



## Research article

# Efficient control of the fungal pathogens *Colletotrichum gloeosporioides* and *Penicillium digitatum* infecting citrus fruits by native soilborne *Bacillus velezensis* strains

Tao Xuan Vu<sup>a</sup>, Tram Bao Tran<sup>a</sup>, Minh Binh Tran<sup>a</sup>, Trang Thi Kim Do<sup>a</sup>,  
Linh Mai Do<sup>a,b</sup>, Mui Thi Dinh<sup>b,c</sup>, Hanh-Dung Thai<sup>b,d</sup>, Duc-Ngoc Pham<sup>d</sup>,  
Van-Tuan Tran<sup>b,d,\*</sup>

<sup>a</sup> Center for Experimental Biology, National Center for Technological Progress, Ministry of Science and Technology, C6 Thanh Xuan Bac, Thanh Xuan, Hanoi, Viet Nam

<sup>b</sup> Genomics Unit, National Key Laboratory of Enzyme and Protein Technology, University of Science, Vietnam National University, Hanoi (VNU), 334 Nguyen Trai, Thanh Xuan, Hanoi, Viet Nam

<sup>c</sup> Bio-Agritech, Thuong Mo, Dan Phuong, Hanoi, Viet Nam

<sup>d</sup> Department of Microbiology, Faculty of Biology, University of Science, Vietnam National University, Hanoi (VNU), 334 Nguyen Trai, Thanh Xuan, Hanoi, Viet Nam



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## ABSTRACT

Destruction of citrus fruits by fungal pathogens during preharvest and postharvest stages can result in severe losses for the citrus industry. Antagonistic microorganisms used as biological agents to control citrus pathogens are considered alternatives to synthetic fungicides. In this study, we aimed to identify fungal pathogens causing dominant diseases on citrus fruits in a specialized citrus cultivation region of Vietnam and inspect soilborne *Bacillus* isolates with antifungal activity against these pathogens. Two fungal pathogens were characterized as *Colletotrichum gloeosporioides* and *Penicillium digitatum* based on morphological characteristics and ribosomal DNA internal transcribed spacer sequence analyses. Reinfection assays of orange fruits confirmed that *C. gloeosporioides* causes stem-end rot, and *P. digitatum* triggers green mold disease. By the heterologous expression of the green fluorescent protein (GFP) in *C. gloeosporioides* using *Agrobacterium tumefaciens*-mediated transformation, we could observe the fungal infection process of the citrus fruit stem-end rot caused by *C. gloeosporioides* for the first time. Furthermore, we isolated and selected two soilborne *Bacillus* strains with strong antagonistic activity for preventing the decay of citrus fruits by these pathogens. Molecular analyses of 16 S rRNA and *gyrB* genes showed that both isolates belong to *B. velezensis*. Antifungal activity assays indicated that bacterial culture suspensions could strongly inhibit *C. gloeosporioides* and *P. digitatum*, and shield orange fruits from the invasion of the pathogens. Our work provides a highly effective *Bacillus*-based preservative solution for combating the fungal pathogens *C. gloeosporioides* and *P. digitatum* to protect citrus fruits at the postharvest stages.

\* Corresponding author. Genomics Unit, National Key Laboratory of Enzyme and Protein Technology, University of Science, Vietnam National University, Hanoi (VNU), 334 Nguyen Trai, Thanh Xuan, Hanoi, Viet Nam.

E-mail address: [tuantran@vnu.edu.vn](mailto:tuantran@vnu.edu.vn) (V.-T. Tran).

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## 1. Introduction

Citrus species, including oranges, lemons, grapefruits, pomelos, and limes, are the economically important and preferred fruit crops worldwide. The Food and Agriculture Organization of the United Nations (FAO) reported that citrus crops are commercially cultivated in more than 140 countries, with a global citrus production of 143,755,600 tons in 2019 [1]. However, the citrus production industry may be severely affected by the destruction caused by postharvest pathogens [2]. One of the most common *Colletotrichum* species infecting tropical and temperate fruit crops is reported as *C. gloeosporioides* [3–5]. This fungus is a hemibiotrophic pathogen of anthracnose disease in many plant hosts. *C. gloeosporioides* is the major causative agent of leaf blight, leaf spot, and stem-end rot, followed by premature fruit drop and postharvest anthracnose in citrus [4,6,7]. Morphological characteristics of *C. gloeosporioides* are highly similar to those of other *Colletotrichum* species. These features are not reliable enough for fungal identification at the species level because they can be changed under different environmental conditions. Therefore, molecular analysis based on the sequencing of some target genes is usually required to accurately identify *Colletotrichum* species [3,8]. Another severe disease in citrus at postharvest stages is green mold decay caused by *Penicillium digitatum*. This necrotrophic fungus infects citrus fruits through injuries during handling, transportation, storage, and marketing [2]. *P. digitatum* is the most destructive postharvest pathogen on citrus fruits and accounts for approximately 90% of total postharvest losses [9,10].

Synthetic fungicides such as imazalil, thiabendazole, prochloraz, mancozeb, fludioxonil, pyrimethanil, etc., are mainly used to control postharvest diseases, including stem-end rot and green mold in citrus. Applying these fungicides for fruit preservation raises public concerns over their harmful risks to human health and the environment. Additionally, overuse of these chemical agents causes fungicide resistance in numerous fungal strains, especially in the green mold fungus *P. digitatum*. Therefore, biological control of postharvest diseases exhibits a safer and eco-friendly approach. Several strategies based on microbial antagonists and plant-derived compounds have been developed for controlling postharvest pathogens. Among them, antagonistic microbes appear to be a promising source for developing preservative applications in citrus production at the postharvest stages [6,11–14].

