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Volatile organic compounds from *Bacillus subtilis* BE6 inhibit and lyse mycelial growth of *Sclerotium rolfsii*

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Abstract. *Hardiyanti S, Amaria W, Rahayuningsih S, Supriadi, Wartono, Harni R, Korlina E, Widodo, Syabana MA, Wijaya R. 2026. Volatile organic compounds from* Bacillus subtilis *BE6 inhibit and lyse mycelial growth of* Sclerotium rolfsii*. Asian J Agric 10 (1): g100123. https://doi.org/10.13057/asianjagric/g100123. Sclerotium rolfsii* is a devastating soil-borne pathogen responsible for significant crop losses worldwide, highlighting the need for effective control strategies. Antagonistic bacteria provide an eco-friendly biocontrol alternative for managing such plant diseases. This study evaluated the antifungal efficacy of Volatile Organic Compounds (VOCs) produced by eight bacterial isolates belonging to the genera *Bacillus*, *Serratia*, *Brucella*, and *Burkholderia* against *S. rolfsii* under in vitro conditions. Antifungal activity was assessed using a dual-culture assay in divided petri dishes, followed by Scanning Electron Microscopy (SEM) to assess morphological changes, and headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME/GC-MS) for chemical profiling. Among the isolates, indigenous *Bacillus subtilis* BE6 exhibited the strongest inhibitory effect on *S. rolfsii* isolate Sc.lb, achieving a growth inhibition rate of 57.2±10.5% compared to the control. Other notable isolates included *Bacillus amyloliquefaciens* P7 (43.5±7.3%) and *Serratia surfactantfaciens* S108 (42.7±10.9%). Observation through SEM demonstrated that VOCs from *B. subtilis* BE6 induce severe hyphal abnormalities, such as shrinkage, wrinkling, and lysis. GC-MS analysis identified 27 VOCs emitted by *B. subtilis* BE6, with the dominant compounds being cis-2,3-epoxybutane (27.34%), methyl (Z)-N-hydroxybenzenecarboximidate (17.19%), 5-methyl-2-hexanone (14.10%), hexanal (12.46%), and 2,5-dimethylpyrazine (2.89%). These compounds belong to the aldehyde, epoxide, oxime, ketone, and pyrazine groups, all of which are associated with antimicrobial properties. Overall, the results demonstrate that VOCs produced by *B. subtilis* BE6 effectively inhibit and disrupt the mycelial growth of *S. rolfsii*, highlighting their potential as eco-friendly biofumigant agents for the managing of soil-borne plant diseases. Further studies are required to validate their efficacy under in vivo and field conditions, as well as to elucidate the individual and synergistic roles of key VOCs.

Keywords: *Bacillus subtilis*, biocontrol, HS-SPME/GC-MS, hyphal abnormalities, SEM

INTRODUCTION

Sclerotium rolfsii (teleomorph: *Athelia rolfsii*) is a highly destructive soil-borne pathogen causing southern blight, stem rot, and damping-off in over 500 plant species across 100 families, including Solanaceae, Cucurbitaceae, Fabaceae, Poaceae, and Malvaceae (Billah et al. 2017; Yadav et al. 2022). Disease incidence is alarmingly high, reaching up to 55% on *Solanum lycopersicum* in Indonesia, 47% in *Callistephus chinensis*, and 30% in Chinese *Pinellia ternate* (Mahadevakumar et al. 2018; You et al. 2020; Yanti et al. 2021). The pathogen's persistence is driven by its ability to produce brown-to-black sclerotia, which serve as the primary inoculum that can persist in host tissues or soil for extended periods (Paul et al. 2017; Paul et al. 2021). Furthermore, its broad adaptability to various carbon sources (e.g., glucose, sucrose, and starch), pH ranges (5-9), and temperatures (20-37°C) makes its management a significant challenge in diverse agricultural environments (Paul et al. 2021).

