/*v*) balanced with 0.1% formic acid in water (solvent A, *v*/*v*) at a flow rate of 0.3 mL/min: 5% B for 1 min, 5–100% B in 10 min, isocratic 100% B for 5 min, and finally 100–5% B in 1 min. For equilibrium, the column was maintained under 5% B for 4 min at the end of the sequences. The sample injection volume was 5 µL, and the resolution was 70,000. The mass spectra were acquired in the full scan mode set from 400 to 1600 m/*z* using the following conditions: source block temperature of 150 °C, desolvation temperature of 500 °C, capillary voltage of 2.5 kV, and cone

voltage of 20 V. Nitrogen was used as a desolvation gas (800 L/h).

#### Development of a modified medium to enhance antagonistic activity

The amount of antagonistic compounds present in the fermentation filtrate determines its antagonistic efficacy against plant pathogens [26]. Therefore, the fermentation filtrate was screened by evaluating the MIC against Xap, which was chosen as an indicator pathogen. The basic culture medium, TSB, was composed of glucose (10.0 g), casein peptone (17.0 g), soy peptone (0.3 g), sodium chloride (5.0 g), and dipotassium phosphate (2.5 g) per liter of distilled water. The impact of thirteen carbon sources (10.0 g/L each) on the antimicrobial activity of *B. velezensis* 8–2 was estimated by substituting the C source in TSB with monosaccharides, such as glucose, fructose, and galactose, disaccharides, such as sucrose, maltose, and lactose, sugar alcohols, such as sorbitol, xylitol, and mannitol, and starch, such as soluble starch, corn starch, potato starch, and sweet potato starch, while the other constituents in TSB remained unchanged. Similarly, the influence of nine nitrogen sources (20.0 g/L), including organic nitrogen sources, such as casein peptone, soy peptone, sodium caseinate, corn steep liquor, soybean meal, yeast extract, and beef extract, and inorganic nitrogen sources, such as ammonium nitrate and sodium nitrate, were also estimated with the selected carbon source. The impact of varying carbon (1–5%) to nitrogen (0.5–10%) ratios was evaluated. *B. velezensis* 8–2 (1% *v*/*v*) was inoculated in 500 mL Erlenmeyer flasks with 100 mL of each culture medium and grown at 30 °C and 150 rpm for 3 days. Subsequently, the MIC value of the filtrates from *B. velezensis* 8–2 cultured in each medium was determined. The medium components exhibiting the highest antibacterial activity were selected to compose the modified medium for further studies.

#### Influence of agitation speed on polyketides and cyclic lipopeptides production

For lab-scale fermentation, 1% of the seed culture was inoculated into a 5 L jar fermenter (BioCnS, Daejeon, Korea) containing 3.3 L modified medium and incubated at 30 °C for 72 h. The total airflow rate was 1 vvm, and pH was not controlled during the submerged culture. Three levels of agitation speed, 300, 400, and 500 rpm, were tested. Culture broth was collected every 4 h to analyze biomass, sporulation, metabolite production, and substrate consumption patterns. Then, the correlation between sporulation and PKs and CLP productivity was analyzed. Cultivations were performed in triplicate.

#### Preparation of suspension concentration formulation

The fermentation broth (1 L) cultured under optimal culture conditions was mixed with oxidized starch (100 g) and then dried using a YC-500 laboratory spray dryer (Shanghai Pilotech Instrument Equipment Co., Ltd. Shanghai, China). The spray-dry (SD) conditions were used as follows: atomizer rate, 10,000 rpm; inlet temperature, 190–198 °C; and outlet temperature, 90–98 °C. The SD (10 g) was added to distilled water with xanthan gum (0.1 g) as a suspending agent, sodium dioctyl sulfosuccinate and tristyrylphenol ethoxylates (3.0 g) as wetting agents, polydimethylsiloxane (0.1 g) as a defoaming agent, propylene glycol (5.0 g) as a humectant, and sodium benzoate (0.2 g) as a preservative to prepare 100 g of suspension concentration (8–2 SC10; 10% a.i.) formulation.

#### Disease control efficacy against shot-hole disease under the field condition

The disease control efficacy of 8–2 SC10 against SH disease in flowering cherry trees was evaluated in a field located at Chonnam National University (CNU, Gwangju, Korea). The SH disease was prevalent in the selected location, because the trees had been severely damaged by Xap and Mc in previous seasons [4]. The field experiment was conducted in 2.3 m × 30 m plots with a 2 m spacing between each plant, resulting in a planting density of 0.3 plants/$m^2$. Each group consisted of three 15-year-old plants, which were 4–5 m in height. The test formulation was diluted 500-fold with tap water, while the positive control, Daisen M45 (MCZ 75%; Farm Hannong Co., Seoul, Korea), was diluted 1000-fold. Tween 20 (250 μg/mL) was used as a negative control. The treatments were foliar sprayed at a rate of 100 mL per plant with three branches containing 30–40 leaves every 2 weeks from May to June. After four treatments, the number of shothole spots (NS) per leaf was counted in July [27]. The disease incidence was investigated according to Eq. 4, and the control value and disease symptoms reduction were calculated using Eqs. 5 and 6, respectively:

$$Disease\ incidence(DI, \%) = \left( \frac{The\ number\ of\ diseased\ leaves\ per\ branch}{Total\ number\ of\ leaves\ per\ branch} \right) \times 100 \quad (4)$$

$$Control\ value(\%) = \left(\frac{DI\ of\ control - DI\ of\ treatment}{DI\ of\ control}\right) \times 100 \quad (5)$$

$$Disease\ syptomes\ reduction(\%) = \left(\frac{NS\ ofcontrol - NS\ of\ treatment}{NS\ of\ control}\right) \times 100 \quad (6)$$

**Statistical analysis**

All statistical analyses were conducted using IBM SPSS statistical software (Ver. 20; SPSS Inc., USA). The experimental data were expressed as the mean ± standard deviation of replicates. Statistical differences were evaluated by one-way analysis of variance (ANOVA) using Tukey's HSD test ($p < 0.05$). The MIC value was compared with media compositions using Student's $t$ test at a significance level of $P < 0.05$.

