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Antifungal Activity of Volatile Organic Compounds from *Bacillus velezensis* CE 100 against *Colletotrichum gloeosporioides*

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Abstract: *Colletotrichum gloeosporioides* is the most prevalent phytopathogen, causing anthracnose disease that severely affects the production of various fruit trees, including walnut and jujube. In this study, the volatile organic compounds (VOCs) from *Bacillus velezensis* CE 100 disrupted the cell membrane integrity of *C. gloeosporioides* and reduced the spore germination by 36.4% and mycelial growth by 20.0% at a bacterial broth concentration of 10%, while the control group showed no antifungal effect. Based on the headspace solid-phase microextraction/gas chromatography-mass spectrometry (HS-SPME/GC-MS) analysis, seven VOCs were identified from the headspace of *B. velezensis* CE 100. Out of the seven VOCs, 5-nonylamine and 3-methylbutanoic acid were only detected in the headspace of *B. velezensis* CE 100 but not in the control group. Both 5-nonylamine and 3-methylbutanoic acid showed significant antifungal activity against the spore germination and mycelial growth of *C. gloeosporioides*. Treatment with 100 µL/mL of 5-nonylamine and 3-methylbutanoic acid suppressed the spore germination of *C. gloeosporioides* by 10.9% and 30.4% and reduced mycelial growth by 14.0% and 22.6%, respectively. Therefore, 5-nonylamine and 3-methylbutanoic acid are the potential antifungal VOCs emitted by *B. velezensis* CE 100, and this is the first report about the antifungal activity of 5-nonylamine against *C. gloeosporioides*.

Keywords: antagonistic bacteria; 5-nonylamine; 3-methylbutanoic acid; anthracnose disease; spore germination; mycelial growth

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1. Introduction

Plant pathogenic fungi are among the most common causes of plant diseases, with more than 10,000 species reported to cause infections in various plant hosts, leading to enormous losses in yield and quality of plant produce [1,2]. *Colletotrichum* species are among the top 10 plant pathogens of scientific and economic importance [3]. The phytopathogen *C. gloeosporioides* is one of the most problematic and economically harmful phytopathogens that cause anthracnose diseases, especially in the tropic and subtropic regions of the world [4–7]. Recently, anthracnose diseases caused by *C. gloeosporioides* were reported to cause anthracnose diseases in fruit trees, including walnut (*Juglans regia* L.), and jujube (*Zizyphus jujuba* Miller var. *inermis Rehder*) in Korea [7,8]. Especially in walnut, anthracnose disease causes dieback and black spots on leaves, rotten flowers, and fruits, and results in reduced productivity [6,7,9]. *Colletotrichum* spp. are well-known hemibiotrophic pathogens that initially display biotrophic characteristics for a short period before causing necrotic lesions [3]. The conidial fungi are the principal source of infection and are commonly spread by irrigation water, and rainwater drops (splashes) [10]. Chemical fungicides, such as dithiocarbamate (mancozeb), chlorothalonil, azoxystrobin,

carbendazim, benzimidazoles benomyl, fludioxonil, chlorothalonil, dodine, and fluazinam, are the most extensively used measures to control anthracnose diseases caused by *C. gloeosporioides* in various plants [6,11–14]. However, the use of chemical fungicides could lead to toxic residues in food products, pose health risks to humans and animals, and pollute the environment, which affects the ecosystem [15,16]. Moreover, some fungicides have a slow biodegradation process that leads to long-term exposure to sub-lethal doses and could pose a risk of fungicide resistance [13,17–23].

Due to improved awareness about the health and environmental risks associated with chemical use in food production systems, there has been an increase in demand for fungicide-free products and a subsequent change in crop production strategies that aim at reducing the use of agrochemicals [24,25]. For instance, the use of plant-growth-promoting bacteria (PGPB) and their metabolites is a promising strategy for plant disease management and is an environmentally friendly alternative to chemical fungicides [7,23,26]. The PGPB prevents field and post-harvest plant fungal diseases through the secretion of hydrolytic enzymes [7,8], and by producing bioactive compounds, including the emission of volatile organic compounds (VOCs) [26–29]. Bacterial VOCs are generally lipophilic, with low molecular weight (<300 Da), low polarity, and high vapor pressure, which enables their diffusion to distant targets [26,30–32].

Many research studies have reported the production of antifungal VOCs/gaseous metabolites from *Bacillus* species [29,30,33]. PGPB produce VOCs in response to environmental signals (info-chemicals) during their interactions with other organisms to influence microbial populations and communities (ecosystems). For instance, PGPB can emit VOCs as biocontrol factors or a deterrent against plant pathogenic fungi and other competing bacteria species to protect the host plant [27,29,30]. They could also act as autoinducers to enhance the proliferation of other beneficial microbes [34–37]. Recent advances in analytical science such as the headspace solid-phase microextraction/gas chromatography-mass spectrometry (HS-SPME/GC-MS), have enabled the extraction, identification, and characterization of VOCs emitted by PGPB and other bacterial species [30,38,39]. Based on the previous studies, *Bacillus* spp. emit different VOCs, including organic acids, aldehydes, alcohols, ketones, esters, phenols, N-containing compounds, S-containing compounds, and other hydrocarbons that have the potential for antifungal activity [30,39,40]. Gao et al. [41] found that the VOCs produced by *B. velezensis* ZSY-1 could effectively inhibit spore germination of *B. cinerea* and mycelial growth of *Alternaria solani*. In their study, the main VOCs that were detected using HS-SPME/CG-MS include pyrazine (2,5-dimethyl), benzothiazole, 4-chloro-3-methyl, and phenol-2,4-bis (1,1-dimethyl ethyl) [41]. Some of the VOCs that are notably known for antifungal activity against plant pathogens include primary amines [42], and organic acids such as 3-methyl butanoic acid and 2-methylbutanoic acid [39,43]. These antifungal VOCs can disrupt the hyphal morphology and lowers the cell membrane stability, which causes desiccation and cell invasion by water-soluble toxins, due to increased cell membrane permeability [33]. This antagonist activity of VOCs reduces the spore germination and mycelial growth of phytopathogenic fungi, which lowers in turn the rate of infection and plant damage [29,33,39]. In addition, some VOCs can also induce systemic resistance in plants, which enables plants to resist adverse effects from fungal pathogen infections [29,33,39].