To date, several non-pathogenic *Bacillus* species with strong antifungal activity, including *B. subtilis*, *B. amyloliquefaciens*, and *B. velezensis* have been exploited as biocontrol agents. These Gram-positive, aerobic, endospore-forming bacteria can inhibit a broad spectrum of fungal plant pathogens [13,15,16]. However, the inhibitory effects of the *Bacillus* species on the pathogenicity of both citrus pathogens, *C. gloeosporioides* and *P. digitatum*, still need to be further investigated for potential preservative applications in the future. In the present study, we identified fungal postharvest pathogens causing citrus stem-end rot and green mold in a specialized citrus cultivation region in Northern Vietnam as *C. gloeosporioides* and *P. digitatum*, respectively. Furthermore, two native soilborne *B. velezensis* isolates with strong antifungal activity against both *C. gloeosporioides* and *P. digitatum* were selected as potential bioagents for preserving citrus fruits against postharvest diseases.

## 2. Materials and methods

### 2.1. Sample collection

Orange fruits with stem-end rot or green mold disease symptoms and soil samples were collected from a specialized citrus cultivation region of Ha Giang province in Northern Vietnam. Samples were stored in sterile bags and delivered immediately to the laboratory for microbial isolation.

### 2.2. Isolation of fungal pathogens infecting citrus fruits

Fungal materials were collected from the infected orange fruits of stem-end rot or green mold by sterile toothpicks, diluted in sterile distilled water (SDW), and streaked on potato dextrose agar (PDA) medium. PDA was supplemented with chloramphenicol (100 µg/mL) to prevent bacterial contamination. The plates were incubated at 25 °C for 2–3 days. Fungal isolates were purified by single spore isolation and identified by morphological analyses under a microscope (Krüss, Hamburg, Germany).

To confirm the citrus pathogenicity of fungal strains, orange fruits were cleaned with SDW and 70% ethanol. For the *P. digitatum* infection, artificial wounds (10 mm long and 2 mm deep) were made on the surface of the oranges using a sterile tool, while smaller wounds (5 mm long and 1 mm deep) were generated on the fruit pedicel for the *C. gloeosporioides* infection. A volume of 10 µL of fungal spore suspension (10<sup>6</sup> spores/mL) was inoculated to the respective wounded position on the fruit. SDW was used for the uninfected control fruits (mock) instead of the spore suspensions. The orange fruits were incubated in sterile plastic boxes at 25 °C for 3–10 days.

### 2.3. Isolation of native soilborne *Bacillus* strains with antifungal activity

Soil samples were resuspended and successively diluted in SDW before being heated at 100 °C for 10 min. The bacterial suspensions were then spread on Luria Bertani (LB) agar medium, and the plates were incubated at 30 °C for 1 day. Single colonies appearing on the plates were purified for morphological analysis under microscopy. The antifungal activity of the *Bacillus* isolates against the stem-end rot and green mold pathogens was examined using dual-culture assays.

To examine the antifungal activity of liquid cultures, the *Bacillus* strains were cultivated in Erlenmeyer flasks containing the LB medium at 30 °C for 1 day. The cultures were centrifuged at 12000 rpm for 10 min at 4 °C, and the supernatants were collected for antifungal activity assays by the agar well diffusion method. A volume of 50 µL of fungal spore suspension (10<sup>6</sup> spores/mL) was spread on a PDA plate using sterile glass beads. The plate was kept in a sterile cabinet at room temperature for 30 min to dry the agar surface to

avoid the spreading growth of residual *Bacillus* cells in the supernatants after centrifugation. Next, one well was made in the center of the agar plate, and 30  $\mu\text{L}$  of a bacterial supernatant was added to the well. SDW and the LB medium were used for the control plates instead of bacterial culture supernatants. Plates were incubated at 4 °C for 4 h to promote the diffusion of antifungal substances into the agar medium and then at 25 °C for 5 days to facilitate the fungal growth. The diameters of the inhibition zones were measured to calculate the fungal inhibition as previously described [17]. The experiments were performed in triplicate.

#### 2.4. Molecular identification of microbial strains

Fungal strains were cultivated in potato dextrose broth (PDB) medium at 25–28 °C for 3 days using a shaking incubator. Meanwhile, bacterial isolates were grown in the LB liquid medium at 28–30 °C for 1 day. Total DNA samples were prepared from bacterial biomass or fungal mycelial biomass using our in-house chemical protocols or G-spin™ Total DNA Extraction Mini Kit (iNtRON Biotechnology, Gyeonggi-do, South Korea). DNA samples were directly used for PCR amplifications or stored at –20 °C for later use.

PCR amplification of the fungal internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was performed with the universal primer pair ITS1 (TCCGTAGGTGAACCTGCGG)/ITS4 (TCCTCCGCTTATTGATATGC). Bacterial sequences of 16 S rRNA and *gyrB* were amplified with the universal primer pairs fD1 (AGAGTTTGATCCTGGCTCAG)/rP1 (ACGGTTACCTGTTCAGACTT) and UP-1 (GAAGTCATCATGACGTTCTGCAYGCNNGGNGNAARTTYGA)/UP-2r (AGCAGGGTACGGATGTGCGAGCCRTCACRTCNACRTCNCGTCNGT-CAT), respectively [18–20]. Phusion™ high-fidelity DNA polymerase (Thermo Scientific, Massachusetts, USA) was used for the PCR amplifications. PCR thermal cycle includes 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s; 72 °C for 3 min. PCR products were purified with MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, Gyeonggi-do, South Korea) for sequencing by the 1st BASE company (Singapore). DNA sequences were analyzed with BioEdit 7.2 and compared to the GenBank database using the BLAST tool. Phylogenetic analyses were performed with MEGA X using the statistical neighbor-joining method and bootstrap values of 1000 replicates.

#### 2.5. *Agrobacterium tumefaciens*-mediated transformation of the fungal pathogens *C. gloeosporioides* and *P. digitatum*

*C. gloeosporioides* HGC201 and *P. digitatum* HGP01 were cultivated on PDA plates at 25 °C for 5–7 days to prepare spore suspensions. Fungal spore suspensions as starting materials for genetic transformation were collected from the plates with SDW and adjusted to a concentration of 10<sup>6</sup> spores/mL. The binary vector pGreen2 [21] was employed for the GFP tagging of both fungal pathogens. The steps of *Agrobacterium tumefaciens*-mediated transformation (ATMT) were implemented as previously reported [18]. Some parameters were adjusted for the genetic transformation of *C. gloeosporioides*. Mainly, the co-culture process was performed at 22 °C for 60 h, and the hygromycin concentration of 300  $\mu\text{g}/\text{mL}$  was used for selecting transformants.

