Effectiveness of a Novel Endophyte *Bacillus velezensis* Strain B1 in the Biocontrol of Pear Postharvest Ring Rot

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Abstract: Pear ring rot caused by *Botryosphaeria dothidea* severely affects the quality and storage life of pear fruit. Plant endophytes are potential new sources of biocontrol agents due to their abilities to produce novel bioactive products. In this work, we focused on an endophytic strain B1, which was isolated from roots of *Dendrobium huoshanense*. Notably, strain B1 exhibited strongly inhibitory effects against the growth of *Botryosphaeria dothidea* with an inhibition rate of 73.2% after 1 week of co-cultivation. Furthermore, it displayed a broad-spectrum inhibitory activity. Strain B1 also effectively delayed the onset of ring rot caused by *B. dothidea* in pear fruit and reduced the lesion diameter by 73.4%. Strain B1 was identified as *Bacillus velezensis* based on core-genome phylogeny. Genome mining with antiSMASH revealed 13 potential gene clusters involved in antimicrobial metabolites. Three main groups of lipopeptides (surfactin, iturin and fengycin) were identified with MALDI-TOF-MS, and the lipopeptides in the inhibition zone were greatly upregulated by *B. dothidea*; especially, fengycin isoforms were detected with higher abundance. These results proved that lipopeptides produced by strain B1 had significant antagonistic effect on *B. dothidea*. In conclusion, our results suggest that strain B1 has potential applications in the biocontrol of *B. dothidea* in postharvest fruit.

Keywords: *Botryosphaeria dothidea*; pear ring rot; antagonism; *Bacillus velezensis*; MALDI-TOF-MS

1. Introduction

Postharvest diseases caused by plant pathogens have a detrimental impact on fruit yield, resulting in significant economic losses [1]. Pear is one of widely cultivated fruits in the world, and China is the largest producer and consumer of pears [2]. Throughout the cultivation process, pear is susceptible to various pests and diseases. *Botryosphaeria dothidea* is one of the most destructive pathogens in pear production. This pathogen can infect not only the fruits but also leaves and trunks of pear trees, causing pear ring rot and canker and dieback diseases on the tree [3]. Notably, *B. dothidea* exhibits no host preference and has been reported to affect a wide range of plants important in agriculture, forestry and horticulture, making it a global concern [3]. Furthermore, *B. dothidea* have occasionally infected humans. For instance, Noguchi et al. [4] showed that the causal agent of ungual phaeohyphomycosis was *B. dothidea*. A recent study also demonstrated that *B. dothidea* tended to accumulate compounds toxic to mammalian cells [5].

Currently, chemical fungicides are widely used to control *B. dothidea*, causing environmental and public health issues. Hence, there is a requirement to find an alternative to chemical pesticides that are both safe for food production and environmentally friendly. The utilization of microbial agents for the biological control of pathogens presents a promising strategy. In recent years, microbial agents have been reported to produce bioactive compounds with biocontrol properties [6–10]. While most studies have focused on bacteria and fungi from plant rhizospheres, plant endophytes have gained much more attention.
Plant endophytes encompass a diverse array of microorganisms residing inside plants without causing any sign of infection [15]. They occupy a similar ecological niche to phytopathogens, making them viable candidates for biocontrol agents [16]. Endophytes have been detected in every plant sampled; although there are nearly 300,000 plant species on Earth, only a fraction of them have been thoroughly investigated relative to endophytes. Therefore, great efforts to study endophytes from diverse plant species in various ecosystem could bring us the opportunity to obtain new and valuable endophytes with potential applications in medicine and agriculture.