## Results and discussion

Among the various microorganisms present in the plant rhizosphere, *Bacillus* spp. play important roles as biocontrol agents that can directly inhibit the growth of pathogens by producing secondary metabolites or by promoting the growth of host plants [28]. Many studies have reported the antagonistic effect of bacterial strains on phytopathogens in vitro, but only a few studies have successfully controlled plant diseases under field conditions. In particular, urban trees, which hold high value to humans, are often subjected to stress due to damage, drought, improper planting depth in unsuitable soil, and nutrient deficiency [29]. As a result, these trees are more susceptible to plant disease infestations. In particular, controlling SH disease, which affects flowering cherry trees, is challenging, because it is caused by a combination of both bacterial and fungal infections. To the best of our knowledge, this is the first report demonstrating effective control of SH disease in flowering cherry trees in field conditions.

### Antagonistic activity of *Bacillus* velezensis 8–2 against various plant pathogens

#### *Dual-culture assay*

Dual-culture assays were performed between *B. velezensis* 8–2 and 14 fungal strains to screen the antifungal activities (Fig. S1). Especially, the mycelial growth of Vc and Cp was distinctly inhibited by more than 70% (supplementary Table 1). The mycelial growth of Ou, Et, Pi, Rq, and Bd was moderately retarded with 60% inhibition. The growth of Ca, An, Bc, Fg, Fo, Fv, and Mc was slightly delayed by less than 60% when they were co-cultured with *B. velezensis* 8–2. These results suggested that the antifungal activity of *B. velezensis* 8–2 was highly variable depending on the fungus species. *B. velezensis* 8–2 inhibited the mycelial growth of ascomycetes among fungi (data not shown). The strong antifungal activity of *B. velezensis* 8–2 is of great significance, particularly against fungal strains causing tree disease, such as Vc causing

**Table 1** Effect of culture medium on antagonistic activity of *Bacillus velezensis* 8–2 against various plant pathogenic bacteria

| Plant pathogenic bacteria | Minimum inhibitory concentration | | |
|---|---|---|---|
| | TSB (%) | Modified medium (%) | Streptomycin sulfate (μg/mL) |
| *Acidovorax avenae* subsp. *cattleyae* (Aac) | 5.00* ± 0.00 | 0.63 ± 0.00 | > 200.00 |
| *Agrobacterium tumefaciens* (At) | 1.25 ± 0.00 | 0.16 ± 0.00 | 100.00 ± 0.00 |
| *Burkholderia glumae* (Bg) | 10.00 ± 0.00 | 0.63 ± 0.00 | 25.00 ± 0.00 |
| *Claviabacter michiganensis* subsp. *michiganensis* (Cmm) | 7.50 ± 2.50 | 0.94 ± 0.00 | 41.67 ± 7.22 |
| *Erwinia amylovora* (Ea) | 10.00 ± 0.00 | 1.25 ± 0.00 | 3.13 ± 0.00 |
| *Erwinia pyrifoliae* (Ep) | > 10.00 | 5.00 ± 0.00 | 3.13 ± 0.00 |
| *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) | > 10.00 | 2.50 ± 0.00 | 7.29 ± 1.80 |
| *Pectobacterium chrysanthemi* (Pc) | > 10.00 | 10.00 ± 0.00 | > 200.00 |
| *Pseudomonas syringae* pv. *actinidiae* (Psa) | 5.00 ± 0.00 | 0.63 ± 0.00 | 0.78 ± 0.00 |
| *Ralstonia solanacearum* (Rs) | 5.00 ± 0.00 | 0.16 ± 0.00 | 3.13 ± 0.00 |
| *Xanthomonas arboricola* pv. *pruni* (Xap) | 1.25 ± 0.00 | 0.12 ± 0.04 | 12.50 ± 0.00 |
| *Xanthomonas campestris* pv. *citri* (Xcc) | 1.25 ± 0.00 | 0.16 ± 0.00 | 6.25 ± 0.00 |
| *Xanthomonas euvesicatoria* (Xe) | 1.25 ± 0.00 | 0.31 ± 0.00 | 12.50 ± 0.00 |

* Mean ± standard deviation from three determinations

valsa canker in apple trees, Cp causing chestnut blight, and Ou causing Dutch elm disease. Studies on the biocontrol of tree diseases, including cherry trees, are lacking compared to studies on diseases of herbaceous and annual plants [30]. *B. velezensis* 8–2 could be a promising biological control agent (BCA) for tree diseases.

#### *In vitro antibacterial activity*

The antagonistic activity of the fermentation filtrate of *B. velezensis* 8–2 cultured in TSB and a modified medium was tested against 13 plant pathogenic bacteria (Table 1).

The MIC values decreased significantly when the modified medium was used as the culture medium instead of TSB ($t=7.6$, $P<0.001$). The filtrate of *B. velezensis* 8–2 cultured in the modified medium exhibited approximately 4–32 times higher antibacterial activity against all phytopathogenic bacteria than the filtrate of TSB culture. In the assessment of the antibacterial activity of the TSB fermentation filtrate, At, Xap, Xcc, and Xe exhibited the highest sensitivity, with MIC values of 1.25%, followed by Aac, Psa, and Rs with MIC values of 5%. The MIC values of fermentation filtrate for Bg, Cmm, and Ea ranged from 7.5% to 10% indicating the relative resistance of these bacteria. Moreover, three pathogenic bacteria, Ep, Pcc, and Pc, demonstrated high tolerance levels. In contrast, the fermentation filtrate from the modified medium showed potent inhibition against Xap, evident from MIC value of 0.12%, followed by At, Rs, and Xcc with MIC values of 0.16%. In addition, this filtrate exhibited strong antibacterial activity against eight bacterial strains, with MIC values ranging from 0.31% to 5%, while Pc remained relatively insensitive.