Fungal colonies appearing on the filter membranes were transferred to the PDA plates containing the hygromycin selection agent (200–300  $\mu\text{g}/\text{mL}$ ) to confirm the resistance. Hygromycin-resistant transformants were purified by single spore isolation and cultivated in the PDB medium for total DNA extraction. GoTaq® Green Master Mix (Promega, Wisconsin, USA) was used for PCR amplification to confirm fungal transformants. Primer pairs, including HPH-F/HPH-R and GFP-F/GFP-R [18], were recruited for the specific amplification of the hygromycin resistance cassette or the *GFP* reporter gene from genomes of fungal transformants, respectively. The GFP expression in fungal transformants was examined under the Axioplan fluorescence microscope (Carl Zeiss, Oberkochen, Germany) using the slide culture method [18].

To follow the host colonization process, spores of the GFP-tagged strains were inoculated to wounded sites on orange fruits. The infected fruits were incubated at 25 °C for 3–5 days. After the incubation, thin orange tissue slices of the infected areas were prepared with a sharp blade. Samples were then observed under fluorescence microscopy.

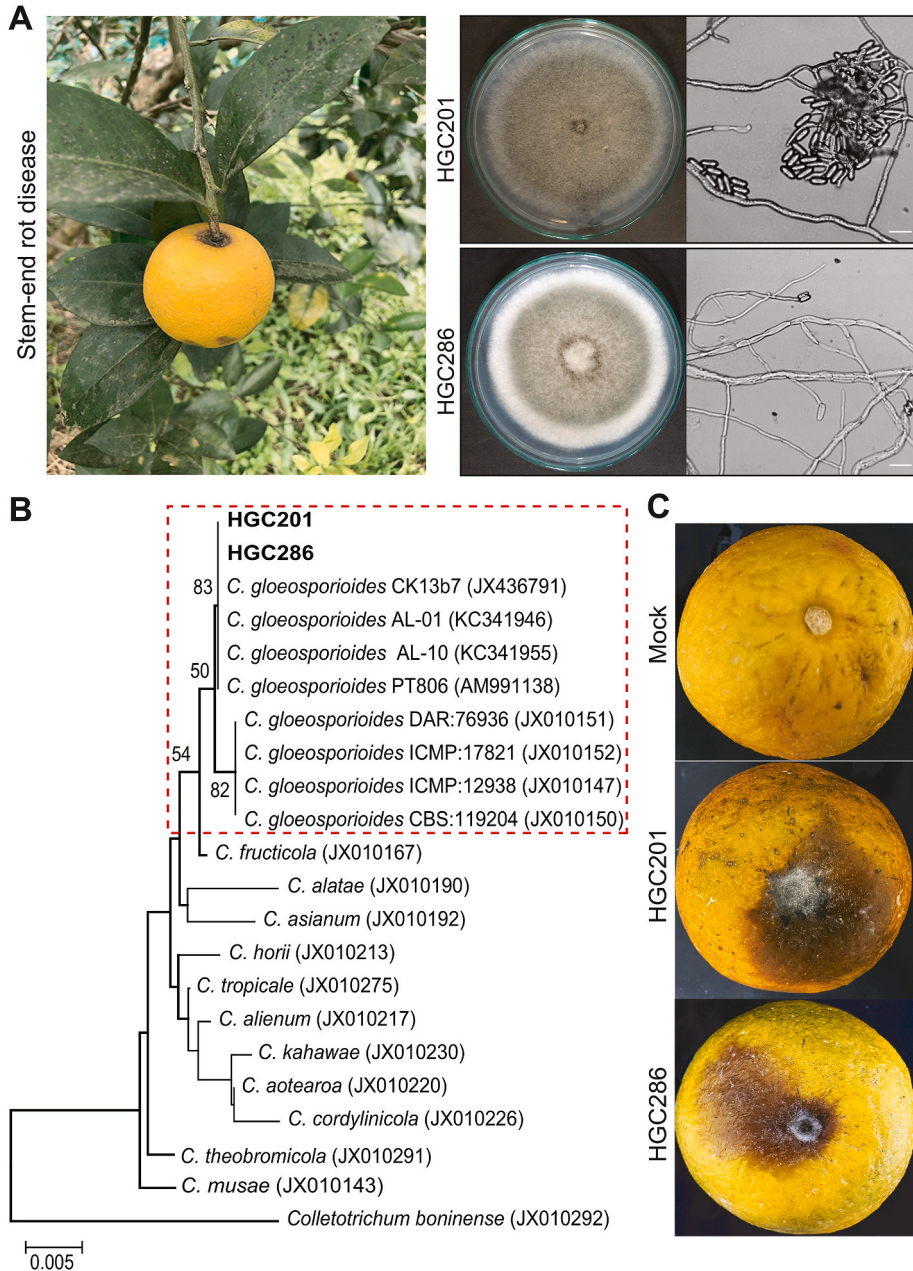
#### 2.6. Evaluating effects of temperature and filtration on antifungal activity of bacterial cultures

Bacterial culture supernatants obtained from centrifugation were incubated at different temperatures, including 30 °C, 40 °C, 60 °C, 80 °C, and 100 °C, for 30 min using a block heater (Corning, Arizona, USA). The heat-treated supernatants were used for antifungal activity assays against *C. gloeosporioides* and *P. digitatum*. These supernatants were further filtered through sterile syringe filters with a pore size of 0.22  $\mu\text{m}$  or 0.45  $\mu\text{m}$  to remove bacterial cells completely. Cell-free filtrates were evaluated for antifungal activity by the agar well diffusion method or the microdilution method [17]. For microdilution assays, 1% or 5% of each filtrate was supplemented to PDA before pouring this medium into Petri plates. Spore suspensions were prepared at 10<sup>4</sup>–10<sup>6</sup> spores/mL by serial dilutions, and 10  $\mu\text{l}$  of each diluted spore suspension was inoculated onto the culture filtrate-containing PDA plates. Filtrate-free PDA plates were used for the controls. Plates were incubated at 25–28 °C for 5 days.