An effective strategy to mitigate this problem is the implementation of integrated disease management practices. Among the various strategies, biological control using antagonistic microorganisms has emerged as a sustainable and environmentally friendly method compared to chemical control (Dwivedi and Prasad 2016). Numerous studies have reported the utilization of antagonistic microbial agents against *S. rolfsii*, including *Pseudomonas* spp., *Bacillus* spp., and *Serratia* spp. (Kamensky et al. 2003; Karthikeyan et al. 2006; Chen et al. 2020; Hardiyanti et al. 2024). Recently, Meena et al. (2024) showed that the treatment of *Trichoderma harzianum*, *Trichoderma viridae*, and *Pseudomonas fluorescens* inhibited mycelial growth of *S. rolfsii* of groundnut in vitro, while in planta, the seed and

soil applications of a dual formulation of *T. harzianum* and *P. fluorescens,* followed by *T. viride* and *P. fluorescens*, reduced disease incidence, as the result increased dry weight and pod yield of groundnut under in vitro and in vivo (Meena et al. 2024). These antagonistic microorganisms employ several mechanisms to suppress plant diseases, such as inducing plant resistance, competing for nutrients and space, hyperparasitism, and the production of antimicrobial metabolites (Köhl et al. 2019). While many biocontrol agents rely on direct contact, recent attention has shifted toward the role of Volatile Organic Compounds (VOCs). Hardiyanti et al. (2024) demonstrated that trapped VOCs from *Serratia surfactantfaciens* S108 and *Brucella intermedia* BE60 can inhibit the radial growth of *S. rolfsii*, suggesting that these gaseous metabolites are potent tools for pathogen suppression.

VOCs are small molecular-weight substances (<300 Da) with high vapor pressure, allowing them to diffuse through air-filled soil pores and facilitate long-distance microbe-microbe interactions (Garbeva and Weisskopf 2019). Beyond their role as biopesticides, VOCs enhance plant health by inducing systemic resistance and promoting growth (Poveda 2021). A notable example is 2,4-di-tert-butylphenol (2,4-DTBP) produced by *Bacillus subtilis* 0618A, which effectively penetrates soil up to 2 cm depth to inhibit sclerotia germination and enhance peanut defense enzyme activities, as well as increase soil bacteria and actinomycetes populations and improve sucrase activity (Cheng et al. 2025). The interaction between VOCs and pathogenic fungi has recently attracted increasing attention due to their role in suppressing soil-borne pathogens. Management strategies for utilizing VOCs in agricultural disease control include fumigant application, organic amendments, microbial inoculants, and cropping practices, with biocontrol inoculation being the most used for managing soil-borne fungal diseases (Boer et al. 2019). Previous studies have demonstrated that VOCs can inhibit the growth of soil-borne pathogenic fungi such as *Phytophthora capsici, Rhizoctonia solani, S. rolfsii, Fusarium* sp., and *Verticillium dahliae* (Syed-Ab-Rahman et al. 2019; Munif and Asmoro 2021; Montes-Osuna et al. 2022).

Despite increasing evidence that bacterial VOCs play an important role in suppressing soil-borne fungal pathogens, the specific VOC profiles of indigenous antagonistic bacteria and their direct morphological impacts on *S. rolfsii* remain insufficiently characterized. We hypothesized that VOCs produced by antagonistic bacteria, particularly *B. subtilis* BE6, significantly inhibit the mycelial growth of *S. rolfsii* through VOC-mediated mechanisms that induce structural damage to fungal hyphae, and that this inhibitory activity is associated with a distinct VOC profile dominated by bioactive aldehydes, ketones, epoxides, oximes, and pyrazine derivatives.