The antibacterial activity of the PKs, difficidin and its oxidized form, oxydifficidin, was first reported in 1987 [31]. Difficidin and oxydifficidin exert antibacterial activity by destroying the cell membrane and cell wall of pathogens and inhibiting their motility, polysaccharide production, and cellulose activity [32]. The broad-spectrum antibacterial activity of difficidin has been previously reported. Difficidin, produced by *B. amyloliquefaciens*, reduced the disease severity of the rice blight caused by Xoo [33] and the rice leaf streak caused by *X. oryzae* pv. *oryzicola* [34]. In addition, difficidin derivatives exhibited antibacterial activity against multidrug-resistant pathogens [35]. Im et al. [25] reported that difficidin and oxydifficidin isolated from *B. velezensis* DR-08 exhibited broad antibacterial activity against various plant pathogenic bacteria, and their susceptibility was different for each pathogen. One of the SH pathogens, Xap, was three times more sensitive to oxydifficidin than to difficidin, with MIC values of 3.3 and 10 μg/mL, respectively.

Although limited, there are reports on the direct antibacterial activity of CLPs produced by *Bacillus* strains [36, 37]. Zeriouh et al. [38] constructed an iturin-deficient mutant of *B. subtilis* and confirmed the antibacterial activity of iturins against *X. campestris* pv. *cucurbitae*. Medeot et al. [39] confirmed the antibacterial activity of fengycins produced by *B. amyloliquefaciens* $MEP_2$18 against *X. axonopodis* pv. *vesicatoria* with MIC of 25 μg/mL and against *Pseudomonas aeruginosa* PA01 with MIC of 200 μg/mL. Fan et al. [40] constructed *srfAB* deletion mutant and demonstrated that surfactin is the main antibacterial compound of *B. subtilis* 9407. The antibacterial activity of antagonistic compounds can vary depending on the bacterial pathogen. In this study, iturin A and fengycin were not antagonistic against Xap at a concentration of 500 μg/mL, whereas surfactin exhibited antibacterial activity with an MIC value of 125 μg/mL. Oxydifficidin and difficidin exhibited antibacterial activity against Xap, but their MIC values were 38 and 12.5 times higher than surfactin, respectively. Therefore, the antibacterial activity against Xap is likely to depend more on the concentration of PKs than CLPs.

#### *In vitro antifungal activity*

The in vitro antifungal activity of the fermentation filtrate of *B. velezensis* 8–2 cultured using TSB and modified medium was assessed against seven fungal strains (Table 2). Although the antifungal effect did not increase as much as the antibacterial activity, a significant difference in mycelial growth inhibition was observed depending on the medium composition ($t=56.0$, $P<0.001$). For all fungal strains except Fg, mycelial growth was inhibited with less than 10% fermentation filtrate from TSB.

Compared to the TSB fermentation filtrate of *B. velezensis* 8–2, the modified medium filtrate showed two times higher antifungal activity against all tested fungal

**Table 2** Antagonistic activity of *Bacillus velezensis* 8–2 against various plant pathogenic fungi according to medium compositions

| Plant pathogenic fungi | Minimum inhibitory concentration (%) | |
|---|---|---|
| | TSB | Modified medium |
| *Collectotrichum acutatum* | 10.00* ± 0.00 | 5.00 ± 0.00 |
| *Cryphonectria parasitica* | 2.50 ± 0.00 | 1.25 ± 0.00 |
| *Fusarium graminearum* | > 10.00 | > 10.00 |
| *Mycosphaerella cerasella* | 10.00 ± 0.00 | 5.00 ± 0.00 |
| *Raffaelea quercus–mongolicae* | 10.00 ± 0.00 | 5.00 ± 0.00 |
| *Sclerotinia homoeocarpa* | 2.50 ± 0.00 | 1.25 ± 0.00 |
| *Valsa cerasperma* | 10.00 ± 0.00 | 5.00 ± 0.00 |

* Means ± standard deviations from three determinations

strains, except for Fg. The MIC values for the modified medium filtrate ranged from 1.25% to 5%, whereas those for the TSB filtrate ranged from 2.5% to 10%.

Several studies have reported the disease control efficacy of CLPs produced by *B. velezensis* against fungal strains. The antifungal effects of CLPs are direct, through pore formation and osmotic imbalance in the cell membrane of fungal pathogens, or indirect, by contributing to the formation of a biofilm on the host plant, or by promoting rhizosphere colonization [41–43]. *B. velezensis* HC6 inhibited the growth of pathogenic fungi in maize, such as *A. flavus, A. parasiticus, F. graminearum,* and *F. oxysporum*, and reduced mycotoxin production [15]. At a concentration of 8 mg/mL, CLPs extract obtained from *B. velezensis* XT1 reduced the disease severity of gray mold caused by Bc in grapes and tomatoes [44].

### Effects of cyclic lipopeptides on the inhibition of fungal mycelial growth

The antifungal activity of CLPs, including iturin A, fengycin, and surfactin, was evaluated in vitro (Table 3). Iturin A exhibited the strongest antifungal activity with an MIC value of 7.81–62.50 µg/mL, followed by fengycin with an MIC value of 31.25–500.00 µg/mL. In contrast, fungal growth was not inhibited by surfactin, even at a concentration of 500 µg/mL. Similar inhibitory effects of CLPs on fungal strains were previously reported [45]. Moreno-Velandia et al. [43] described that iturin and fengycin reduced germination and mycelial growth of *F. oxysproum* f. sp. *physali* in a concentration-dependent manner, but surfactin did not. In addition, Liu et al. [15] extracted CLPs from *B. velezensis* HC6, and found inhibitory activity of iturin A and fengycin in the range of 125–1000 µg/mL and 3.1–12.5 µg/mL, respectively. In contrast, surfactin did not inhibit the growth of pathogenic fungi even at a concentration of 1000 µg/mL. However, surfactin was shown to have antibacterial activity, protecting tomatoes from bacterial wilt caused by Rs [46] and bacterial canker caused by Cmm [47]. Surfactin treatment in this study exhibited similar effects. Surfactin did not inhibit the tested fungal strains even at a concentration of 500 µg/mL (Table 3), but exhibited antibacterial activity against Xap with an MIC value of 125 µg/mL (data not shown).