In the present work, we investigated the antifungal activity of VOCs produced by *B. velezensis* CE 100 against *C. gloeosporioides*, which cause walnut anthracnose disease [7]. Previously, *B. velezensis* CE 100 exhibited antifungal activity against the mycelial growth and spore germination of *C. gloeosporioides* through the production of cyclic tetrapeptide [28], and lytic enzymes activity [7]. However, the antifungal activity of VOCs produced by *B. velezensis* CE 100 against *C. gloeosporioides* has not been described. Therefore, the aim of this study was to determine the antagonistic effect of the VOCs produced by *B. velezensis* CE 100 against the germination of spores and to reduce the mycelial growth of *C. gloeosporioides*. In addition, the study aimed to identify the VOCs produced by *B. velezensis* CE 100 using

HS-SPME/GC-MS and to evaluate their respective antifungal effect against the germination of spores and reducing the rate of mycelial growth of *C. gloeosporioides*.

2. Materials and Methods

2.1. Antagonistic Bacteria and Pathogenic Fungi

B. velezensis CE 100 was isolated from the soil used for the tomato experiment [44], and the bacterial stock culture was stored at −80 °C in a tryptone soy broth (TSB) medium (Neogen Corporation, Lansing, MI, USA) with 50% (*v*/*v*) glycerol for further experiments. The fungal pathogen *C. gloeosporioides* WL-11 (accession number PRJNA849159), was isolated from the black-spotted leaf of the walnut tree and was previously confirmed for the pathogenicity of anthracnose disease in walnut [7]. The fungus was sub-cultured on potato dextrose agar (PDA) medium (Difco Laboratories, Detroit, MI, USA) at 25 °C for 7 days and used in this study.

2.2. Antifungal Activity of VOCs from Bacillus velezensis CE 100 against Spore Germination and Mycelial Growth of Colletotrichum gloeosporioides

The inhibitory effect of VOCs from *B. velezensis* CE 100 on the germination of spore and mycelial growth was tested using the double petri-dish assay [33]. The phytopathogen *C. gloeospoioides* was grown on a PDA medium at 25 °C for 7 days. Then, 10 mL of sterilized distilled water was flooded on a fungal plate and gently scrubbed with a spatula. The resulting spore suspension was then filtered through four layers of sterile cheesecloth, and the suspension was adjusted to 10^6 conidia/mL using sterilized distilled water. Then, *B. velezensis* CE 100 (10^7 Colony-forming unit (CFU)/mL) was grown in a pink broth (PB) medium [7], for 7 days at 28 °C. Then, *B. velezensis* CE 100 broth culture was diluted using sterilized distilled water to different treatment concentrations: 1%, 3%, 5%, 7%, and 10%. Then, 100 µL of the different concentrations of *B. velezensis* CE 100 broth culture (1, 3, 5, 7, and 10%) were placed on a PB solid medium plate, and another PDA plate inoculated with 100 µL (10^6 conidia/mL) of *C. gloeosporioides* spore suspension was inverted on top and tightly sealed with two layers of parafilm tape. For the control group, PB solid medium plates without bacterial treatment were used. Three replicates were set up for each experimental group. The plates were tightly sealed with two layers of parafilm tape and incubated at 25 °C for 6 h. The germination of spores in each group was observed on light microscopy (Olympus BX41, Tokyo, Japan) at 200× magnification. When the spore germination rate in the control group exceeded 90%, then the spore germination rate of all treatment groups was measured, and the reduction rate in spore germination was calculated using the following formula, GR (%) = ((C − T)/C) × 100, where GR is the rate of reduction in spore germination, C is the percentage of spore germination in the control group, and T is the percentage of spore germination in the treatment group.

The antifungal effect of VOCs produced by *B. velezensis* CE 100 against the mycelial growth of *C. gloeosporioides* was conducted following a previously described method [33]. The fungus was grown on a PDA medium at 25 °C for 7 days. Then, a plug (5 mm) from the fungal agar was punched and placed at the center of a fresh PDA medium. The fungal plates were inverted onto the PB solid medium containing100 µL of the different concentrations of *B. velezensis* CE 100 broth culture (1%, 3%, 5%, 7%, and 10%). The fungal plates inverted on a PB medium without bacterial treatment were used as control. The plates were tightly sealed with two layers of parafilm and incubated at 25 °C for up to 7 days. Each treatment was prepared in three replicates. The rate of reduction in mycelial (MR) was calculated using the formula MR (%) = ((C − T)/C) × 100, where MR is the rate of reduction in mycelial growth, T is the rate of mycelium growth on the treatment plate, and C is the rate of mycelium growth on the control plate. Then, small pieces of mycelial growth were examined for hyphae deformation. The mycelia were placed on a glass slide with a drop of water, covered with cover glasses, and examined for changes in hyphae morphology under a light microscope (Olympus BX41, Tokyo, Japan) at a magnification of 200× [45,46].