#### 2.7. In vivo assays for antifungal activity against the pathogens on citrus fruits

Two orange varieties were used in the study, including *Citrus sinensis* and *Citrus reticulata* × *sinensis* (a citrus hybrid originating in Vietnam with the name “Cam Sanh”), which were also grown in Ha Giang province, Northern Vietnam. Orange fruits were cleaned with SDW and subsequently with 70% ethanol. The fruits were soaked into the bacterial cultures for 30 min. Afterward, the fruits were air-dried at room temperature before being infected with either *C. gloeosporioides* or *P. digitatum*. For uninfected control fruits (mock), SDW was used instead of fungal spore suspensions. Ten orange fruits were used for each treatment, and the experiment was performed in triplicate. The GFP-tagged strains of *C. gloeosporioides* and *P. digitatum* were also recruited to verify this assay. The fungal invasion

was examined under fluorescence microscopy with the GFP signal. The fungal strains were re-isolated by placing thin slices of infected orange tissue on PDA supplemented with chloramphenicol (100 µg/mL) to inhibit bacterial growth. The inoculated fruits were kept separately in sterile plastic boxes at 25 °C for 3–10 days. Symptomatic progression of fruit decay was initially observed after 3 days.

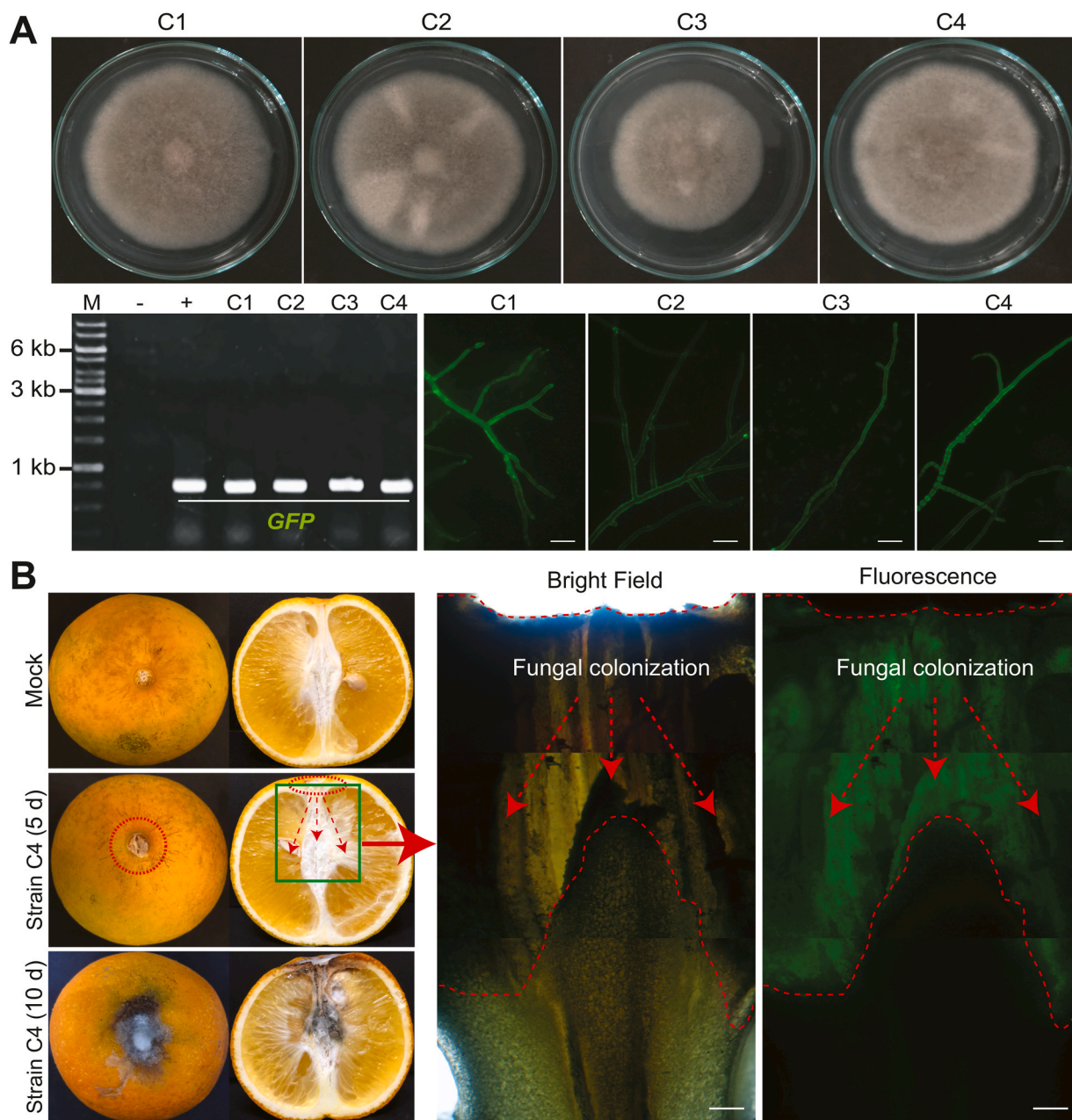


**Fig. 1.** Identification of the stem-end rot pathogen in citrus fruits. (A) Two fungal strains (HGC201, HGC286) resembling *Colletotrichum* fungi were isolated from stem-end rot orange fruits. Fungal strains were cultivated on PDA at 25 °C for 5 days for morphological analyses. (B) Molecular identification of strains HGC201 and HGC286 by ITS rDNA sequencing. The ITS sequences were compared to GenBank using the BLAST tool. The phylogenetic tree was constructed with MEGA X using the neighbor-joining method and 1000 bootstrap replications. Statistical support values at branch nodes of the tree, genetic distance scale, and accession numbers for the ITS sequences from other fungi are indicated. The ITS sequences for two *C. gloeosporioides* strains (HGC201 and HGC286) are deposited in GenBank with accession numbers OL739236–OL739237. (C) Reinfection of the isolated strains on citrus fruits to confirm their pathogenicity. Fungal spore suspensions (10<sup>6</sup> spores/mL) were employed for the fruit infection assays. Negative control fruits (mock) were inoculated with SDW. The fruits were incubated in sterile plastic boxes for 10 days. Scale bars indicate the sizes of the images. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



## 2.8. Statistical analysis

Experiments were performed in triplicate, and data were expressed as mean  $\pm$  standard deviation (SD). Statistical analyses were conducted with GraphPad Prism 8 using one-way ANOVA. A statistically significant difference was considered when  $p < 0.05$ .