Furthermore, inhibitory activity was assessed, and the $FIC_3$ score was calculated to investigate the synergistic effect of the three CLP combinations on antifungal efficacy. For individual assessment, iturin A, fengycin, and surfactin were tested at 500 µg/mL concentrations each, whereas the content of each CLP in the mixture of the same concentration was 167 µg/mL. The mixture of these three CLPs exhibited a $FIC_3$ value of 0.13–0.88 against seven fungal strains. The $FIC_3$ value of $<1$ indicates a synergistic effect of the three CLPs [23]. Kim et al. [48] reported that iturin A strongly inhibited spore germination of Fg, whereas no inhibitory effect was evident with individual treatments of fengycin or surfactin, but a mixture of the three CLPs exerted a synergistic inhibitory effect. The synergistic effect of the CLPs mixture can increase the cell permeability of fungal hyphae. Although the antifungal effect of surfactin is weak, its combination with iturin [49] or fengycin [50] has been reported to have a synergistic effect and improve the antifungal efficacy.

### Identification of antimicrobial compounds

Standard CLPs, including iturin A, fengycin, and surfactin, were purchased from Sigma-Aldrich, while PKs such as difficidin and oxydifficidin were isolated and purified according to a previous study [25].

**Table 3** Minimum inhibitory concentration (MIC) values of surfactin, iturin A, fengycin, and their mixture (1:1:1, v/v) against several plant pathogenic ascomycetes fungi and the fractional inhibitory concentration (FIC) index

| Plant pathogenic fungi | Minimum inhibitory concentration (µg/mL) | | | | $FIC_3$* |
|---|---|---|---|---|---|
| | Iturin A | Fengycin | Surfactin | Mix** | |
| *Collectotrichum acutatum* | 15.63*** ± 0.00 | 62.50 ± 0.00 | > 500.00 | 15.63 ± 0.00 | 0.42 ± 0.00 |
| *Cryphonectria parasitica* | 7.81 ± 0.00 | 62.50 ± 0.00 | > 500.00 | 7.81 ± 0.00 | 0.38 ± 0.00 |
| *Fusarium graminearum* | 15.63 ± 0.00 | 500.00 ± 0.00 | > 500.00 | 31.25 ± 0.00 | 0.70 ± 0.00 |
| *Mycosphaerella cerasella* | 15.63 ± 0.00 | 250.00 ± 0.00 | > 500.00 | 15.63 ± 0.00 | 0.36 ± 0.00 |
| *Raffaelea quercus–mongolicae* | 7.81 ± 0.00 | 62.50 ± 0.00 | > 500.00 | 15.63 ± 0.00 | 0.76 ± 0.00 |
| *Sclerotinia homoeocarpa* | 15.63 ± 0.00 | 31.25 ± 0.00 | > 500.00 | 3.91 ± 0.00 | 0.13 ± 0.00 |
| *Valsa cerasperma* | 62.50 ± 0.00 | 250.00 ± 0.00 | > 500.00 | 125.00 ± 0.00 | 0.88 ± 0.00 |

* FIC, fractional inhibitory concentration

** Iturin A, fengycin, and surfactin were dissolved in 100% methanol (MeOH) at a concentration of 5000 µg/mL, and then a 1:1:1 (*v/v/v*) mixture of the three standards was prepared. The mixture at 5000 µg/mL contains 1666 µg/mL of iturin A, fengycin, and surfactin

*** Means ± standard deviations from three determinations

UPLC-Q–Orbitrap–MS analysis was conducted to identify these five antimicrobial compounds, comparing the mass spectra between the standards and crude samples. When analyzing crude CLPs and PKs extracts in negative ion mode, the mass spectrum presented an $[M-H]^-$ ion peak at *m/z* 543.27850, representing the molecular formula $C_{31}H_{45}O_6P$, and at *m/z* 559.27307, representing the molecular formula $C_{31}H_{45}O_7P$. These two formulas were identified as difficidin (Fig. S2A) and oxydifficidin (Fig. S2B), respectively. In positive ion mode analysis, the results indicated an $[M+H]^+$ ion peak at *m/z* 1043.5524 ($C_{48}H_{74}O_{14}N_{12}$), representing iturin A (Fig. S2C). The $[M+H]^+$ ion peak was detected at *m/z* 1463.8036 ($C_{72}H_{110}O_{20}N_{12}$), representing fengycin (Fig. S2D). Moreover, the $[M+H]^+$ ion peak at *m/z* 1036.6906 ($C_{53}H_{93}O_{13}N_7$) represented surfactin (Fig. S2E).

*B. velezensis* is well-known for producing bioactive molecules with antimicrobial activity against plant bacterial and fungal pathogens, demonstrating biocontrol potential against plant diseases [51]. Specifically, *B. velezensis* is capable of producing PKs, i.e., difficidin, bacillomycin, and macrolactin, exhibiting antibacterial activity, and CLPs, i.e., iturins, fengycins, and surfactins, which possess antifungal activity [52]. Plant diseases caused by various bacteria and fungi worldwide often co-occur [53]. Typically, Xap invades flowering cherry tree leaves through stomata or wounds, particularly under conditions of high temperature and humidity [54]. Concurrently, pathogens like Mc, which cause SH disease, also infect cherry trees. Co-infection with pathogens can further increase disease severity due to synergistic interactions between pathogens, and it can also complicate the diagnosis and subsequent management of the disease [55]. Application of separate control agents for bacteria and fungi may cause persistent infections to occur due to the inability to achieve effective control against complex infections. Therefore, in this study, we aimed to control SH disease in cherry trees effectively using *B. velezensis* 8–2, which can produce both antibacterial and antifungal active substances.

#### Medium composition modification to improve the antimicrobial activity of *Bacillus* velezensis 8–2

Composition of the culture medium was modified using various carbon and nitrogen sources to enhance the antagonistic activity of *B. velezensis* 8–2. Among the 13 carbon and 9 nitrogen sources tested, maximum inhibition of Xap growth was observed when fructose and yeast extract were used as the carbon and nitrogen sources, with an MIC values of 0.63% and 0.31%, respectively (data not shown). Furthermore, the optimal C:N ratio of the selected carbon and nitrogen sources was confirmed. The C:N ratio of 4:3 showed the highest antibacterial activity with an MIC value of 0.12%, which was more than ten times greater than the activity of the TSB filtrate. Based on these findings, *B. velezensis* 8–2 was cultured in a modified medium containing 4% fructose and 3% yeast extract at 30 ℃ and 150 rpm for 72 h. In another study, Kumar et al. [56] reported that the yield of antimicrobial compounds and antimicrobial activity against *P. expansum* and *Escherichia coli* were improved when fructose and yeast extract were used as carbon and nitrogen sources, respectively.