MATERIALS AND METHODS

Biological material

A total of eight antagonistic bacterial isolates were utilized (Table 1). These specific isolates were chosen based on their previously observed superior antagonistic activity in preliminary screenings against a range of fungal pathogens (Amaria et al. 2024; Hardiyanti et al. 2024). Prior to the antifungal assays, a biosafety assessment was conducted to ensure the safety of the bacterial isolates. The results from the hemolysis and hypersensitivity tests demonstrated that all selected strains were non-pathogenic (Amaria et al. 2023). The *S. rolfsii* strain Sc.lb was isolated from *Aloe vera*. Bacterial genera of *Bacillus*, *Brucella*, and *Burkholderia* were obtained from the collection of the National Research and Innovation Agency in Cibinong, West Java, Indonesia, while *S. surfactantfaciens* S108 was sourced from the Plant Protection Department, IPB University. Stock cultures from the working collection were refreshed on solid media. Tryptic Soy Agar (TSA, Difco) was used for bacterial cultures, while Potato Dextrose Agar (PDA, Himedia) was used for *S. rolfsii.*

Antifungal volatile assay of antagonistic bacteria

The antifungal activity of bacteria VOCs was evaluated using a divided petri dish (two compartments). Each bacterial isolate was streaked onto TSA medium on one side of the compartment, while a 5 mm plug of *S. rolfsii* was placed on PDA medium in the opposite compartment (Fialho et al. 2011). Control plates consisted of TSA-only medium opposite the *S. rolfsii* plug. To prevent the escape of VOCs, the petri dishes were double sealed with a cut plastic wrap of 3 cm width and incubated at uncontrolled room temperature (25-27°C) for 5 days. The radial growth of *S. rolfsii* was measured daily to evaluate the effect of the VOCs. The Inhibition Rate (IR) was calculated using:

$$\text{IR}\ (\%) = \frac{(\text{D1} - \text{D2})}{\text{D1}}\ \text{x}\ 100\%$$

Where, IR represents inhibition rate, D1 represents the diameter of untreated *S. rolfsii,* and D2 is the diameter of *S. rolfsii* exposed to bacterial VOCs. The experiment was conducted using a completely randomized design with four replicates for each treatment.

Table 1. Potential antagonistic bacteria as biocontrol agents

Bacterial species	Isolate code	Origin	Accession number*
Bacillus subtilis	BE6	Endophyte of ginger	-
Bacillus cereus	BE29	Endophyte of ginger	-
Brucella intermedia	BE60	Endophyte of nutmeg	LC742152
Serratia surfactantfaciens	S108	Rhizosphere of the rubber tree	LC742966
Brucella intermedia	T2	Endophyte of patchouli	LC741402
Bacillus amyloliquefaciens	P7	Endophyte of patchouli	LC739181
Brucella intermedia	S018	Rhizosphere of the rubber tree	LC742969
Burkholderia cepacia	SS19.7	Rhizosphere of cocoa	-

Note: *- indicates isolates that have not been registered in the NCBI GenBank database

Microscopic analysis of pathogenic mycelium

To analyze the structure of the mycelium exposed to VOCs, a scanning electron microscope (Thermo Scientific Prisma E) was used to observe surface morphology. Hyphal segments were selectively excised from the peripheral regions of colonies exhibiting morphological abnormalities, including hyphal thinning, mycelial swelling, and restricted growth. Square mycelial sections (1×1 cm) were aseptically excised using a sterile scalpel. To ensure representative sampling, at least triplet random fields of view were assessed for each of the three replicates, focusing on hyphal integrity and apical development; sample preparation, which included cleaning, pre-fixation, fixation, dehydration, drying, and coating. Samples were immersed in cacodylate buffer for 2 hours and agitated in an ultrasonic cleaner for 5 minutes. The samples were subsequently fixed in 2.5% glutaraldehyde, followed by immersion in 2% tannic acid overnight to enhance structural contrast. After fixation, samples were washed four times for 5 minutes with cacodylate buffer to remove excess fixative. Dehydration was performed through a graded ethanol series of 50% (4×5 minutes), 70% (20 minutes), 85% (20 minutes), 95% (20 minutes), and absolute ethanol (2×10 minutes) at room temperature. The dehydrated samples were then immersed in tert-butanol for 2×10 minutes, frozen, and dried using a freeze dryer or vacuum dryer until completely dry. Dried specimens were mounted on aluminum stubs using conductive adhesive, then coated with a thin layer of gold (Au) using an ion coater to improve conductivity and imaging quality for SEM observation. The SEM observation was performed in triplicate and conducted at magnifications of 2500× and 3000×.