**Fig. 2.** GFP tagging of the fungal pathogens by ATMT for inspecting citrus fruit colonization. **(A)** The transformants generated from *C. gloeosporioides* HGC201 were confirmed for the presence of the *GFP* gene in their genomes by PCR and examined for the *GFP* expression under fluorescence microscopy. **(B)** Orange fruits were infected with spores of the transformants of *C. gloeosporioides* for 5–10 days at 25 °C. Thin slices of the infected fruits were prepared to detect the host colonization by the fungus via the *GFP* signal. Scale bars indicate the sizes of the images. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

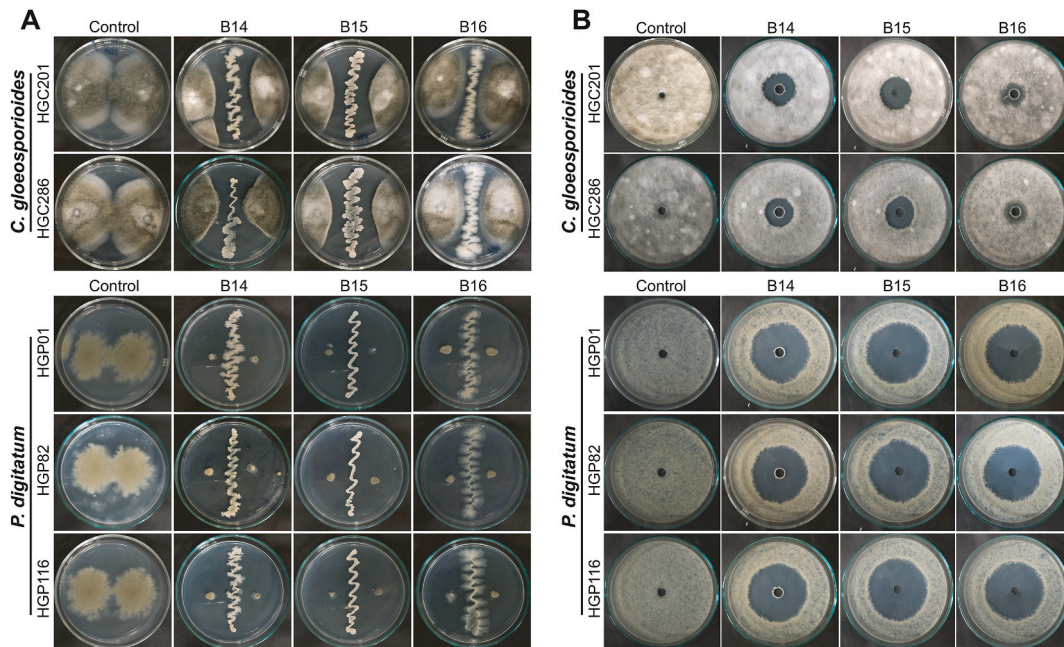
### 3. Results and discussion

#### 3.1. The virulent strains of *C. gloeosporioides* and *P. digitatum* isolated in Northern Vietnam are the main causative agents of stem-end rot and green mold diseases on orange fruits

*C. gloeosporioides* is the causative agent of anthracnose on a wide range of plant hosts, including mango, papaya, apple, pomegranate, citrus, etc. This pathogenic fungus produces a lot of asexual spores (conidia) for dispersal in nature by rain and wind. The anthracnose disease is symptomatically characterized by dark spots or lesions on different parts of plants, including leaves, shoots, twigs tip, petals, and fruits [3,7,22]. Regarding citrus crops, *C. gloeosporioides* were reported to cause anthracnose on citrus hosts in Italy, Spain, Portugal, Greece, Malta, Australia, New Zealand, USA, Tunisia, and Mexico [14,22–25]. The moldy fungus was also revealed as the infectious agent of citrus post-bloom fruit drop in Brazil [4]. In this study, we collected orange fruits with the symptoms of stem-end rot and green mold diseases directly at a specialized orange crop cultivation region in Ha Giang, a mountainous province in Vietnam. Fungal materials separated from the infected fruit tissues were diluted with SDW and spread on the PDA plates supplemented with chloramphenicol to obtain single fungal colonies. Dominant fungal colonies were selected for further analyses. From orange fruits with decay symptoms around the fruit pedicel, we isolated two fungal strains designated as HGC201 and HGC286. The morphological characteristics of these strains are similar to those of *Colletotrichum* species [8]. They possess light grey mycelia after 7 days of cultivation on PDA at 25 °C. Under microscopy, fungal mycelia are branched with single cylindrical conidia at hyphal tips (Fig. 1A). Although the ITS region of rDNA is unreliable for resolving relationships within *Colletotrichum* genus, it allows the separation of *C. gloeosporioides* from all other species [8]. Our molecular analyses based on the rDNA ITS sequences classified HGC201 and HGC286 as *C. gloeosporioides* (Fig. 1B). Reinfection assays confirmed that HGC201 and HGC286 cause the black rot symptom around the pedicel of the infected orange fruits (also called stem-end rot) within 10 days at 25 °C (Fig. 1C).