The composition and concentration of the carbon and nitrogen sources could exert a decisive influence on the production of antimicrobial compounds [57]. The choice of carbon source is reported to affect productivity and the type of antimicrobial substances [58]. Glucose as a carbon source is rapidly metabolized and accelerates cell growth, but it could inhibit the synthesis of secondary metabolites [59]. It has been shown that slowly assimilated carbon sources, other than glucose, can enhance secondary metabolite productivity [57]. Nitrogen sources are involved as precursors in antimicrobial metabolite production pathways and can significantly impact their synthesis [56, 60]. Yeast extract, abundant in amino acids, polypeptides, vitamins, and minerals, can enhance the cell growth and metabolic activity of *Bacillus* species [61, 62].

#### Effect of fermentation conditions on the production of polyketides and cyclic lipopeptides

To further improve the PKs and CLPs titers, fermentation parameters such as temperature, aeration, agitation speed, and feed substrate concentration were evaluated in a 5-L jar fermenter. The highest antagonistic activity of *B. velezensis* 8–2 was observed at 30 ℃ and 1 vvm, and feeding had no effect on the antagonistic activity of strain 8–2 (data not shown). Among the fermentation parameters tested, agitation speed was the most decisive factor. The agitation speed was varied in the range of 300, 400, and 500 rpm in a 5 L fermenter. The highest PKs concentrations of 150.0 μg/mL of oxydifficidin and 86.6 μg/mL of difficidin were obtained at 400 rpm within 72 h of fermentation, followed by oxidifficidin and difficidin yields of 142.3 μg/mL and 8.2 μg/mL at 500 rpm, and 65.9 μg/mL and 84.1 μg/mL at 300 rpm, respectively (Fig. 1). Time-course profiles of *B. velezensis* 8–2 revealed that cell growth was rapidly accelerated with an increase in agitation speed. In addition, cell growth and PKs production followed similar patterns, with increased PK synthesis during the log phase. Increasing the agitation speed increased the volumetric oxygen transfer coefficient, thereby facilitating oxygen delivery to the microbial cultivation system [63]. Sufficient oxygen supply must be ensured, because oxygen deficiency may limit carbon

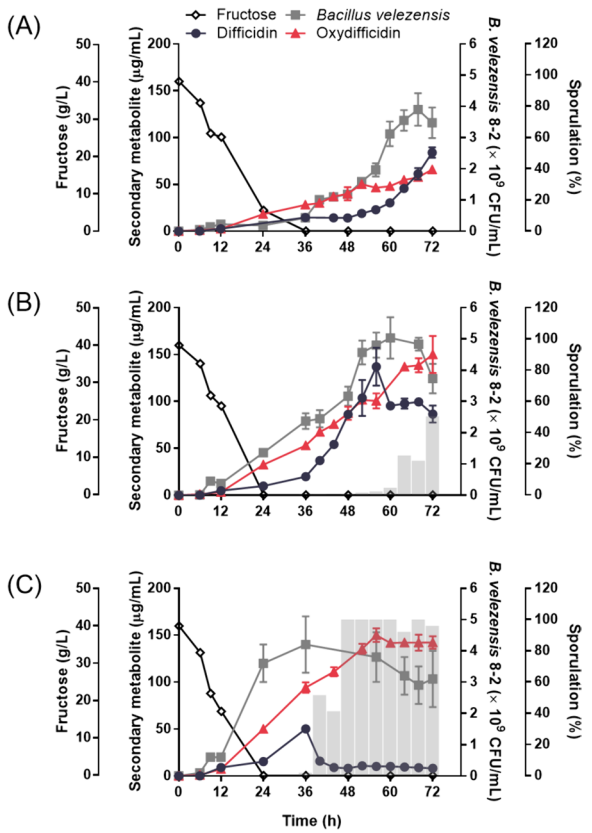



**Fig. 1** Time-course profiles of cell growth and polyketides production during fermentation of *Bacillus velezensis* 8–2 at (**A**) 300, (**B**) 400, and (**C**) 500 rpm. Values represent mean ± standard error of three replicates

and energy metabolism in submerged fermentation of the genus *Bacillus* [64]. An increase in dissolved oxygen can accelerate the rate of glycolysis and increase ATP synthesis, providing more energy for material synthesis [64].

However, PKs synthesis decreased rapidly after sporulation, and these two factors were inversely correlated (Fig. S3). Oxydifficidin production increased initially due to oxidation of the precursor difficidin, but when the sporulation rate increased, it tended to converge to zero, similar to that of difficidin. Sporulation increased with agitation speed, and at 500 rpm, PKs productivity decreased sharply after 36 h of fermentation (Fig. 2). Previous studies have also found a rapid decrease in the productivity of metabolites when sporulation was promoted by increased oxygen supply [65, 66]. High agitation speed can cause a shear effect on microbial cells [63], and excess oxygen supply can result in direct oxidation and substrate loss, reducing productivity [67]. At 300 rpm agitation, no spores were formed throughout the culture, but cell growth seemed to be slow, resulting in low PK production rates. Controlling oxygen supply through agitation speed could be a strategy to maintain cells in vegetative state, leading to improved metabolite productivity. For *B. velezensis* 8–2, the optimal agitation speed for PK production was found to be 400 rpm, where the ratio of vegetative cells and spores was appropriately adjusted.

The trends in CLP production varied across all conditions (Fig. 3). The maximum surfactin yield of 550.2 μg/mL was obtained at 400 rpm during the 72 h fermentation period. Conversely, the concentration of iturin A exhibited an upward production trend with increasing agitation speed, reaching a concentration of 153.9 μg/mL at 500 rpm. Meanwhile, the concentration of fengycin averaged 1037.8 μg/mL, and was unaffected by the agitation speed.