Antifungal volatile analysis of antagonistic bacteria using HS-SPME GC-MS

The antagonistic bacteria that exhibited the highest inhibition against *S. rolfsii* colonies were selected for volatile metabolite profiling using Headspace Solid-Phase Microextraction (HS-SPME) coupled with Gas Chromatography-Mass Spectrometry (GC-MS). The selected bacteria were cultured in a 22 mL SPME vial tube containing 4 mL of TSA medium. This medium-to-vial volume ratio (~1:5) was maintained to provide sufficient headspace for optimal volatile equilibrium while ensuring adequate nutrient availability for microbial growth.

VOC extraction was performed following the protocol described by Gao et al. (2017) with minor modifications. Before extraction, the cultures were incubated for 5 days. For sampling, the vials were equilibrated at 40°C for 30 minutes. A 2 cm DVB/CAR/PDMS fiber was then exposed to the headspace during heating to adsorb the volatile compounds. This fiber type was selected for its broad-spectrum sensitivity, particularly its efficiency in capturing both polar and non-polar volatile metabolites across a wide range of molecular weights.

The captured VOCs were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) (GC Agilent 7890A coupled with MS Agilent 5975C XL EI/CI). The injector was operated at 250°C in splitless mode, with helium as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The oven temperature program was initiated at 50°C (held for 2.0 min), increased at 4°C min^{-1} to 150°C (held for 1.0 min), and finally ramped at 5°C min^{-1} to 210°C. Separation was achieved using an HP-5MS column (30 m × 250 µm × 0.25 µm). The interface, MS source, and MS quadrupole temperatures were maintained at 250°C, 230°C, and 150°C, respectively. The mass scan range was set at 29-550 amu. Compound identification was performed by comparing the obtained mass spectra with those in the NIST14 mass spectral library.

Statistical analysis

The antifungal effects of volatile organic compounds against *S. rolfsii* in vitro were analyzed using one-way Analysis of Variance (ANOVA) to evaluate the antifungal effects of bacterial VOCs against *S. rolfsii*. The normality of the data and the homogeneity of variances were verified using the Shapiro-Wilk and Levene's tests, respectively, to ensure that the assumptions for parametric analysis were met.

Statistical analysis was performed using R software version 4.5.1 within the RStudio IDE version 2025.05.1 (Posit Software, PBC). Treatment means were compared using Fisher's Least Significant Difference (LSD) test at a significance level of $P<0.05$ (Zhang et al. 2020). The LSD test was selected for post-hoc comparisons to maintain high sensitivity in identifying significant differences between the antagonistic treatments, as these comparisons were established a priori based on the study objectives. The experiment followed a Completely Randomized Design (CRD) with four replicates for each treatment.

RESULTS AND DISCUSSION

Volatile antifungal effects of antagonistic bacteria against *S. rolfsii*

S. rolfsii displayed variations in mycelial growth when exposed to the VOCs produced by different antagonistic bacteria compared to the control (without bacterial VOC exposure). The control colony exhibited typical morphology, characterized by dense, uniform, and spreading white mycelia. In contrast, colonies exposed to bacterial VOCs showed marked morphological alterations, including reduced mycelial density, a nearly transparent appearance, and stunted hyphal development. The observed morphological changes are attributed to exposure to VOCs released by *B. subtilis* BE6 and *B. intermedia* T2 (Figures 1.B and C).

After verifying that the data fulfilled the parametric analysis assumptions (Shapiro-Wilk test, p=0.853; Levene's test, p=0.378), the influence of bacterial VOCs on *S. rolfsii* mycelial growth was assessed. A one-way ANOVA subsequently revealed a highly significant impact of the antagonistic bacterial isolates on fungal growth (F(8, 27)=14.31, p<0.001; Table S1). The mycelial growth of *S. rolfsii* was significantly inhibited by all bacterial VOCs compared to the control (p<0.05). According to Fisher's LSD test, *B. subtilis* BE6 exhibited the most potent

antifungal activity, reducing the mycelial growth by 2.05±0.5 cm, which was significantly lower than all other treatments. *B. amyloliquefaciens* P7 (2.71±0.3 cm) and *S. surfactantfaciens* S108 (2.75±0.5 cm) followed as the next most effective antagonists. In contrast, the weakest inhibition was observed in the *B. intermedia* T2 exposed (3.78±0.6 cm), although it remained significantly lower than the untreated control (4.80±0.1 cm) (Figure 2.A).