*Dendrobium huoshanense*, a unique medicinal plant, has significant medicinal value and health benefits. Its endophytic bacteria and fungi exhibit rich and diverse [17,18]. The dominant bacterial endophytes are *Sphingomonas*, *Acinetobacter*, *Enterococcus*, *Bacillus* and *Methylobacterium* [17]. *Streptomyces* is the largest genus of *Actinobacteria* and is well known for producing various secondary metabolites. An endophytic *Streptomyces* sp. HS-3-L-1 from *D. huoshanense* is capable of biosynthesis of three new polyketide dimmers known as huoshanmycins A, B and C, and two of these (A and B) show moderate cytotoxicity against MV4–11 human leukemia cells [19]. *Stagonosporopsis oculihominis*, *Alternaria eichhorniae*, *Phyllosticta aristolochiicola*, *Aspergillus flavus* and *Fusarium lactis* are the dominant fungi endophytes of *D. huoshanense* which exhibit anti-inflammatory and antibacterial effects similar to those of the host plant [18]. In the present study, we aimed to isolate and screen endophytic bacteria with antagonistic activity from *D. huoshanense*, assessing the protective effect of the isolated strains against *B. dothidea* on postharvest pears. Additionally, we conducted genomics analysis to mine biosynthetic gene clusters encoding antimicrobial metabolites with antiSMASH. Furthermore, the production of these active metabolites was confirmed with MALDI-TOF-MS. These results could unravel the genetic and metabolic bases of the biocontrol traits exhibited by the antagonistic endophyte. This knowledge will enable us to develop rational strategies for its application in agriculture.

2. Materials and Methods

2.1. Isolation of Antifungal Bacterial Strains

*Dendrobium huoshanense* sampled in Anhui Province, China, were used to isolate root endophytic bacteria using the methods described by Wu et al. [20]. Briefly, roots were washed thoroughly with tap water and surface-sterilized in 75% ethanol for 30 s and in 3% NaClO for 3 min, then rinsed in sterile water 5 times. Sterilized roots were cut into 1 cm pieces, placed in sterile Petri dishes containing beef extract peptone medium (BPN medium) and incubated at 28 °C. Both endophytic bacteria and fungi emerged from the roots in a Petri dish. Bacteria with obvious antagonistic activities towards fungi were isolated and purified, then stored at −80 °C in 20% glycerol.

2.2. Screening of Antagonistic Bacteria against *Botryosphaeria dothidea*

Pear ring rot pathogen *Botryosphaeria dothidea* was isolated from diseased pear fruit and preserved in our laboratory. To assess the antagonistic abilities of isolated endophytes against *B. dothidea*, a dual-culture method was used [21]. Each treatment consisted of three replicate plates and incubated at 28 °C for 7 d. The antagonistic effect was evaluated by measuring the inhibition zones. Among 9 isolated strains, B1 showed the strongest antagonistic activity and was selected for further characterization. The antagonistic effect of B1 on mycelial growth of *B. dothidea* was observed under a light microscope (Olympus CX-40), and the inhibition rate (IR) of the pathogen was calculated using the formula as follows: \[ IR = \left[ \frac{(C2 - C1)}{C2} \right] \times 100\% \], where C2 is the colony radius of the control and C1 is the average colony radius of the treatment.

2.3. Evaluating the Inhibitory Effect of Culture Filtrate of Strain B1

To evaluate the production of antimicrobial compounds by strain B1 in liquid culture against *B. dothidea*, the well-diffusion method on PDA was used. Strain B1 was grown
in liquid BPN. Overnight bacterial cultures (500 µL) were inoculated into 250 mL flasks containing 50 mL of liquid BPN. The flasks were incubated on a rotary shaker at 30 °C and 150 rpm. After incubation for 72 h, the culture was centrifuged at 13,800 × g for 15 min at 4 °C. The obtained cells were washed with sterilized water three times then resuspended in sterilized water to obtain cell suspensions (CSs), while the resulting supernatant was filtered through 0.22 µm filters to obtain cell-free supernatants (CFSs).

The inhibition of fungal growth was assessed on PDA plates, around the periphery of which wells (0.6 cm) were made equidistantly, and 100 µL CS, CFS, or sterilized BPN broth (control) was added to the wells. Then, mycelia plugs (0.6 cm diameter) taken from the edge of the 7-day-old colony of B. dothidea were inoculated in the center of the same plates. The plates were incubated at 28 °C for seven days, then inhibition zones were measured. The experiment was conducted two times in a completely randomized design with three replicates.

2.4. Inhibition Spectrum of Strain B1

Several important plant pathogens purchased from the Agricultural Culture Collection of China were kept in our laboratory. Antagonistic ability of strain B1 against these pathogens was tested as described in Section 2.2.