2.3. Collection and Identification of VOCs Emitted by Bacillus velezensis CE 100 Using HS-SPME/GC-MS

The VOCs from *B. velezensis* CE 100 were analyzed by using headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography and mass spectrometry (GC-MS), following the procedure described in Mendez-Bravo et al. [47]. Briefly, 5 mL of PB agar medium was placed into 20 mL headspace vials and autoclaved for 15 min. Then, 100 µL of *B. velezensis* CE 100 (10^7 CFU/mL) broth culture was inoculated on the surface of the PB agar medium in the vials. The vials were tightly capped with polytetrafluoroethylene (PTFE)/silicone septa and incubated for 7 days at 28 °C in the dark. For the control group, vials containing only PB medium without bacterial inoculation were used. Three replicates were used for each group. Then, an HS-SPME fiber assembly, HS-20 (Shimadzu, Kyoto, Japan), was inserted into the headspace for VOC extraction. Before every extraction, the fiber was conditioned at 250 °C for 30 min. After extraction, SPME fibers were injected into the port of GC-MS-QP2010 Ultra (Shimadzu, Kyoto, Japan), and VOCs were thermally desorbed at 250 °C. The separation and detection of peaks were carried out using gas chromatography. Helium gas was used as a carrier gas (1.5 mL/min, constant flow) at a split ratio of 10:1, and an Elite-5ms column (30 m, 0.25 mm inside diameter, and 0.25 µm film thickness) was used. Each run was performed for 24 min. The initial oven temperature of 33 °C was held for 3 min, and then ramped up to 180 °C at a rate of 10 °C/min, and then further to 240 °C for 4 min (at an increment rate of 40 °C/min). The mass spectrometer was operated in the electron ionization (EI) mode at an ionizing voltage of 70 eV with a source temperature of 220 °C, interface temperature of 220 °C, injection volume of 1.0 mL, and a mass range of 50 m/z to 500 m/z was used. Then the mass spectra of VOCs obtained were compared with those in the NIST/EPA/NIH Mass Spectrometry Library with respect to the spectra in the Mainlib and Replib databases to identify the gases produced by *Bacillus velezensis* CE 100 [48].

2.4. Antifungal Activity of the VOCs Produced by B. velezensis CE 100 against the Germination of Spore and Mycelial Growth of Colletotrichum gloeosporioides

After analysis by HS-SPME/GC-MS, the VOCs that were only identified from *B. velezensis* CE 100 headspace vails and not detected in the control (5-nonylamine and 3-methylbutanoic acid) were purchased from Daejung Chemicals (Siheung-si, Korea). Then, the individual compounds of the identified VOCs were tested for antifungal activity (reduction in the rate of spore germination and mycelial growth) against *C. gloeosporioides*. The VOCs, 5-nonylamine, and 3-methylbutanoic acid were separately prepared at different concentrations of 10, 30, 50, 70, and 100 µL/mL with dimethyl sulfoxide (DMSO). The antifungal effect of each VOC against *C. gloeosporioides* was evaluated by measuring the rate of reduction in spore germination and mycelial growth after treatment. For each treatment, 100 µL of the different concentrations of 5-nonylamine and 3-methylbutanoic acid were used. The double petri-dish method as described above was used, with the fungal plate inverted on the treatment plate. The plates were tightly sealed together with two layers of parafilm to avoid gaseous escape. For the control group, the PB medium was treated with only DMSO without VOC. Three replicates were used in each group.

2.5. Statistical Analysis

The statistical analysis was performed using statistical analysis software (SAS) version 9.4 software (SAS Institute, Cary, NC, USA). Means were compared using the least significant difference (LSD) test of analysis of variance (ANOVA) at $p = 0.05$.

3. Results

3.1. Antifungal Activity of the VOCs from Bacillus velezensis CE 100 against Spore Germination and Mycelial Growth of Colletotrichum gloeosporioides

The VOCs produced by the different concentrations of *B. velezensis* CE 100, exhibited a significant antifungal efficacy against the spore germination of *C. gloeosporoides* (Figure 1).

The VOCs from the different concentrations: 1%, 3%, 5%, 7% and 10% of *B. velezensis* CE 100, reduced the spore germination of *C. gloeosporioides* by 15.4%, 18.9%, 25.4%, 30.0% and 36.4%, respectively (Figure 1A). The antifungal activity of the VOCs produced at each of the concentrations of *B. velezensis* CE 100, remarkably reduced the germ tube elongation compared to normal germ tube elongation observed in the control group (Figure 1B).