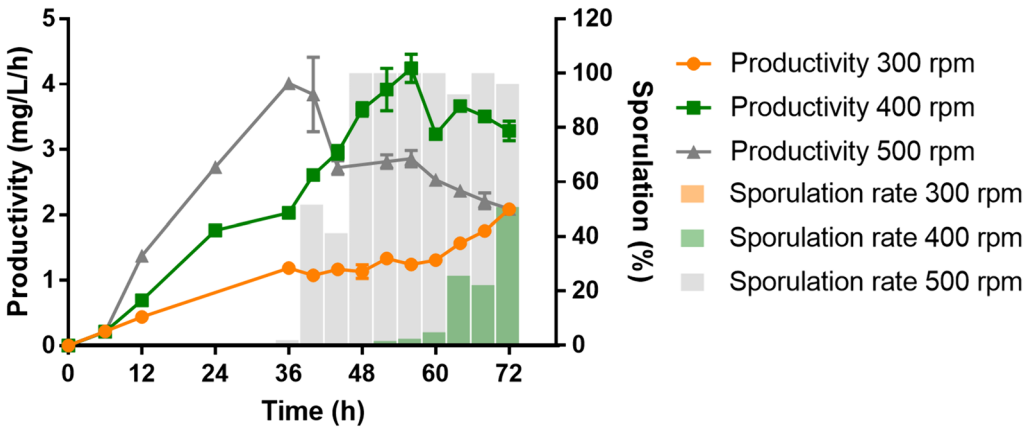



**Fig. 2** Analysis of polyketides (PKs) productivity and sporulation rate by *Bacillus velezensis* 8–2 at different agitation speed (300, 400, and 500 rpm). Values represent mean ± standard error of three replicates

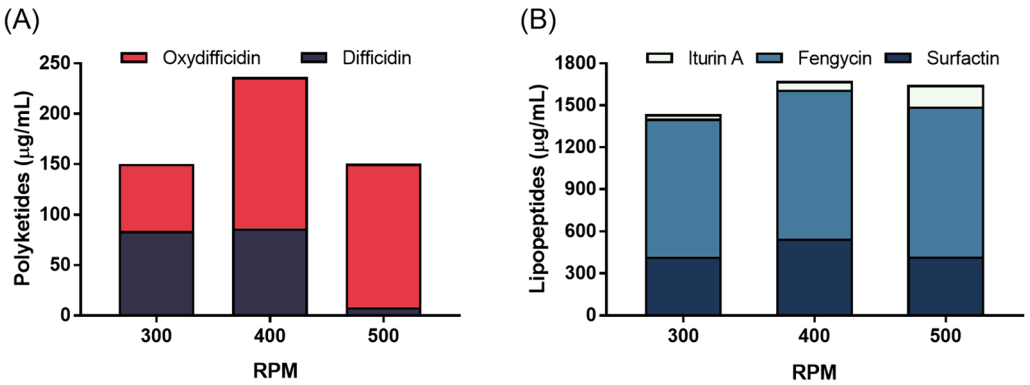



**Fig. 3** Effect of different agitation speeds on (**A**) polyketides (oxydifficidin and difficidin) and (**B**) cyclic lipopeptides (iturin A, fengycin, and surfactin) production by *Bacillus velezensis* 8–2 after 72 h fermentation

**Table 4** Antimicrobial activity of *Bacillus velezensis* 8–2 cultured in flask scale using TSB and the modified medium and 5 L scale using the modified medium against plant pathogenic bacteria and fungi causing shot hole disease on flowering cherry tree

| Species | Plant pathogens | Minimum inhibitory concentration (%) | | |
|---|---|---|---|---|
| | | Flask | | 5 L fermenter |
| | | TSB | Modified medium | Modified medium |
| Bacterium | *Xanthomonas arboricolar* pv. *pruni* | 1.25* ± 0.00 | 0.16 ± 0.00 | 0.04 ± 0.00 |
| | *Burkholderia contaminns* | 5.00 ± 0.00 | 2.50 ± 0.00 | 0.31 ± 0.00 |
| | *Pseudomonas syringae* pv. *syringae* | 5.00 ± 0.00 | 0.63 ± 0.00 | 0.47 ± 0.22 |
| Fungus | *Micosphaerella cerasella* | 10.00 ± 0.00 | 5.00 ± 0.00 | 5.00 ± 0.00 |
| | *Epicoccum tobaicum* | 7.50 ± 3.54 | 5.00 ± 0.00 | 1.56 ± 1.33 |

* Means ± standard deviations from three determinations

### Disease control efficacy of *Bacillus* velezensis 8–2 against shot-hole disease under the field condition

Optimization of medium composition and oxygen supply in submerged culture of *B. velezensis* 8–2 enhanced antagonistic activity against pathogenic bacteria (Xap) and fungi (Mc) that cause SH disease on flowering cherry trees. When *B. velezensis* 8–2 was cultured in modified medium in a 5 L fermenter, the antibacterial activity was 31.3 times higher and the antifungal activity was 2 times higher than when cultured with TSB in a flask (Table 4).

The disease control efficacy of the SC10 formulation prepared from *B. velezensis* 8–2 fermentation broth with MIC values of 0.04% and 5% against Xap and Mc, respectively, was evaluated under the field conditions (Fig. 4). The formulation effectively inhibited the development of the SH disease in flowering cherry leaves and reduced the disease incidence to 25.4% (Fig. 4A). The application of 500-fold diluted 8–2 SC10 resulted in 66.6% disease control. In contrast, the positive control, 1,000-fold diluted Daisen M45 (a.i. mancozeb 75%), exhibited a disease control efficacy of 34.2% (Fig. 4B). In addition, the 500-fold diluted 8–2 SC10 successfully reduced the number of shot-holes per leaf (Fig. 4C). The disease symptoms were reduced by 90.2% and 73.0% for 8–2 SC10 and Daisen M45, respectively (Fig. 4D). Furthermore, in the 8–2 SC10 treatment group, the size of the shot-hole was significantly reduced compared to the Daisen M45 treatment group (Fig. 4E).