Furthermore, from decayed oranges with green mold symptoms, three other fungal strains (HGP01, HGP82, HGP116) were isolated. Colonies of these strains are turned from white into olive green after 3 days of growth on PDA at 25 °C. Microscopic examinations revealed that fungal mycelia are irregularly branched, and conidiophores possess terverticillate structures with chains of oval conidia at tips (Fig. S1A). These morphological features resemble *P. digitatum* PdVN1, which was previously reported [18]. Analyses of the ITS sequences using the universal primer pair ITS1/ITS4 indicated that all three strains, HGP01, HGP82, and HGP116, belong to *P. digitatum* (Fig. S1B). Pathogenicity assays confirmed that these strains trigger the green mold disease on orange fruits within 7 days at 25 °C (Fig. S1C).

Our reinfection results indicated that the strains of each fungal pathogen are quite similar in their virulence (Fig. 1 & S1). Therefore,



**Fig. 3.** Antifungal activity of the *Bacillus* isolates. The dual-culture and agar well diffusion methods were used to inspect the antifungal activity of *Bacillus* isolates against two stem-end rot *C. gloeosporioides* strains (A) and three green mold *P. digitatum* strains (B). For the dual-culture method, three *Bacillus* strains (B14, B15, B16) were cultivated on the PDA plates in the presence of the fungal pathogens. Control plates were inoculated with only fungal strains. For the agar well diffusion method, the *Bacillus* strains were grown in conical flasks containing the LB medium at 30 °C for 1 day. Culture supernatants collected by centrifugation were added to agar wells on the PDA plates fully covered with fungal spores. For control plates, SDW was used instead of the culture supernatants. The plates were incubated at 25–28 °C for 5 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



we selected just one representative strain of each fungus, including *C. gloeosporioides* HGC201 and *P. digitatum* HGP01, for further investigation.

### 3.2. GFP tagging approach allows following the orange fruit colonization of the stem-end rot pathogen *C. gloeosporioides* and the green mold pathogen *P. digitatum*

To inspect the invasive progress of citrus fruits by *C. gloeosporioides* and *P. digitatum*, the strain HGC201 of *C. gloeosporioides* and HGP01 of *P. digitatum* were employed for the GFP tagging using the ATMT method. Fungal transformants were selected on PDA supplemented with hygromycin. The results showed that the transformants could grow well under the selection pressure of the high hygromycin concentrations (200–300 µg/mL). Molecular investigations by PCR analysis using the specific primer pairs indicated that both the cassettes for the hygromycin resistance (*HPH*) and the *GFP* expression were present in the genomes of these transformants. Furthermore, the strong expression of the *GFP* reporter gene under the control of the *Aspergillus nidulans* *gpdA* promoter could be easily detected in the transformants generated from *C. gloeosporioides* and *P. digitatum* (Fig. 2A and S2). Fungal colonization of citrus fruits via the *GFP* expression has been recently reported for *P. digitatum* [18,26]. In this study, the GFP-tagged *C. gloeosporioides* (strain C4) with the wild-type like phenotype was employed for visualizing the fungal colonization process and decay of the central cores in the infected orange fruits. Compared to the negative control (mock), the severe disease symptom of stem-end rot was detected by direct observation inside and outside the infected fruits after 10 days of spore inoculation. Under fluorescence microscopy, the invasion of this fungal pathogen could be seen at the earlier stage of 5 days, in which the GFP-tagged *C. gloeosporioides* infected the host by mycelial colonization through the central core of orange fruit (Fig. 2B). This is the first report describing the disease progression of citrus stem-end rot caused by the fungal pathogen *C. gloeosporioides*.

### 3.3. The soilborne *B. velezensis* isolates possess strong antifungal activity against both fungal pathogens infecting citrus fruits

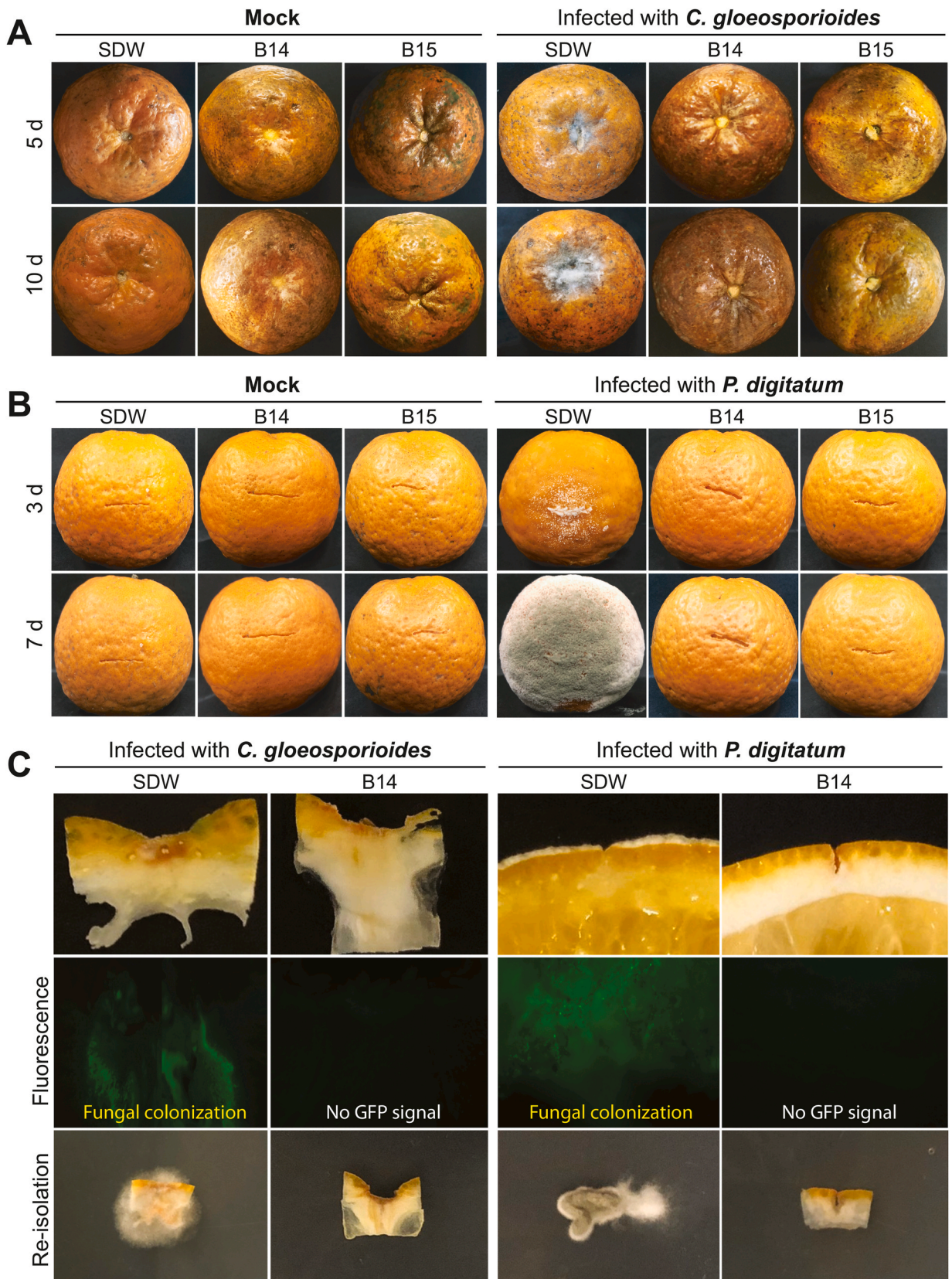
To prevent the attack of postharvest pathogens, synthetic fungicides are often used to preserve citrus fruits. However, the continuous use of fungicides may pose potential risks to the environment and human health. Additionally, it also promotes fungicide resistance in fungal pathogens. Therefore, biological control is a promising approach to replacing synthetic fungicides in fruit preservation [10,13]; Wang et al., 2020). From native soil samples, we detected three bacterial strains possessing antifungal activity against both the citrus pathogens *C. gloeosporioides* and *P. digitatum*. These bacterial isolates were designated as B14, B15, and B16. The results from the assays using the dual-culture method indicated that B14 and B15 exhibited vigorous antifungal activity against the *C. gloeosporioides* strains, while B16 displayed weak antifungal activity toward this pathogen. However, these bacterial isolates presented robust activity against all tested *P. digitatum* strains (Fig. 3A). Inspections of culture supernatants using the agar well diffusion method confirmed the strong activity of B14 and B15 against *C. gloeosporioides* and *P. digitatum* with inhibition zones in diameters of 21–22 mm and 50–56 mm, respectively (Fig. 3B).