The inhibition rate of *S. rolfsii* was analyzed using one-way ANOVA. Preliminary diagnostic tests confirmed that the data satisfied the assumptions of normality (Shapiro-Wilk, p=0.433) and homogeneity of variance (Levene's test, p=0.751). The ANOVA results indicated a highly significant effect of the different antagonistic bacterial VOCs on the inhibition of fungal growth (F (7, 24) = 7.62, p < 0.001; Table S2). VOCs emitted from eight antagonistic bacteria significantly inhibited the growth of *S. rolfsii* colonies, with an inhibition rate ranging from 21% to 57%. Among the antagonistic bacteria, VOCs from *B. subtilis* BE6 exhibited the greatest inhibition of *S. rolfsii* mycelial growth, followed by a group of moderately effective isolates, including *B. amyloliquefaciens* P7, *S. surfactantfaciens* S108, and *B. intermedia* S018. In contrast, *B. cereus* BE29 and *B. intermedia* T2 showed significantly lower inhibitory activity (Figure 2.B). These findings suggest that each antagonistic bacterium can produce unique volatile antifungal compounds that differentially affect the growth of pathogenic fungal colonies.

Antifungal effect of volatile emitted by *B. subtilis* BE6 against *S. rolfsii*

Based on an in vitro assay of eight antagonistic bacteria, *B. subtilis* BE6 produced VOCs with the strongest inhibiting mycelial growth of *S. rolfsii* and induced malformations in fungal hyphae and mycelia. Consequently, Scanning Electron Microscopy (SEM) was used to observe hyphal abnormalities in *S. rolfsii* colonies exposed to these VOCs. The treated hyphae showed surface irregularities, lost their characteristic cylindrical form, appearing highly distorted and shriveled. The presence of flattened, "ribbon-like" hyphae suggests that the VOCs may have triggered the degradation of cell wall components (Figure 3.A). Severe localized contractions were observed, where hyphae appeared folded and collapsed upon themselves. In some regions (indicated by red arrows), the mycelial filaments exhibited abnormal coiling and twisting (Figure 3.B). This shrinkage suggests a significant loss of internal cytoplasmic content and a drop in turgor pressure, likely caused by the disruption of the plasma membrane's semipermeability (Figure 3.C). However, the unexposed hyphae exhibited healthy, typical fungal architecture. The hyphae maintained a turgid, cylindrical shape with smooth, intact surfaces and uniform diameter, indicating high cellular turgor pressure and structural integrity (Figure 3.D). These morphological characteristics provide visual evidence that the VOCs emitted by *B. subtilis* BE6 affect the fungal cell wall. This disintegration suggests a putative mechanism involving membrane destabilization or the induction of oxidative stress that eventually leads to the observed mycelial disintegration.

Profiling of antifungal volatiles emitted by *B. subtilis* BE6

The identity and abundance of VOCs produced by antagonistic bacteria play a crucial role in supporting their mechanisms as biological control agents. The HS-SPME GC-MS method employed in this study was qualitative. The reported values of area (%) provided valuable insights into the relative abundance of VOCs produced by the bacterial isolates. However, rather than representing the actual amounts of the chemicals in the headspace, the area (%) represents relative abundance obtained from a single, uncalibrated injection. Several variables, including temperature and fiber affinity, influence the SPME technique. The Total Ion Chromatogram (TIC) of VOCs produced by *B. subtilis* BE6 is shown in Figure 4 and Table S3. Peaks appeared well-distributed from 1.2 to ~38.0 minutes, indicating a broad spectrum of volatile compounds with diverse chemical properties. The diversity and abundance of detected compounds suggest that *B. subtilis* BE6 produces a complex set of VOCs potentially responsible for its antifungal effects against *S. rolfsii*.