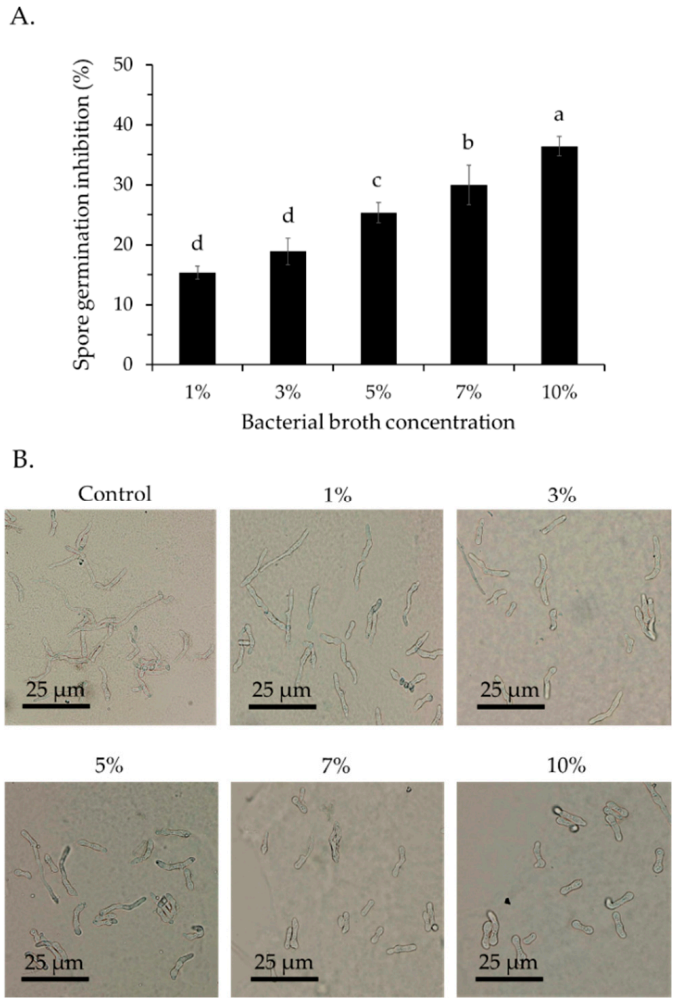

Figure 1. The antifungal effect of VOCs produced by the different concentrations of *Bacillus velezensis* CE 100 against the spore germination of *Colletotrichum gloeosporioides*. Percentage reduction in spore germination (**A**), and suppression of spore germination and germ tube elongation (**B**). The bacterial broth concentration of 1% is equivalent to a bacterial cell concentration of 0.02 OD_{600}. Error bars represent the standard deviation of the mean (n = 3). Means with different letters are significantly different ($p < 0.001$).

Moreover, the VOCs produced from different concentrations of *B. velezensis* CE 100 reduced the mycelial growth of *C. gloeosporioides* (Figure 2). The highest rate of reduction in mycelial growth of 20.0% was observed at a bacterial concentration of 10%, while the lowest reduction rate of 7.4% was recorded at a concentration of 1% (Figure 2A). The antagonistic activity VOCs from each of the different concentrations (1%, 3%, 5%, 7%, and 10%) of *B. velezensis* CE 100 broth culture caused hyphae abnormalities such as coiling and discoloration, which led to a reduction in the rate of mycelial growth of *C. gloeosporioides* (shown by the arrows). However, the mycelia in the control groups exhibited normal growth, with straight growing hyphae (Figure 2B). The difference between the hyphae morphologies of *C. gloeosporioides* exposed to the treatment and the control group indicates a notable antifungal effect of VOCs from *B. velezensis* CE 100.

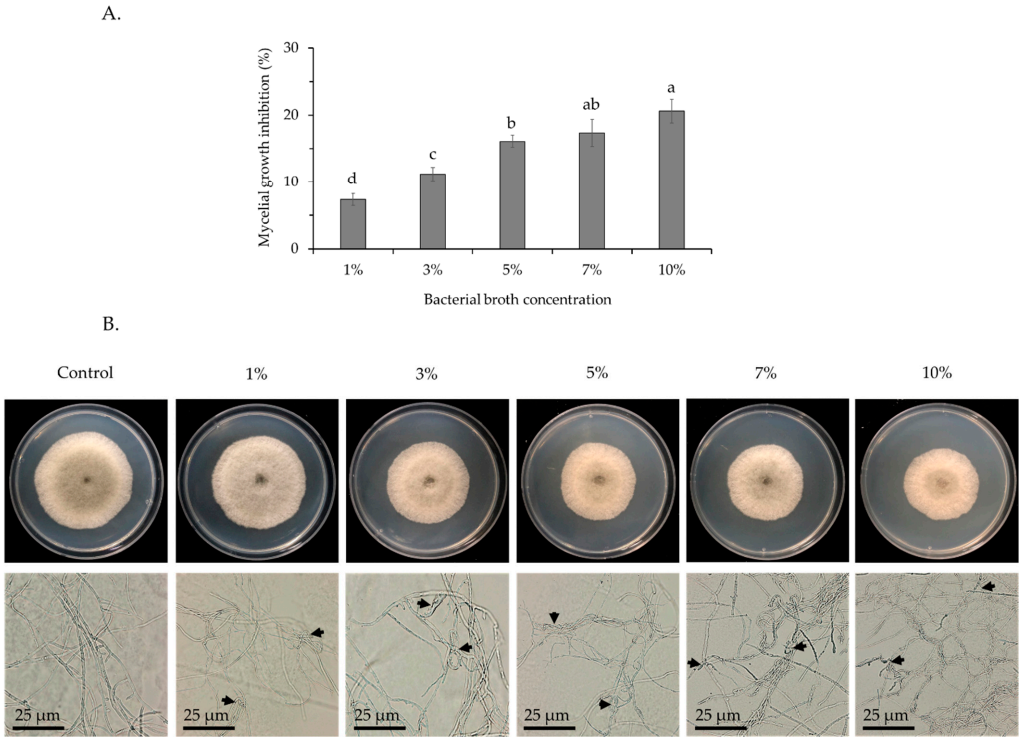

Figure 2. The antifungal effect of VOCs produced by the different concentrations of *Bacillus velezensis* CE 100 against the mycelial growth of *Colletotrichum gloeosporioides*. Percentage reduction in mycelial growth (**A**), and reduction in mycelial growth and hyphal morphology (**B**). The bacterial broth concentration of 1% is equivalent to a bacterial cell concentration of 0.02 OD_{600}. Arrows indicate deformed hyphal after exposure to VOCs from the different concentrations of *B. velezensis* CE 100. Error bars represent the standard deviation of the mean (n = 3). Means with different letters are significantly different ($p < 0.001$).