Han et al. [4] investigated the in vitro and in vivo antagonistic activity of fermentation filtrates of *B. velezensis* JCK-1618 and JCK-1696 against pathogenic bacteria and fungi that cause SH disease in cherry trees. The MIC values against Xap, Bc, Pss, Mc, and Et were 0.31%, 2.5%, 5%, 1.25%, and 1.25%, respectively. The fivefold diluted filtrate of these strains reduced disease symptom severity by more than 90%, which was not significantly different from the 1000-fold diluted Daisen M45 activity in vivo. In this study, medium composition modification and scale-up were performed to enhance the antagonistic activity compared to a previous study [4]. As a result, the antibacterial activity against SH pathogens increased approximately 7.8–10.6 times compared with previous in vitro studies, and 8–2 SC10 effectively reduced disease incidence and the number of shot-holes per leaf in cherry tree fields compared with Daisen M45.

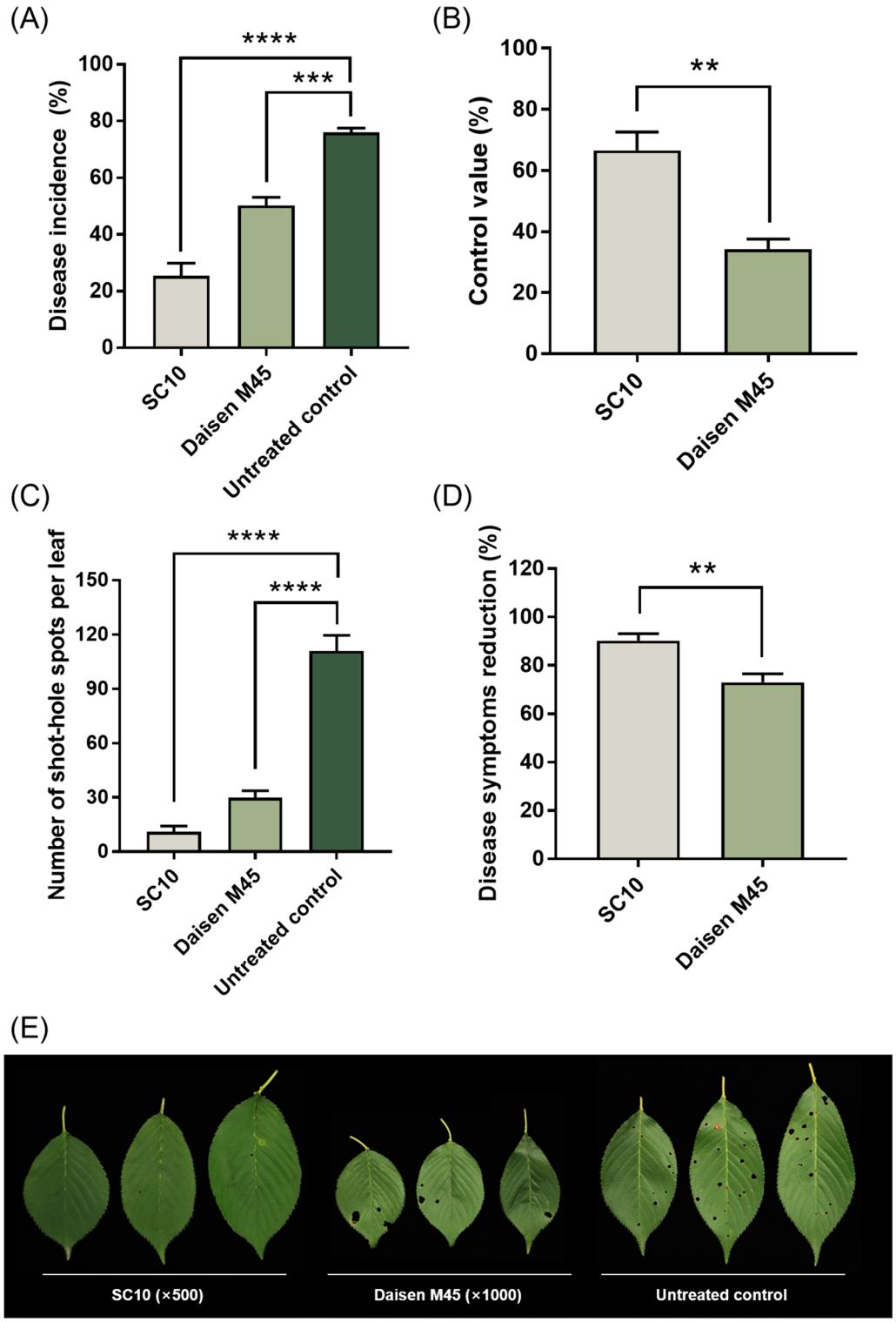



**Fig. 4** Control efficacy of the suspension concentration (SC) formulation of *Bacillus velezensis* 8–2 and Daisen M45 (a.i. mancozeb 75%) against shot-hole (SH) disease on flowering cherry leaves in field experiment. The formulation was diluted 500-fold and Daisen M45 used as a positive control was diluted 1000-fold. Tween 20 (250 µg/mL) was used as a negative control. SH disease occurred naturally in the cherry fields. **A** Disease incidence, **B** control value, **C** the number of shot-holes and leaf spots per leaf, **D** disease symptoms reduction, and **E** disease symptom development. Values represent mean ± standard error of three runs, with three replicates each. In (**A**, **C**), One-way ANOVA and Tukey's HSD test were used (***$p < 0.005$, ****$p < 0.0001$). In (**B**, **D**), independent sample *t* test was used to indicate differences between two groups ($\bar{x}$, $n = 3$)

Several reports have documented potential biological control candidates in both in vitro and in vivo studies, but there are few instances of exceptional efficacy, especially in the control of tree diseases, in field conditions [30]. The efficacy of biocontrol agents in the field is reduced compared to in vitro and in vivo assays [68]. The field environment is susceptible to abiotic (ultraviolet light, rainfall, and wind) or biotic factors (hosts and pathogens), which are known to affect the efficiency of BCAs, especially reducing their persistence [69]. In this study, SC formulation was developed by incorporating additives to address challenging field conditions and enhance the sustainability of the BCAs. Formulation can enhance the efficiency of BCAs by improving their physical properties through the use of additives devoid of medicinal properties [70]. Formulation can enhance the wettability, adhesion, retention, and penetration capabilities of BCAs, allowing them to be exposed to the host for an extended period and enhancing the persistence of BCAs against diseases. The efficacy of 8–2 SC10 against SH disease in the field trials exhibited a significant improvement, suggesting that 8–2 SC10 can be effective in reducing SH disease in cherry trees under field conditions.