Microscopic examinations indicated that B14 and B15 are Gram-positive bacteria with rod-shaped cell morphology (data not shown). When cultured on the LB agar, colonies of these antagonistic bacteria possess a milky white phenotype with a wrinkled surface (B14) or jagged edges (B15) (Fig. S3A). Molecular analyses showed that the 16 S rRNA sequences from B14 and B15 are almost identical (99.6–100%) to those of *B. velezensis*, and very closely related to those of *B. amyloliquefaciens* and *B. subtilis* deposited in GenBank (Fig. S3B). To ensure the accurate taxonomy of these strains, the *gyrB* gene encoding DNA gyrase subunit B was recruited as the second signature for the identification. Based on the *gyrB* sequences, the data confirmed that B14 and B15 belong to the *B. velezensis* species (Fig. S3C). By DNA sequencing of some target genes, several antagonistic *Bacillus* strains used as a vital component of commercial bioproducts for plant protection have been recently re-identified as *B. velezensis* instead of *B. subtilis* or *B. amyloliquefaciens* [27]. Therefore, a combination of morphological and DNA analyses is required for discriminating *B. velezensis* from other closely related *Bacillus* species.

A prominent role of *Bacillus* species in biological control against phytopathogens has been recently documented [15,28,29]. Many isolates of *B. velezensis* possess antimicrobial activity against pathogenic bacteria, fungi, and nematodes [29,30]. The *B. velezensis* TS3B-45 strain was shown to have inhibitory activity *in vitro* against the anthracnose pathogen *C. gloeosporioides*, similar to the synthetic fungicide prochloraz [31]. The volatile organic compounds produced by *B. velezensis* strains exhibited antifungal activity against postharvest fungal pathogens, including *P. digitatum* [32]. Two strains of *B. velezensis* isolated in this study could suppress the citrus pathogens *C. gloeosporioides* and *P. digitatum*. These antagonistic isolates may also inhibit the growth of other pathogenic fungi. Further analyses of the strains *B. velezensis* B14 and B15 still need to be investigated.

### 3.4. Antifungal activity of the bacterial liquid cultures was decreased by heat treatment or sterile filtration

*B. velezensis* has emerged as a vital member of plant microbiomes for agricultural applications. Numerous studies have shown that *B. velezensis* and other closely related *Bacillus* species possess gene clusters involved in the biosynthesis of secondary metabolites, which play significant roles in suppressing microbial pathogens. These bioactive metabolites include surfactin, iturin A, bacillomycin D, fengycin, bacillibactin, macrolactin, bacillaene, and difficidin [15,29–31,33,34]. Furthermore, volatile organic compounds from *B. velezensis* strains were reported to inhibit the growth of *P. digitatum* by up to 28% [32]. Antifungal compounds from *Bacillus* species are usually stable after treating with abiotic stresses [35,36]. To assess the thermostability of bacterial liquid cultures for antifungal activity, B14 and B15 were grown in LB, and culture supernatants were collected by centrifugation. After treatment at different temperatures, the supernatants were tested for antifungal activity. Our results showed that heat-treated supernatants maintained



(caption on next page)



**Fig. 4.** *In vivo* assays of the antagonistic *B. velezensis* strains in protecting citrus fruits. Oranges were soaked in bacterial cultures for 30 min and air-dried at room temperature. The fruits were wounded and infected with fungal spores from (A) *C. gloeosporioides* HGC201 or (B) *P. digitatum* HGP01. The infected oranges were incubated in sterile boxes at 25 °C for 3–10 days. (C) The antifungal effects of bacterial cultures on citrus fruit infection were evaluated using the GFP-tagged strain of *C. gloeosporioides* HGC201 or *C. gloeosporioides* HGC201. The fungal strains were re-isolated from the infected fruit tissues using PDA supplemented with chloramphenicol (100 µg/mL) to eliminate bacterial cells and facilitate fungal growth. The plates were incubated at 25 °C for 5 days.

significant ability against *C. gloeosporioides* and *P. digitatum*. However, antifungal activity against *P. digitatum* of the supernatants treated at 100 °C was strongly decreased for B14, and even lost for B15 (Fig. S4).