2.5. Biocontrol of Pear Fruit Ring Rot by Strain B1

Pear fruit (Pyrus pyrifolia Nakai cv. Shuijing) with uniformity of size and no mechanical injury and infection were selected, sterilized with 75% alcohol for 1 min and air dried. The fruit were wounded according to the method of Wu et al. [21]. To evaluate the biocontrol efficacy of strain B1, each wound of fruit was inoculated with 30 µL of B1 (5 × 10^7 cfu/mL), and sterilized distilled water served as a control. After 12 h, mycelia plugs (6 mm in diameter) of B. dothidea taken from the margins of the 7-day-old colony were also applied to the wounds. The treated pears were incubated in climatic chambers at 25 °C and 85% relative humidity for 7 d, with three replicates and 8 pears per replicate. Disease incidence and lesion diameter of pear fruit were determined, and the inhibition rate was calculated as follows:

\[
\text{Inhibition rate (％) = } \left( \frac{\text{Rot diameter of control} - \text{Rot diameter of treatment}}{\text{Rot diameter of control}} \right) \times 100\%
\]

2.6. Genome Sequencing, Annotation and Phylogenetic Analysis

The genomic DNA of strain B1 was extracted with the SDS method. Genome sequencing was performed on the Illumina HiSeq 2500-PE125 platform with massively parallel sequencing (MPS) Illumina technology. The reads were quality trimmed to the Q30 confidence level, and the de novo genome assembly was performed using SOAPdenovo (http://soap.genomics.org.cn/soapdenovo.html) URL (accessed on 26 March 2018). The genome was annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP). The full genome sequence of strain B1 was deposited in NCBI under the GenBank accession number CP097895.

Various Bacillus strains were downloaded from the NCBI database. Genomic alignment between the B1 genome and reference genomes were performed using the MUMmer [22] and LASTZ [23] tools. Core and specific genes were analyzed by the CD-HITsoftware V4.6.8 [24], employing a threshold of 50% pairwise identity and a 0.7 length difference cutoff in amino acids. A pan- and core-genome plot was generated, while MUSCLE was used for aligning the core genes [25]. The phylogenomic tree based on the core genome was constructed using TreeBeST [26] with the neighbor-joining method.

2.7. Genome Mining of Secondary Metabolites Biosynthetic Gene Clusters

AntiSMASH is a comprehensive bioinformatic tool for the automatic genome mining of biosynthetic gene clusters (BGCs) involved in antimicrobial compounds, such as NRPs, PKs, NRPs-PKs hybrids, bacteriocins and terpenes. The B1 genome was analyzed using the
mass spectrometry analysis of secondary metabolites produced by strain B1

Statistical analyses were performed using SPSS (SPSS Statistics 20.0). The values are expressed as means of three replicates ± the standard deviation (SD). Differences between mean values were compared using Student’s t test, with p < 0.05 considered as a significant level.

3. Results

3.1. Isolation and Screening of Antagonistic Endophytes

The growth of B. dothidea was markedly inhibited by strain B1 (Figure 1B), with an inhibition rate of 73.2% after 1 week incubation compared with the control (Figure 1A). Light microscopic observations revealed that the mycelial morphology of B. dothidea was severely distorted by strain B1. The hyphae structure exhibited irregular thickness, with some hyphae forming swollen bubbles that eventually ruptured, resulting in leakage of the intracellular material (Figure 2A). In contrast, the control mycelium was uniform in thickness and smooth in surface, characterized by vigorous growth (Figure 2B). These results indicated that strain B1 severely damaged the mycelium morphology and inhibited the growth of B. dothidea.

Figure 1. In vitro inhibition of Botryosphaeria dothidea by strain B1 after seven days of co-incubation on PDA. (A): control, (B): B. dothidea co-incubation with strain B1.
Figure 2. Light microscopy observations of the changes in hyphal morphology of *B. dothidea* mycelium after seven days of co-incubation with B1 on PDA. (A): Treated mycelium of *B. dothidea* with strain B1, (B): control mycelium.

### 3.2. Inhibitory Effect of Cell Suspension and Cell-Free Supernatants on Growth of *B. dothidea*

Strain B1 was examined for the production of antimicrobial compounds against *B. dothidea* in liquid culture, and the well-diffusion method was used to compare the antifungal properties of cell suspensions (CSs) and cell-free supernatants (CFSs). After 1 week incubation, the hyphal extension of *B. dothidea* was significantly inhibited on PDA medium (Figure 3). Notably, CS was more effective compared to CFS, producing a wider zone of inhibition (Table 1). The higher level of antagonistic activity of CS could be linked to additional biosynthesis of antifungal metabolites by the actively growing B1 cells on PDA medium compared to the CFS.