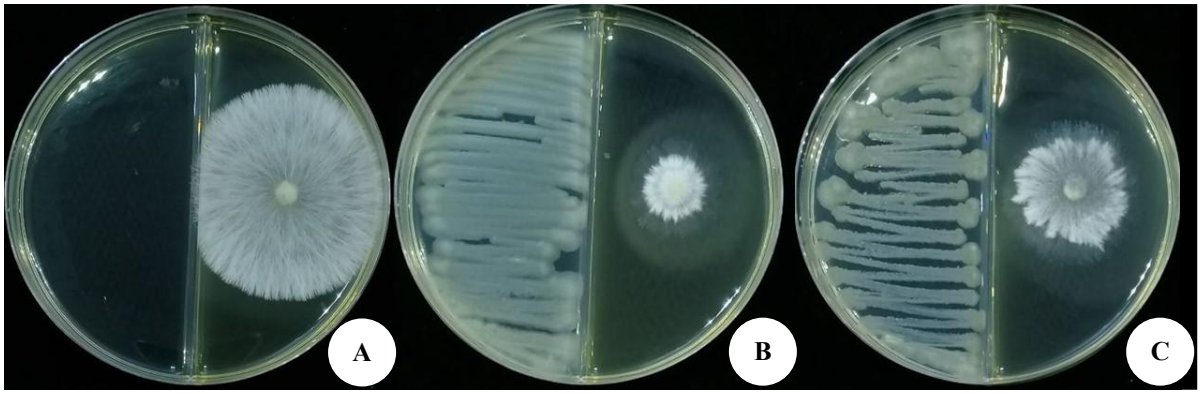

Figure 1. Antifungal activity of Volatile Organic Compounds (VOCs) produced by antagonistic bacteria against *S. rolfsii*. A. Control colony of *S. rolfsii* showing typical dense and uniform mycelial growth, B. Colony exposed to *B. subtilis* BE6 VOCs exhibiting the highest radial growth inhibition and reduced mycelial density, C. Colony exposed to *B. intermedia* T2 VOCs showing moderate inhibition and altered morphology

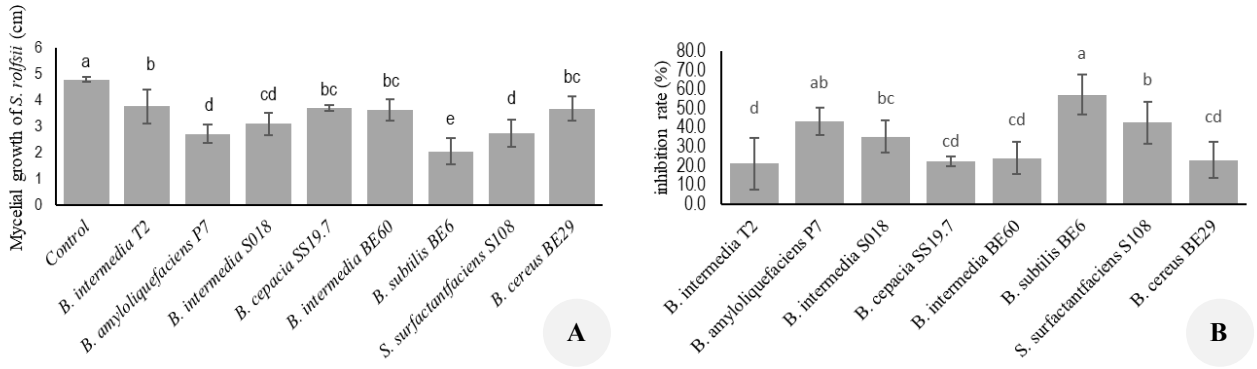

Figure 2. Antifungal efficacy of VOCs produced by antagonistic bacteria against *S. rolfsii*. A. Mycelial growth (cm) and B. Inhibition rates (%) of *S. rolfsii* after 5 days of exposure. Each treatment consisted of four replicates (n=4) arranged in a completely randomized design. Different letters above bars indicate statistically significant differences based on the post hoc LSD test at the 5% significance level. Error bars represent standard deviation values