Removing bacterial cells from a liquid culture by sterile filtration to obtain a cell-free filtrate is essential for conducting antifungal assays [37]. Strains B14 and B15 were cultivated in the liquid medium, and the cultures were filtered through sterile syringe filters with a pore size of 0.22 µm or 0.45 µm. The cell-free culture filtrates were examined for antifungal activity against the pathogens by the agar well diffusion method and the microdilution method. The results showed that the antifungal activity of the filtrates was significantly reduced for *P. digitatum* and even lost entirely for *C. gloeosporioides* (Fig. S5). Our data suggest that potential antifungal molecules produced by *B. velezensis* B14 and B15 might be somehow retained on the membranes of the syringe filters during sterile filtration. Therefore, it is worthwhile to consider the direct use of liquid cultures of the *B. velezensis* strains to maximize antagonistic activity against the citrus fungal pathogens.

### 3.5. Bacterial cultures as preservatives to protect orange fruits from the attacks of the pathogens

Like other closely related *Bacillus* species, including *B. subtilis* and *B. amyloliquefaciens*, *B. velezensis* is also a beneficial and safe microorganism for humans and the environment [30,33]. When mixed with cultivation media, the culture filtrate of *B. velezensis* CE 100 effectively suppressed *C. gloeosporioides*. A cyclic tetrapeptide from *B. velezensis* CE 100 was indicated to inhibit the growth of this pathogen *in vitro* even more effectively than benzoic acid [38]. In this work, we examined whether bacterial cultures from the *B. velezensis* strains (B14 and B15) could serve as preservatives to protect citrus fruits from stem-end rot and green mold pathogens. The wounded oranges were soaked in bacterial liquid cultures or SDW before being infected with either *C. gloeosporioides* HGC201 or *P. digitatum* HGP01. The uninfected control experiments (mock) were also conducted similarly but without fungal inoculation. The results showed that the cultures prepared with B14 or B15 suppressed symptomatic disease development on the treated fruits that resembled the uninfected control fruits. In contrast, the fruits soaked in SDW and infected with the fungi displayed severe symptoms of stem-end rot or green mold (Fig. 4A and B).

Further inspections using the GFP-tagged strains of *C. gloeosporioides* HGC201 or *P. digitatum* HGP01 also resulted in supportive pieces of evidence. In this assay, the liquid culture of B14 also effectively protected the wounded orange fruits from the invasion of the GFP-tagged strains. Under fluorescence microscopy, no green signal of the GFP reporter protein could be detected in the fruits treated with the B14 culture suspension before fungal infection. Inversely, in the control fruits soaked in SDW, the systemic colonization of fungal mycelia inside the orange tissues could be observed with clear GFP signals. The GFP-tagged strains were easily re-isolated from the infected tissues (Fig. 4C).

We also performed additional antifungal tests to compare the antifungal ability of the *B. velezensis* strains with the common synthetic fungicide mancozeb. As the commercial name AN-K-ZEB 80WP, this synthetic fungicide contains 80% of the antifungal agent mancozeb and is widely distributed in Vietnam for controlling fungal plant pathogens. Our results revealed that mancozeb strongly inhibited the growth of both fungal pathogens on the agar medium at a low concentration of 0.001%. However, it was not as effective in protecting citrus fruits from fungal infection as the antagonistic *B. velezensis* strains, even at the recommended fungicide concentration of 0.25% (Fig. S6). Recently, the metabolite bacillomycin D from *B. velezensis* HN-2 has been demonstrated to possess strong activity against *C. gloeosporioides* compared to the commonly used fungicide mancozeb [39]. The potential of *B. velezensis* as a probiotic in animal feeds and bioproducts for agriculture has also been reviewed [29,40]. Our data showed that the soilborne *B. velezensis* strains, B14 and B15, can serve as biological agents for citrus fruit preservation instead of synthetic fungicides. However, additional analyses still need to be conducted to identify antifungal metabolites, the safety, and other biological characteristics of these antagonistic *B. velezensis* strains.

## 4. Conclusions

In this work, we reported two predominant fungal pathogens causing stem-end rot and green mold diseases on citrus fruits cultivated in Ha Giang province of Northern Vietnam. Based on morphological and DNA sequencing analyses, these postharvest pathogens were identified as *C. gloeosporioides* and *P. digitatum*. Further, we provided experimental evidence for the citrus fruit infection process by *C. gloeosporioides* using a GFP-tagged strain to observe fungal colonization directly under fluorescence microscopy. To search for potential bioagents to control these fungal pathogens, two *B. velezensis* strains with strong antifungal activity were isolated from the native soil samples. These bacterial isolates efficiently antagonized the citrus causal agents *C. gloeosporioides* and *P. digitatum* *in vitro* and *in vivo*. We also indicated that the direct application of bacterial liquid cultures was more effective in preventing the pathogens from attacking citrus fruits. The present study revealed that the selected *B. velezensis* strains are promising candidates for developing biofungicides to prevent postharvest losses in citrus crops due to stem-end rot and green mold diseases. These *B. velezensis* strains may also be exploited as bioagents to replace synthetic fungicides in controlling other phytopathogenic fungi.

## Declarations

### Author contribution statement

Tao Xuan Vu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Tram Bao Tran, Minh Binh Tran, Trang Thi Kim Do, Linh Mai Do, Mui Thi Dinh, Hanh-Dung Thai, Duc-Ngoc Pham: Performed the experiments; Analyzed and interpreted the data. Van-Tuan Tran: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### Data availability statement

Data included in article/supplementary material/referenced in article.

## Declaration of competing interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2023.e13663>.

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