![Figure 3](image-url)

Figure 3. Effect of antifungal compounds from strain B1 on *B. dothidea* growth. CK: BPN broth; CS: cell suspension; CFS: cell-free supernatant. Photographs were taken following seven days incubation on PDA medium at 28 °C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inhibition Zones (cm) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>1.30 ±0.07 a</td>
</tr>
<tr>
<td>CFS</td>
<td>0.52 ± 0.06 b</td>
</tr>
</tbody>
</table>

Different letters in the same column indicate significantly different results (*p < 0.05*). * The value represented the mean ± standard deviation of three replicates.

### 3.3. Antifungal Spectrum of Strain B1

Other important fungal pathogens were applied to test the antifungal activity of B1 using the same methods as above, and remarkable antagonistic activity was observed (Table 2). Strain B1 showed a broad inhibitory spectrum.
Table 2. Assessment of antifungal spectrum of strain B1.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Inhibition Zone/cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria astroemeriae</td>
<td>1.5 ± 0.14</td>
</tr>
<tr>
<td>Verticillium dahiae</td>
<td>2.4 ± 0.28</td>
</tr>
<tr>
<td>Rhizoctonia solani</td>
<td>1.2 ± 0.13</td>
</tr>
<tr>
<td>Alternaria brassicae</td>
<td>1.3 ± 0.12</td>
</tr>
<tr>
<td>Botrytis cinerea</td>
<td>1.2 ± 0.21</td>
</tr>
<tr>
<td>Fusarium graminearum</td>
<td>1.3 ± 0.22</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>0.4 ± 0.02</td>
</tr>
<tr>
<td>Pyricularia oryzae</td>
<td>2.0 ± 0.23</td>
</tr>
</tbody>
</table>

3.4. Biocontrol Effect of Strain B1 on B. dothidea In Vivo

The incidence of pear fruit ring rot caused by B. dothidea was significantly reduced in the B1 treatment group compared to the control group (Figure 4, Table 3). After 3 days of inoculation, ring rot started to appear in the control pear group, whereas the B1 treatment could completely suppress the disease incidence in pear fruit until 5 days of inoculation. Subsequently, ring rot symptoms initiated in the B1 treatment group. After 7 days of inoculation, the control group displayed over 50% of the fruit area affected by rot, with the average lesion diameter of 4.31 cm. In contrast, the lesion diameter of pear fruit in the B1 treatment group was 1.3 cm on average, 73.4% lower than the control (Table 3). Strain B1 effectively slowed down the occurrence and development of ring rot of pear fruit, achieving a good control effect.

![Figure 4. Biocontrol activity of strain B1 against B. dothidea on wounded pears. Wounded pear fruit treated with strain B1 (B1) and water (CK). Photographs were taken after 7 days of incubation at 25 °C.](image)

Table 3. Incidence and severity of ring rot in wounded pear fruit treated with strain B1 and water (CK). The trials were performed at 25 °C, and disease was assessed 3, 5 and 7 days after B. dothidea inoculation. Different letters in the same column indicate significant difference (p < 0.05) between treatments according to Student’s t test. All data were recorded as means ± standard deviation of three replicates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3d Rot Diameter (cm)</th>
<th>3d Inhibition Rate (%)</th>
<th>5d Rot Diameter (cm)</th>
<th>5d Inhibition Rate (%)</th>
<th>7d Rot Diameter (cm)</th>
<th>7d Inhibition Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>1.3 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>2.81 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>4.31 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.4 ± 18.6</td>
</tr>
<tr>
<td>B1</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>1.3 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

3.5. Genome General Characterization and Phylogenetic Analysis of Strain B1

The strain B1 genome had a sequencing coverage of 25×. The generated raw data were assembled and approximately 4.0 Mbp in length, consisting of 1 contig with a GC
value of 46.54%. This genome harbored 3725 predicted protein-coding genes, 27 rRNA operons and 86 tRNAs (Table S1).