3.2. HS-SPME/GC-MS Analysis of VOCs Emitted by Bacillus velezensis CE 100

The analysis of the headspace of *B. velezensis* CE 100 and the control reveals a diverse profile of VOCs in each group after 7 days of incubation. The VOCs in the bacterial group include organic acids, the methyl group, the amine group, and esters, while the control comprised of the esters group and a methyl group (Table 1).

The HS-SPME/GC-MS identification of VOCs emitted by *B. velezensis* CE 100 revealed that only two out of seven compounds, 5-nonylamine (amine) and 3-methylbutanoic acid (organic acid), were not present in the control groups (Table 1). The cyclotrisiloxane esters and estragole (methyl) were common in the headspace of both *B. velezensis* CE 100 and the control. Specifically, 5-nonylamine was the most abundant component of the volatiles produced by *B. velezensis* CE 100, with a relative peak area of 39.5%. On the other hand, 3-methylbutanoic acid only showed a relative peak area of 2.92% on a mass spectrum.

Table 1. The identified volatile organic compounds from the headspace of *Bacillus velezensis* CE 100, at 28 °C in PB medium.

Family	Identified Compounds	Molecular Formula	CAS Number	RT (min)	Relative Peak Area (%)	Control/Blank
Amine	5-Nonylamine	$C_9H_{21}N$	112−20−9	1.70	39.5	Nd
Acid	3-Methylbutanoic acid	$C_5H_{10}O_2$	140−67−0	1.96	2.92	Nd
Ester	Cyclotrisiloxane hexamethyl	$C_6H_{18}O_3Si_3$	503−74−2	5.45	3.21	*
	Cyclotetrasiloxane octamethyl	$C_8H_{24}O_4Si_4$	541−05−9	8.96	16.22	*
	Cyclopentasiloxane decamethyl	$C_{10}H_{30}O_5Si_5$	556−67−2	11.66	14.05	*
	Cyclohexasiloxane dodecamethyl	$C_{12}H_{36}O_6Si_6$	514−02−6	14.25	4.2	*
Methyl	Estragole	$C_{10}H_{12}O$	140−67−0	12.69	19.9	*

RT: retention time in minutes, Nd: not detected in the control, and *: detected in the control. Samples were prepared in headspace vials and volatile organic compounds were identified by SPME/GC-MS.

3.3. Antifungal Activity of 5-Nonylamine and 3-Methylbutanoic Acid against Spore Germination and Mycelial Growth of Colletotrichum gloeosporioides

After identification, 5-nonylamine and 3-methylbutanoic acid were separately evaluated for the antifungal activity against *C. gloeosporioides* to determine the activity of each component of the VOCs emitted by *B. velezensis* CE 100. Both 5-nonylamine and 3-methylbutanoic acid exhibited significant antifungal activity against *C. gloeosporioides*, causing a reduction in spore germination and reducing the rate of mycelial growth (Figure 3).