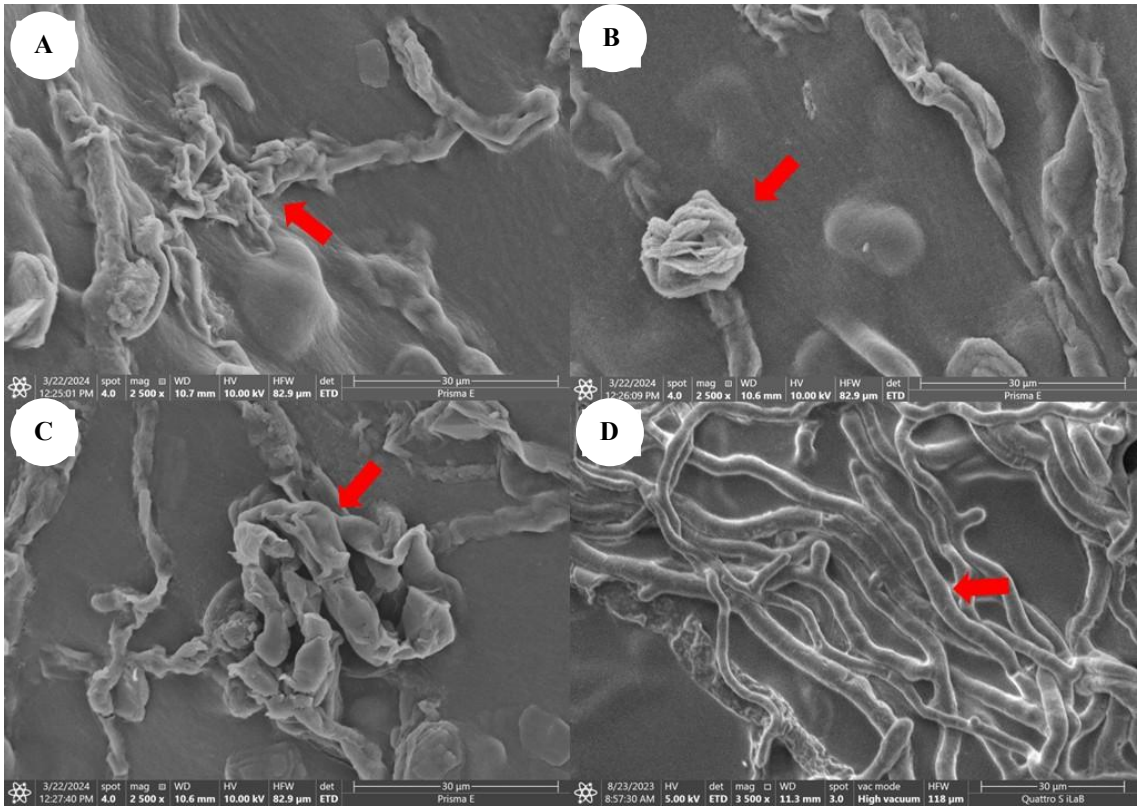

Figure 3. SEM images of *S. rolfsii* treated with VOCs from *B. subtilis* BE6. Arrowheads highlight hyphal deformation and cell wall damage, including A. Lysis and wrinkled, B. Shortened and rolled, and C. folded, D. Control hyphae show smooth and intact structures

The area values indicate the relative abundance of each compound, providing insights into the major volatile metabolites produced by *B. subtilis* BE6. A total of 27 VOCs were identified based on their retention time (RT), chemical identity, and relative abundance (peak area). The most abundant compounds identified were cis-2,3-epoxybutane (27.34%), methyl (Z)-N-hydroxybenzenecarboximidate (17.19%), 5-methyl-2-hexanone (14.10%), hexanal (12.46%), and 2,5-dimethylpyrazine (2.89%), respectively. These compounds belong to several functional groups, including aldehydes, epoxides, oximes, ketones, and pyrazines. In addition, minor components such as 4,6-dimethylundecane (0.01%) and 1-phenylethanone (0.02%) were also detected. Among these, aldehydes, ketones, and pyrazines are the most frequently reported VOCs with antifungal potential and are considered key contributors to the control of fungal phytopathogens. Nevertheless, certain compounds, including aliphatic hydrocarbons and polycyclic aromatic hydrocarbons, have not previously been documented as antifungal agents against plant pathogenic fungi (Table 2).