To infer the phylogenomic relationship between strain B1 and its closely related species, an unrooted phylogenomic tree was constructed based on the core genome (Figure 5). Strain B1 was most closely related to *Bacillus amyloliquefaciens* LFB112 and formed a distinct branch with *B. velezensis* and *B. amyloliquefaciens* strains including *B. amyloliquefaciens* FZB42 (Figure 5). *B. amyloliquefaciens* species were previously separated into two taxa “*B. amyloliquefaciens* subspecies *amyloliquefaciens*” and “*B. amyloliquefaciens* subspecies *plantarum*” [28], and the well-known *B. amyloliquefaciens* FZB42 was described as the type strain of this “*plantarum*” subspecies. *B. amyloliquefaciens* FZB42 and LFB112 have been re-categorized as *B. velezensis* [29]. Due to their high phenotypic and 16S rRNA similarities, it was difficult to differentiate *B. velezensis*, *B. amyloliquefaciens* and *B. siamensis* [30]. Dunlap et al. [31] demonstrated that *B. velezensis* NRRLB-41580 T, *B. methylotrophicus* KACC 13105 T, ”*B. oryzicola*” KACC 18228 and *B. amyloliquefaciens* subsp. *plantarum* FZB42 T were conspecific through comparative genomics, DNA–DNA relatedness calculations and phylogenomic analysis based on the core genome. Consequently, *B. velezensis* was considered a heterotypic synonym of *B. amyloliquefaciens* subsp. *plantarum* and was distinguished from its nearest neighbors *B. amyloliquefaciens* and *B. siamensis* [6,31,32]. Consistent with these reports, our phylogenomic analysis placed strain B1 within the *B. velezensis* branch (Figure 5), confirming its identification as *B. velezensis*.

Figure 5. Phylogenomic tree of the strain B1 based on the core genomes.
3.6. Genome Mining of Secondary Metabolites Biosynthetic Gene Clusters (BGCs)

The strong antagonistic activity of well-known biocontrol bacterial strains was mostly due to the production of bioactive secondary metabolites. To better understand the biocontrol properties of strain B1, we aimed to determine genome mining BGCs potentially related to diverse bioactive compounds using antiSMASH. Strain B1 harbored BGCs encoding for 13 secondary metabolites, including NRPSs, polyketide synthases (PKSs), hybrid lipopeptides (NRPS-PKS), terpenes and lanthipeptide (Table S2). Clusters 6, 7, 8, 11, 12 and 13 showed 100% similarity to macrolactin H, bacillaene, fengycin, difficidin, bacillibactin and bacilysin, respectively; cluster 2 showed 86% similarity to surfactin. However, cluster 1 showed 21% similarity to locillomycin/locillomycin B/locillomycin C, and gene cluster 3 showed 7% similarity to butirosin A/butirosin B. Notably, clusters 4, 5, 9 and 10 did not match any known secondary metabolites. BGCs that shared less than 70% amino acid identity against known clusters were regarded as novel, suggesting that clusters 1, 3, 4, 5, 9 and 10 might produce novel compounds.

It is noteworthy that the iturin biosynthetic gene clusters were not found in the strain B1 genome. This is particularly intriguing as gene clusters responsible for the production of iturin were typically present in all the genomes of B. amyloliquefaciens, B. sianensis and B. velezensis [33–35]. Through manual verification of the results from antiSMASH, we found that the fengycin biosynthetic gene cluster actually contains two adjacent gene clusters, related to fengycin (fengycin or fengycin-like compounds plipastatin) and iturin (bacillomycin D or mycosubtilin, iturin-like compounds) biosynthesis respectively. Remarkably, the fengycin and iturin gene clusters exhibited 100% similarity on an amino acid level to the respective B. velezensis FZB42 gene clusters (Figure 6).

![Figure 6](image_url).

**Figure 6.** BGC organization of the bacillomycin D or mycosubtilin and fengycin or plipastatinoperon in strain B1 genome. Gene clusters within the solid rectangle, exhibited 100% similarity to gene cluster synthesizing bacillomycin D or mycosubtilin; Gene clusters within dashed rectangle, exhibited 100% similarity to gene cluster synthesizing fengycin or plipastatin.

AntiSMASH was unable to distinguish between these clusters, possibly due to the close proximity of the fengycin and iturin gene clusters in the genome. Similar observations had been reported previously in B. velezensis FZB42; a large antibiotic DNA island containing the bmy gene clusters involved in the biosynthesis of bacillomycin D was found inserted close to the fen operon [36]. In the genome of B. subtilis ATCC 6633, the BGC responsible for mycosubtilin biosynthesis instead of the bmy gene clusters was found in the same location [37]. These results indicated that the B1 genome contained three lipopeptide BGCs responsible for the synthesis of surfactin, fengycin and bacillomycin D or mycosubtilin and three polyketide gene clusters involved in synthesis of macrolactin, bacillaene and difficidin. These findings suggested that strain B1 had the potential to be used as biocontrol agent.