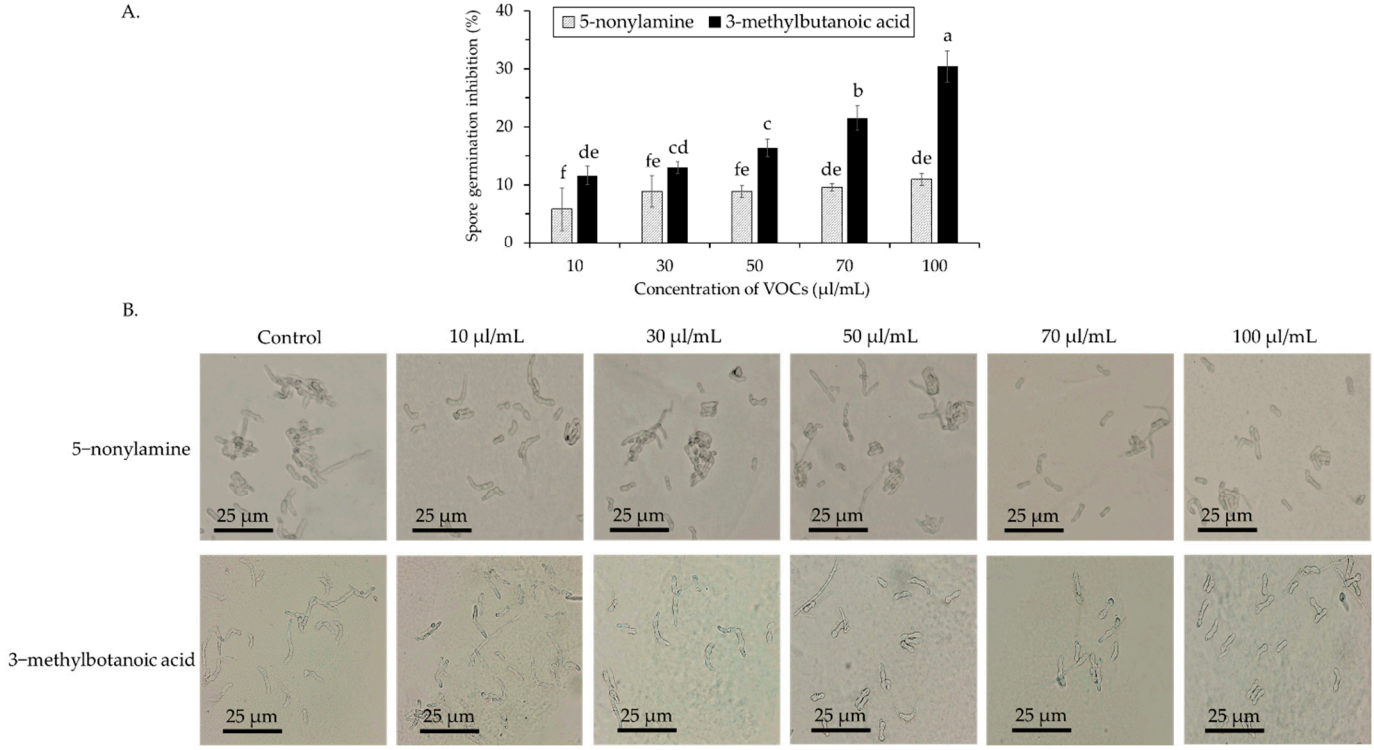

Figure 3. Antifungal activity of 5-nonylamine and 3-methylbutanoic acid against spore germination of *Colletotrichum gloeosporioides*. (**A**) Percentage reduction in spore germination and (**B**) images showing a reduction in spore germination and germ tube elongation of *C. gloeosporioides* exposed to different concentrations of (a) 5-nonylamine, and (b) 3-methylbutanoic acid. Error bars represent the standard deviation of the mean (n = 3). Means with different letters are significantly different ($p < 0.001$).

Treatment with 5-nonylamine at concentrations of 10, 30, 50, 70, and 100 µL/mL, reduced the spore germination of *C. gloeosporioides* rate by 5.8%, 8.9%, 8.9%, 9.6%, and 10.9%, respectively. However, treatment with low concentrations (10 to 50 µL/mL) of 5-nonylamine did not exhibit a notable difference in preventing germ tube elongation of the germinated spores, except at the concentrations of 70 and 100 µL/mL. In addition, treatment with 3-methylbutanoic acid at different concentrations of 10, 30, 50, 70, and 100 µL/mL reduced the spore germination of *C. gloeosporioides* by 11.6%, 13.0%, 16.4%, 21.5% and 30.4%, respectively (Figure 3A). Exposure of *C. gloeosporioides* to 3-methylbutanoic acid caused a significantly higher rate of reduction in spore germination compared to 5-nonylamine. Moreover, the reduction in the rate of spore germination caused by 3-methylbutanoic acid significantly increased with an increase in concentration from 30 to 100 µL/mL. Each concentration of 3-methylbutanoic acid notably reduced the ability of germ tube elongation compared to normal germ tube elongation in the control group (Figure 3B).

Moreover, the different concentrations of 5-nonylamine and 3-methylbutanoic acid also exhibited significant antifungal activity against the mycelial growth of *C. gloeosporioides* (Figure 4A,B). Treatment with 5-nonylamine caused the highest rate of reduction in mycelial

growth of 14.0% at 100 µL/mL and the lowest rate of 6.7% was recorded at 10 µL/mL. Similarly, 3-methylbutanoic acid caused the highest rate of reduction in mycelial growth of 22.6% at 100 µL/mL and the lowest rate of 15.7% at 10 µL/mL (Figure 4A). The mycelia of *C. gloeosporioides* under the different concentrations of 5-nonylamine and 3-methylbutanoic acid exhibited abnormal hyphal morphologies, such as cell wall lysis, fracturing, and coiling of the hyphae and hyphae discoloration, as shown by the arrows (Figure 4C). Treatment with the different concentrations of 5-nonylamine and 3-methylbutanoic acid disrupted the hyphae growth to a varying extent and reduced the mycelial density of *C. gloeosporioides*, compared to the normal hyphae growth in the control group (Figure 4C). At each treatment concentration, 3-methylbutanoic acid showed a significantly higher rate of reduction in the mycelial growth of *C. gloeosporioides* compared to 5-nonylamine.