## Conclusions

This study emphasizes the potential field application of *B. velezensis* 8–2 as a biological control agent and its disease control efficacy against SH disease in cherry. Antimicrobial metabolites, including PKs and CLPs, produced by *B. velezensis* 8–2 exhibit wide ranging activity against plant pathogens. With the optimization of fermentation parameters, the yield of antimicrobial metabolites increased significantly, and 150.0 μg/mL of oxydifficidin, 86.6 μg/mL of difficidin, 62.6 μg/mL of iturin A, 1061.4 μg/mL of fengycin, and 550.2 μg/mL of surfactin, were produced. In the field condition, the SC10 formulation effectively suppressed SH disease, showing 66% control efficacy. To the best of our knowledge, this is the first report describing the successful application of a *Bacillus* species for biological control of SH disease in flowering cherry trees, suggesting that *B. velezensis* 8–2 would be a promising biocontrol agent.

### Abbreviations

| | |
|---|---|
| Aac | *Acidovorax avenae* Subsp. *cattleyae* |
| An | *Aspergillus niger* |
| ANOVA | Analysis of variance |
| At | *Agrobacterium tumefaciens* |
| Bc | *Burkholderia contaminans* |
| Bc | *Botrytis cinerea* |
| BCA | Biological control agent |
| Bd | *Botryosphaeria dothidea* |
| Bg | *Burkholderia glumae* |
| BuOH | N-Butanol |
| Ca | *Colletotrichum acutatum* |
| CFU | Colony forming units |
| CLP | Cyclic lipopeptides |
| Cmm | *Clavibacter michiganensis* Subsp. *michiganensis* |
| Cp | *Cryphonectria parasitica* |
| DiaMOND | Diagonal measurement of *n*-way drug interactions |
| Ea | *Erwinia amylovora* |
| Ep | *Erwinia pyrifoliae* |
| Et | *Epicoccum tobaicum* |
| Fg | *Fusarium graminearum* |
| FIC | Fractional inhibitory concentration |
| Fo | *Fusarium oxysporum* |
| Fv | *Fusarium verticillioides* |
| KACC | Korean Agricultural Culture Collection |
| KCTC | Korea Collection for Type Cultures |
| Mc | *Mycosphaerella cerasella* |
| MCZ | Mancozeb |
| MeOH | Methanol |
| MIC | Minimum inhibitory concentration |
| MS | Mass spectrometry |
| OD | Optical density |
| Ou | *Ophiostoma ulmi* |
| Pc | *Pectobacterium chrysanthemi* |
| Pcc | *Pectobacterium carotovorum* Subsp. *carotovorum* |
| PDA | Potato dextrose agar |
| PDB | Potato dextrose broth |
| Pi | *Penicillium italicum* |
| PKs | Polyketides |
| Psa | *Pseudomonas syringae* Pv. *actinidiae* |
| Pss | *Pseudomonas syringae* Pv. *syringae* |
| Rq | *Raffaelea quercus–mongolicae* |
| Rs | *Ralstonia solanacearum* |
| SC | Suspension concentration |
| Sh | *Sclerotinia homoeocarpa* |
| SD | Spray-dry |
| SH | Shot-hole |
| TSB | Tryptic soy broth |
| UPLC | Ultra-performance liquid chromatography |
| Vc | *Valsa cerasperma* |
| VOCs | Volatile organic compounds |
| Xap | *Xanthomonas arboricola* Pv. *pruni* |
| Xcc | *Xanthomonas campestris* Pv. *citri* |
| Xe | *Xanthomonas euvesicatoria* |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-024-00604-x.

Supplementary Material 1: Fig. S1. Dual-culture assay of *Bacillus velezensis* 8-2 against fourteen fungal strains. (A) *Aspergillus niger,* (B) *Botryosphaeria dothidea,* (C) *Botrytis cinerea,* (D) *Colletotrichum acutatum,* (E) *Cryphonectria parasitica,* (F) *Epicoccum tobaicum,* (G) *Fusarium graminearum,* (H) *Fusarium oxysporum,* (I) *Fusarium verticillioidese,* (J) *Mycosphaerella cerasella,* (K) *Ophiostoma ulmi,* (L) *Penicillium italicum,* (M) *Raffaelea quercus–mongolicae,* (N) *Valsa ceratosperma;* CK, pathogenic fungal strains cultivated alone served as controls; T, co-cultivation of 8-2 with fungal strains.

Supplementary Material 2: Fig. S2. UPLC-quadrupole–Orbitrap–MS spectra, chemical structures, and molecular weights of active compounds derived from *Bacillus velezensis* 8-2. (A) Difficidin ($[M-H]^-$ ion peak at *m/z* 543.27850), (B) oxydifficidin ($[M-H]^-$ ion peak at *m/z* 559.27307), (C) inturin A ($[M+H]^+$ ion peak at *m/z* 1043.5524), (D) fengycin ($[M+H]^+$ ion peak at *m/z* 1463.8036), and (E) surfactin ($[M+H]^+$ ion peak at *m/z* 1036.6906). The chemical structures, LC chromatograms, and mass spectra are shown.

Supplementary Material 3: Fig. S3. Correlation between sporulation rate and polyketides productivity. (A) Difficidin and (B) oxydifficidin produced by *Bacillus velezensis* 8-2. Orange line, productivity at 300 rpm; green line, productivity at 400 rpm; gray line, productivity at 500 rpm.

Supplementary Material 4.

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**Author contributions**
Seulbi Kim: conceptualization, methodology, validation, investigation, writing—original draft. Ho Myeong Kim: methodology, validation, investigation. Jung Eun Yang: methodology, validation, investigation. Seul-Gi Jeong: methodology, validation, investigation. Yeong Yeol Kim: methodology, validation, investigation. In Min Hwang: formal analysis, data curation. Nan Hee Yu: methodology, validation. Jin-Cheol Kim: conceptualization, writing—review and editing. Hae Woong Park: conceptualization, investigation, writing—review and editing, supervision, funding acquisition.

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**Availability of data and materials**
The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
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.

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## Publisher's Note