3.7. MALDI-TOF-MS Analysis of Secondary Metabolites

MALDI-TOF-MS analysis of secondary compounds synthesized by strain B1 in cells and inhibition zones could identify those active compounds with antifungal properties. Two main groups of mass peaks were detected in the MALDI-TOF-MS spectra (Figure 7). The literature search indicated that compounds with the most clusters of peaks could be
attributed to lipopeptides, including surfactin (C14 to C15), iturin (C14 to C17) and fengycin (C14 to C17) [36,38] (Table 4).

Figure 7. MALDI-TOF-MS analysis of lipopeptides from strain B1 after 2 (I) and 4 (II) days of culturing. Lipopeptides from bacterial colony in presence (C) or absence (A) of B. dothidea and from inhibition zone in presence (D) or absence (B) of B. dothidea.
Table 4. Assignments of lipopeptide mass peaks of strain B1 detected with MALDI-TOF-MS [36,38].

<table>
<thead>
<tr>
<th>Mass Peak (m/z)</th>
<th>Family</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1044.3, 1060.3</td>
<td>Surfactin</td>
<td>C_{14}Surfactin [M + Na, K]^+</td>
</tr>
<tr>
<td>1058.3, 1074.3</td>
<td></td>
<td>C_{15}Surfactin [M + Na, K]^+</td>
</tr>
<tr>
<td>1043.3, 1065.3</td>
<td>Iturin</td>
<td>C_{14}Iturin A/C_{14}Mycosubtilin [M + H, Na]^+</td>
</tr>
<tr>
<td>1057.3, 1079.3</td>
<td></td>
<td>C_{15}Iturin A/C_{15}Mycosubtilin [M + H, Na]^+</td>
</tr>
<tr>
<td>1081.3</td>
<td>Iturin</td>
<td>C_{14}Iturin A/C_{14}Mycosubtilin [M + K]^+/C_{16}Bacillomycin D [M + Na]^+</td>
</tr>
<tr>
<td>1095.3</td>
<td></td>
<td>C_{15}Iturin A/C_{15}Mycosubtilin [M + K]^+/C_{17}Bacillomycin D [M + Na]^+</td>
</tr>
<tr>
<td>1109.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1435.5</td>
<td>Fengycin</td>
<td>Ala-6-C14 Fengycin [M + H]^+</td>
</tr>
<tr>
<td>1449.5</td>
<td></td>
<td>Ala-6-C15 Fengycin [M + H]^+</td>
</tr>
<tr>
<td>1463.5, 1485.5, 1501.5</td>
<td>Fengycin</td>
<td>Ala-6-C16 Fengycin [M + H, Na, K]^+</td>
</tr>
<tr>
<td>1477.5, 1515.5</td>
<td></td>
<td>Ala-6-C17 Fengycin [M + H, K]^+</td>
</tr>
<tr>
<td>1491.5, 1529.5</td>
<td></td>
<td>Val-6-C16 Fengycin [M + H, K]^+</td>
</tr>
<tr>
<td>1505.5</td>
<td></td>
<td>Val-6-C17 Fengycin [M + H]^+</td>
</tr>
</tbody>
</table>

In the co-culture of strain B1 and B. dothidea, the antifungal activity was initially observed after 2 d, but the apparent inhibition zone appeared at 4 d. MALDI-TOF-MS spectra of the cell extracts of strain B1 revealed that lipopeptide production remained relatively stable during the growth periods of strain B1, except that relative intensities of iturin isoforms changed (Figure 7[IA,B,IIA,B]). However, the production and diffusion of lipopeptides were significantly altered in response to B. dothidea, especially in the late stage of coculture (Figure 7[IC,D,IIIC,D]). Analyzing the lipopeptide profiles of the inhibition zone, it was noticed that iturins appeared at all times tested (2 and 4 d), while fengycins and surfactins were only detectable at 4 days of co-culture. In addition, the lipopeptides in the inhibition zone were greatly induced by the pathogen; especially, fengycin isoforms were detected with higher abundance at 4 d of co-culture (Table 5), indicating that the strain B1 was able to perceive signals from the fungus and, in response, promoted the production and diffusion of active antimicrobials from the bacteria colony to the inhibition zone. From these results, we found that iturins contributed to the suppression of B. dothidea at the early stage of incubation, and the stronger antifungal activity coincided with the time at which three lipopeptides were produced, demonstrating that lipopeptides produced by strain B1 had a significant antagonistic effect on B. dothidea.