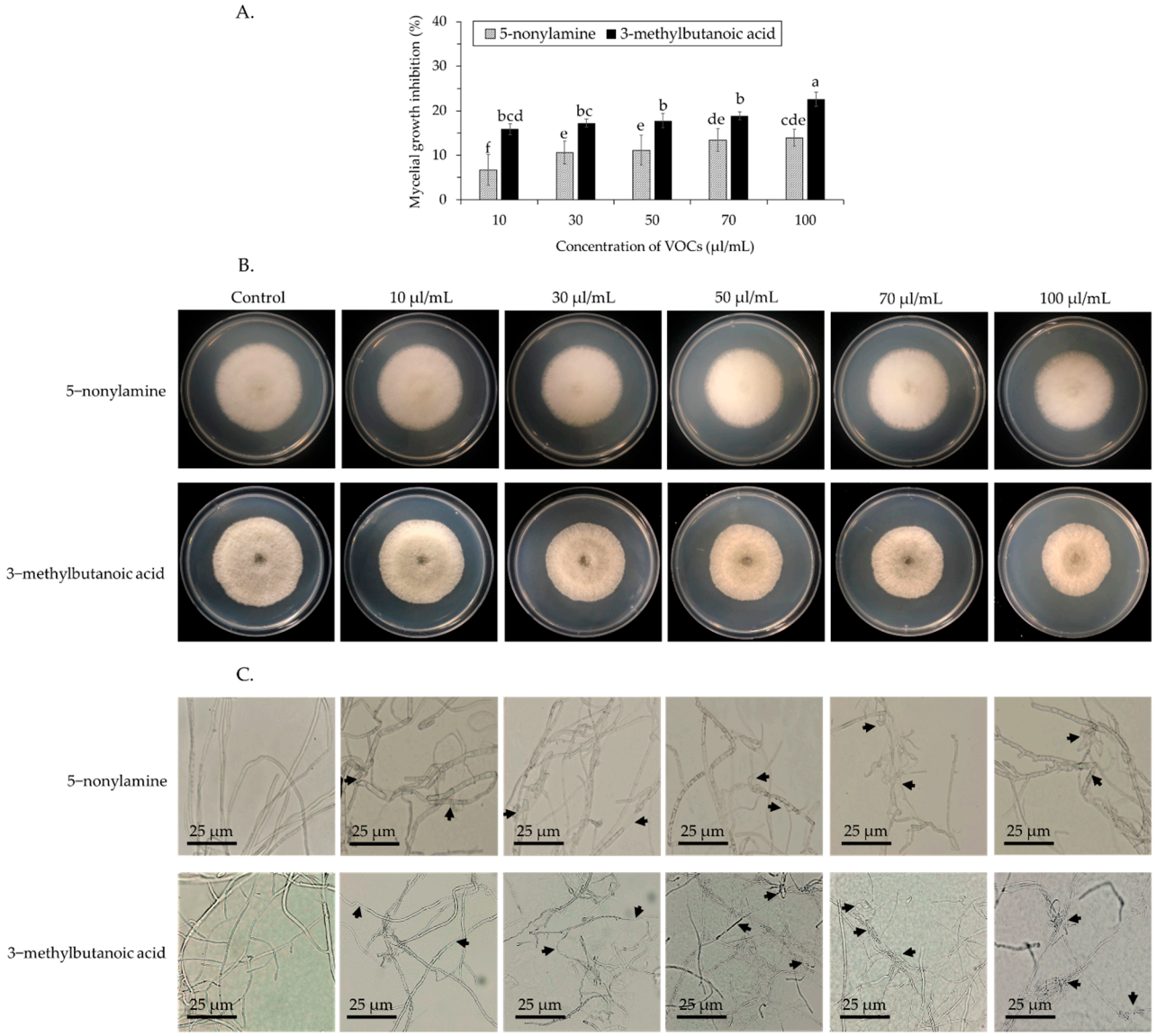

Figure 4. Antifungal activity of different concentrations of 5-nonylamine and 3-methylbutanoic acid against mycelial growth of *Colletotrichum gloeosporioides*. Percentage reduction in mycelial growth (**A**), images showing a reduction in mycelial growth when the fungi were exposed to 5-nonylamine and 3-methylbutanoic acid (**B**), and the deformation of hyphae morphology in 5-nonylamine and 3-methylbutanoic acid (**C**) treatments. Arrows indicate deformed mycelia after treatment with different concentrations of individual VOCs. Error bars represent the standard deviation of the mean (n = 3). Means with different letters are significantly different ($p < 0.001$).

4. Discussion

Bacillus species produce various VOCs, which exhibit antifungal activity against various field and post-harvest phytopathogenic fungi [29,33,40]. In this study, antifungal VOCs emitted by *B. velezensis* CE 100 exhibited the potential to inhibit the spore germination and mycelial growth of *C. gloeosporioides*, which is a notorious phytopathogenic fungus responsible for anthracnose diseases (Figures 1 and 2). Based on HS-SPME/GC-MS technique, the VOCs produced by *B. velezensis* CE 100 were identified as 5-nonylamine (amine), and 3-methylbutanoic acid (organic acid) (Table 1). All the esters (cyclotrisiloxane hexamethyl, cyclotetrasiloxane octamethyl, cyclopentasiloxane decamethyl, and cyclohexasiloxane dodecamethyl), and the methyl compound (estragole) could be components of PB media since they were common in the headspace of both the bacterial and the control groups (Table 1). Thus, these compounds are not responsible for the antifungal activity of the VOCs produced by *B. velezensis* CE 100 (Figures 1 and 2, and Table 1). Evaluation of each of the two identified volatile compounds from *B. velezensis* CE 100 revealed their significant antifungal activity against *C. gloeosporioides* compared to the control group (Figures 3 and 4). The predominant compound detected in the headspace of *B. velezensis* CE 100 was 5-nonylamine with a relative peak area of 39.5%, which is 13.5-fold higher than that of 3-methylbutanoic acid. In this study, 5-nonylamine showed a maximum antifungal activity of 10.9% and 14.0% against spore germination and mycelial growth, respectively (Figures 3A and 4A). Therefore, 5-nonylamine could be a vital component responsible for the antifungal activity of the VOCs emitted by *B. velezensis* CE 100 due to its predominance and activity. However, 5-nonylamine had a significantly lower antifungal efficacy against the germination of spores and mycelial growth of *C. gloeosporioides* compared to 3-methylbutanoic acid (Figures 3A and 4A). The antimicrobial activity of amine and amine derivatives in earlier studies has demonstrated the role of amines in controlling pathogenic microbes, including the activity of surface-active amines against phytopathogenic fungi [42,49,50]. In a comprehensive study examining the antimicrobial activity of 164 amine compounds, several amines were shown to have antimicrobial activity, with the highest bacteriostatic, fungistatic, and algistatic activity being recorded in dodecylamine and dodecylamine acetate compounds [42]. These compounds not only disrupt the fungal hyphae to reduce the rate of mycelial growth, but also reduce the rate of spore germination and, in some cases, restrict the germ tube elongation of the germinated spores [47,48]. Specifically, recent studies have reported the antimicrobial activity of volatile amine derivatives against some important fungal and bacterial pathogens [37,51]. For instance, several soil-born *Bacillus* spp. have been previously reported to produce VOCs during their growth, including volatile amines with antifungal activity [52,53]. The antifungal volatile amines reduce the spore germination and mycelial growth of pathogenic fungi, which suppresses infections and disease severity to mitigate the losses caused by fungal diseases [52]. The results of this study reveal that 5-nonylamine produced by *B. velezensis* CE 100 also reduced the rate of spore germination and mycelial growth of *C. gloeosporioides* and was the most predominant volatile component from the headspace VOCs. The mode of action for the antifungal activity of 5-nonylamine is still unknown but generally, VOCs have been hypothesized to act in multiple modes, either independently or through synergism, to reduce the rate of growth and proliferation of phytopathogenic fungi [31,34,53]. Based on previous findings, the effect of VOCs may include antagonizing the cellular functions, such as disrupting the synthesis of cellular components and cell development, biofilm formation, disrupting the motility of cellular organelles, acting as signals that disrupt inter-and intraspecies interactions and chemical conversations that are vital for microbial survival, acting as virulence factors that could facilitate subsequent infections, and the disruption of microbial nutrient use [31,34,53].