Table 5. Lipopeptide production and diffusion from strain B1 in the inhibition zone after 4 days of culturing.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mass Peak (m/z)</th>
<th>Assignment</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1065.3</td>
<td>C_{14}Iturin A/C_{14}Mycosubtilin [M + Na]^+</td>
<td>1745</td>
</tr>
<tr>
<td></td>
<td>1079.3</td>
<td>C_{15} Iturin A/C_{15}Mycosubtilin [M + Na]^+</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td>1081.3</td>
<td>C_{14}Iturin A/C_{14}Mycosubtilin [M + K]^+/C_{16}Bacillomycin D [M + Na]^+</td>
<td>285</td>
</tr>
<tr>
<td>Dual culture</td>
<td>1044.3</td>
<td>C_{14}Surfactin [M + Na]^+</td>
<td>3976</td>
</tr>
<tr>
<td></td>
<td>1058.3</td>
<td>C_{15}Surfactin [M + Na]^+</td>
<td>893</td>
</tr>
<tr>
<td></td>
<td>1043.3</td>
<td>C_{14}Iturin A/C_{14}Mycosubtilin [M + H]^+</td>
<td>9431</td>
</tr>
<tr>
<td></td>
<td>1057.3</td>
<td>C_{15} Iturin A/C_{15}Mycosubtilin [M + H]^+</td>
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<td>C_{14}Iturin A/C_{14}Mycosubtilin [M + Na]^+</td>
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Table 5. Cont.

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<th>Mass Peak (m/z)</th>
<th>Assignment</th>
<th>Intensity</th>
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<td>Ala-6-C14 Fengycin [M + H]^+</td>
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4. Discussion

The use of biological control agents (BCAs) has emerged as an eco-friendly alternative to the chemical compounds for managing fruit diseases. Plant endophytes have received much attention due to their ability to produce bioactive substances with structural diversity, making them potential antagonists [39–41]. For example, Wicaksono et al. [42] found that endophytes from medicinal plant *Leptospermum scoparium* could be artificially inoculated into kiwifruit plants and control kiwifruit canker disease caused by *Pseudomonas syringae pv. actinidiae*, indicating that endophytes could be transferred to non-host plants and maintain their biocontrol features. Similarly, the endophytic strain *B. subtilis* R31 isolated from the leaves of *Dendrobium orchid* had a significant biocontrol effect on fusarium wilt of susceptible banana varieties, providing another typical example of an exogenous endophyte suppressing non-host banana fusarium wilt [14]. Therefore, endophytes from plants have the potential to be developed as promising biocontrol agents.

In our study, the endophytic bacterium strain B1 exhibited remarkable antagonistic activity against *Botryosphaeria dothidea* and effectively controlled postharvest ring rot in pears. It was identified as *Bacillus velezensis* based on the phylogenomic analysis of core genes (Figure 3). *B. velezensis* is closely related to *B. amyloliquefaciens* and *B. siamensis*, collectively referred to as the “operational group *B. amyloliquefaciens*” [34]. While biosynthetic genes capable of producing surfactin, bacillibactin, amylocyclicin and iturin may be a common genomic feature of the *B. amyloliquefaciens* group [33–35], genes responsible for synthesis of macrolactin, bacillaene and difficidin, LCI and fengycin were only identified from the core genome of *B. velezensis* [33]. Our results were consistent with these reports. *B. velezensis* strain B1 harbored gene clusters responsible for synthesis of lipopeptides (iturin, fengycin, surfactin and bacillibactin), polyketides (macrolactin, bacillaene and difficidin) and bacilysin. These studies indicated that *B. velezensis* strains have the ability to produce a variety of active metabolites with stronger and broader-spectrum antimicrobial properties compared to *B. amyloliquefaciens* and *B. siamensis* strains. Consequently, they have attracted attention as potential BCAs. In accordance with our results, recent studies have verified that endophytic *B. velezensis* was an important source of antagonist. *B. velezensis* strain P2-1, isolated from apple branches, significantly inhibited the growth of *B. dothidea* [43]. *B. velezensis* OEE1, isolated from olive tree roots, showed pronounced inhibition on mycelia growth of *Verticillium dahliae* and effectively controlled fusarium wilt of olive trees under greenhouse and field conditions [44]. *B. velezensis* BLE7, isolated from sisal (*Agave sisalana*), showed a strong biocontrol effect on anthracnose caused by *Colletotrichum musae* in bananas, reducing disease progression up to 97% compared to the positive control [45].
Among the biocontrol mechanisms of this *Bacillus* group, cyclic lipopeptides from *Bacillus* have already been reported as major inhibitors against various phytopathogens [46]. *Bacillus* species were considered to be the most efficient producers of lipopeptides, including the three main families: surfactin, iturin and fengycin, each consisting of closely related isoforms with differences in peptide amino acid sequences and fatty acid chain lengths [47]. Strain B1 exhibited strong antifungal activity against various plant pathogens and caused a significant inhibition zone for pathogens after 7 days of co-culture, indicating that antifungal compounds could be produced and diffused from the bacteria colony into the inhibition zone. MALDI-TOF-MS analysis demonstrated that lipopeptides were the only compounds detected. Strains incapable of producing lipopeptides, such as *B. subtilis* 168, were unable to inhibit the pathogen growth [36]; *Bacillus* mutants lacking the ability to produce surfactin, bacillomycin and fengycin failed to suppress various plant diseases [48]. These findings led us to speculate that one of the antimicrobial mechanisms of B1 might be attributed to the lipopeptide production.