In contrast, the results of this study indicate that 3-methylbutanoic acid had a significantly stronger antifungal activity against *C. gloeospoeioides*, causing a maximum rate of reduction in spore germination of 30.4% (Figure 3A), and a reduction in mycelial growth of 22.6% (Figure 4A) at 100 µL/mL. These results indicate that 3-methylbutanoic acid showed

significantly higher antifungal efficacy against *C. gloeospoeioides* compared to 5-nonylamine. A comparison of the two volatiles identified from the headspace of *B. velezensis* CE 100 at a concentration of 100 µL/mL, indicates the efficacy of 3-methylbutanoic acid against the germination of spores and mycelial growth of *C. gloeosporioides* was higher than that of 5-nonylamine by 2.8-fold and 1.6-fold, respectively. This antifungal 3-methylbutanoic acid or commonly isovaleric acid is a branched-chain alkyl carboxylic acid and is classified as a short-chain fatty acid with high solubility in water [39]. Such properties could enable its adsorption to the surface membrane, resulting in the alteration of membrane properties and compromising the membrane-associated functions [39,43]. Other related fatty acid derivatives have been hypothesized to cause antifungal activity by penetrating and disrupting the lipid bilayer of fungal cell membranes, increasing membrane fluidity, and causing membrane disintegration and the collapsing of cells [54]. It is plausible that 3-methylbutanoic acid (as a short-chain fatty acid) could cause such effects in the target fungal cells depending on the degree of similarities in the function group and the aliphatic chain length. The antifungal activity of 3-methylbutanoic acid has been demonstrated against various phytopathogens, including *Fusarium* spp., *Pyricularia oryzae*, *Rhizoctonia cerealis*, *Alternaria alternata*, *Phytophthora parasitica*, *Cladosporium cladosporioides*, *Penicillium expansum*, *Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp., *Mucor* spp., and *Botryotinia fuckeliana* [39,43,55]. The commonly exhibited antifungal effects of 3-methylbutanoic acid include the reduction in the rate of mycelial growth (reduced mycelial density), reducing the rate of spore germination, and the suppression of germ tube elongation [39,43,55,56]. Despite the comparatively low relative peak area of 2.92% in the headspace composition, 3-methylbutanoic acid could be a vital component for the antifungal activity of the VOCs produced by *B. velezensis* CE 100.

From the microscopic observation, both 5-nonylamine and 3-methylbutanoic acid reduced the spore germination and suppressed germ tube growth (Figure 3B). Both VOCs caused various damage symptoms to fungal mycelia, including hyphal deformation, cell wall lysis, membrane disintegration, as well as the collapsing and coiling of the hyphae (Figure 4C). The fungal cell membrane is enriched with diverse lipids belonging to the class glycerophospholipid, sphingolipid, and sterols, which form the basis for maintaining cell integrity, and cellular materials (organelles), and energy metabolites [57,58]. Thus, the disruption of cell membrane integrity by 5-nonylamine and 3-methylbutanoic acid could cause changes in indexes, and reduce the content of nucleic acids [33,57]. Moreover, both VOCs can diffuse easily through the air and gas-filled soil pore spaces to cause a long-distance microbial interaction [31]. The activity of VOCs could also be related to the functional groups and chemical nature/properties of the individual compounds [31,39,59]. For instance, the hydrophobicity of the compound could affect the depth of penetration into the bilayer membrane of the target fungi to induce changes in the physio-chemical properties of the cell, leading to death or cell malfunction [39,59]. Therefore, the volatile-mediated reduction in the rate of spore germination and mycelial growth of *C. gloeosporioides* by commercially available 5-nonylamine and 3-methylbutanoic acid indicate that both compounds could be responsible for the antifungal activity of the VOCs emitted by *B. velezensis* CE 100. Based on the contrasting relative abundance and antifungal efficacy of 5-nonylamine and 3-methylbutanoic acid, the two VOCs could potentially exert a synergic effect in their antifungal activity against *C. gloeosporioides*.

5. Conclusions

The results of this study provided evidence that VOCs emitted by *B. velezensis* CE 100 could directly inhibit the spore germination and mycelial growth of *C. gloeosporioides.* The two VOCs identified from the headspace of *B. velezensis* CE 100 are 5-nonylamine and 3-methylbutanoic acid. Both compounds exhibited antifungal activity against *C. gloeosporioides*, which was characterized by the reduction in the rate of spore germination, reducing the rate of mycelial growth, and the suppression of germ tube elongation. The HS-SPME/GC-MS analysis of the VOCs and the antifungal assay of identified compounds

revealed that 5-nonylamine was the most abundant VOC emitted by *B. velezensis* CE 100 while 3-methylbutanoic acid had significantly higher antifungal activity. Therefore, 5-nonylamine and 3-methylbutanoic acid emitted by *B. velezensis* CE 100 could potentially exert a volatile-mediated synergic antifungal effect against *C. gloeosporioides*. This is the first report on 5-nonylamine as a volatile antifungal compound produced by *B. velezensis* CE 100 against *C. gloeospoeioides*.

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