Although lipopeptides were not directly related to the growth of the producing bacteria, the synthesis of lipopeptide species could change in different growth periods [36]. Surfactins and bacillomycins were the major lipopeptides in strain FZB42, peaking at different incubation times [36]. Contrary to these observations, our study revealed that lipopeptide production remained relatively stable during the growth periods of strain B1, except for changes in the relative intensities of iturin isoforms. However, the production and diffusion of lipopeptides were significantly induced in response to *B. dothidea*, especially in the late stage of coculture, with all three lipopeptides—surfactins, fengycins and iturins—experiencing significant stimulation. The simultaneous production of three lipopeptides is not entirely usual, as such co-production of multiple yet different lipopeptides is also found in other *Bacillus* species [36,49]. The simultaneous production of three kinds of lipopeptides might synergistically improve their antimicrobial activities. Thimon et al. [50] reported that co-production of surfactin along with iturin and fengycin was an advantageous characteristic because surfactin could increase the effect of the other lipopeptides. However, Koumoutsi et al. [36] revealed that although *B. velezensis* FZB42 could co-produce three kinds of lipopeptides, bacillomycin D was the main compound responsible for antifungal activity against *F. oxysporum*. Similarly, iturin was a primary contributor to inhibited *F. oxysporum* in *B. velezensis* Y6 [51]. Another study reported that in *B. subtilis* UMAF6639 and EA-CB001, fengycin was the major antimicrobial compound against different phytopathogens [52,53]. Therefore, these results revealed that the relative roles of different LPs in antimicrobial activity might be a species-specific reaction between the beneficial microbe and pathogen. To clarify the role of each individual lipopeptide produced by strain B1 in antimicrobial function, further work, such as experiments with the wild type and mutants that are unable to produce lipopeptides, are necessary.

5. Conclusions

The present study demonstrated that the endophytic isolate *Bacillus velezensis* B1 exhibited a strong inhibitory effect against the pear ring rot pathogen *Botryosphaeria dothidea* in vitro and in vivo. Strain B1 harbored gene clusters responsible for the production of iturin, fengycin and surfactin serving as potent biocontrol agents against *B. dothidea*. Notably, the production and diffusion of these lipopeptides in the inhibition zone was significantly enhanced in the presence of *B. dothidea*. Further studies involving experiments with wild-type strains and lipopeptide-deficient mutants are needed to verify the exact role of each individual lipopeptide in antimicrobial function, and the active antimicrobial lipopeptides could be overproduced through engineering approaches such as promotor engineering, condensation domain engineering and adenylation domain engineering. This work may provide a safe biological approach to managing pear ring rot.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9091049/s1. Table S1: General features of the strain B1 genome; Table S2: Secondary metabolite biosynthetic gene clusters identified in the genome of strain B1 with antiSMASH.

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Data Availability Statement: The complete genome sequences of strain B1 have been deposited in the GenBank database under the accession number CP097895.

Conflicts of Interest: The authors declare no conflict of interest.

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