Table 2. Selected volatile organic compounds identified from *B. subtilis* BE6 using HS-SPME GC-MS analysis and their antifungal activities

Compound	Chemical formula	CAS	RT (Min)	Area (%)	Pathogen	Reference
Aldehydes						
Acetaldehyde	C_2H_4O	75-07-0	1.68	8.58	Not reported*	-
Hexanal	$C_6H_{12}O$	66-25-1	3.89	12.46	*Botrytis cinerea, Monilia fructicola, Sclerotinia sclerotiorum, Alternaria alternata,* and *Colletotrichum gloeosporioides*	Dhakshinamoorty et al. (2020)
Furan-2-carbaldehyde	$C_5H_4O_2$	98-01-1	5.14	6.82	*Alternaria mali*	Lam-Gutiérrez et al. (2018)
Furan-3-carbaldehyde	$C_5H_4O_2$	498-60-2	5.50	5.78	*Fusarium oxysporum* f. sp. *lycopersici, Colletotrichum fragariae, B. cinerea, Aspergillus flavus, A. alternata, Fusarium solani,* and *Pseudomonas syringae*	Koitabashi et al. (2004), Peng et al. (2024)
2-Phenylacetaldehyde	C_8H_8O	122-78-1	10.87	0.06	*Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Shigella flexneri, Vibrio cholerae* and *Vibrio parahaemolyticus*	Tanapichatsakul et al. (2018)
Epoxides						
Cis-2,3-Epoxybutane	C_4H_8O	1758-33-4	2.15	27.34	*A. flavus*	Gong et al. (2020)
Ketones						
5-Methyl-2-hexanone	$C_7H_{14}O$	110-12-3	6.08	14.10	*Macrophomina phaseolina, Rhizoctonia solani, Pythium aphanidermatum,* and *S. sclerotiorum*	Rana et al. (2024)
3,6-Dimethyl-5-octen-2-one	$C_{10}H_{18}O$	64165-21-5	10.72	0.06	*S. aureus*	
Octane-2,3-dione	$C_8H_{14}O_2$	585-25-1	11.34	0.03	*B. cinerea*	Zhang et al. (2023)
1-phenylethanone	C_8H_8O	98-86-2	11.69	0.02	*A. flavus*	Shilman et al. (2024)
(1R,4S)-1,7,7-Trimethylbicyclo [2.2.1] heptan-2-one	$C_{10}H_{16}O$	464-49-3	14.38	0.07	Not reported*	-
Pyrazines						
2,5-Dimethylpyrazine	$C_6H_8N_2$	123-32-0	6.81	2.89	Broad-spectrum antimicrobial	Agisha et al. (2019), Vlassi et al. (2020)
3-Ethyl-2,5-dimethylpyrazine	$C_8H_{12}N_2$	13360-65-1	12.11	0.05	*Magnaporthe oryzae*	Patel et al. (2021)
3-(3-Methylbutyl)-2,5-dimethylpyrazine	$C_{11}H_{18}N_2$	18433-98-2	20.22	0.04	Not reported*	-
1,4-dimethylpiperazine	$C_6H_{14}N_2$	106-58-1	6.57	1.65	Not reported*	-
Oximes						
methyl (Z)-N-hydroxybenzenecarboximidate	$C_8H_9NO_2$	67160-14-9	7.50	17.19	*Penicillium digitatum, Penicillium italicum, Rhizopus stolonifer, B. cinerea,* and *M. fructicola*	Cortés et al. (2020)

Note: *: "Not reported" indicates that specific antifungal activity for this compound against the listed pathogens has not been established in the current literature

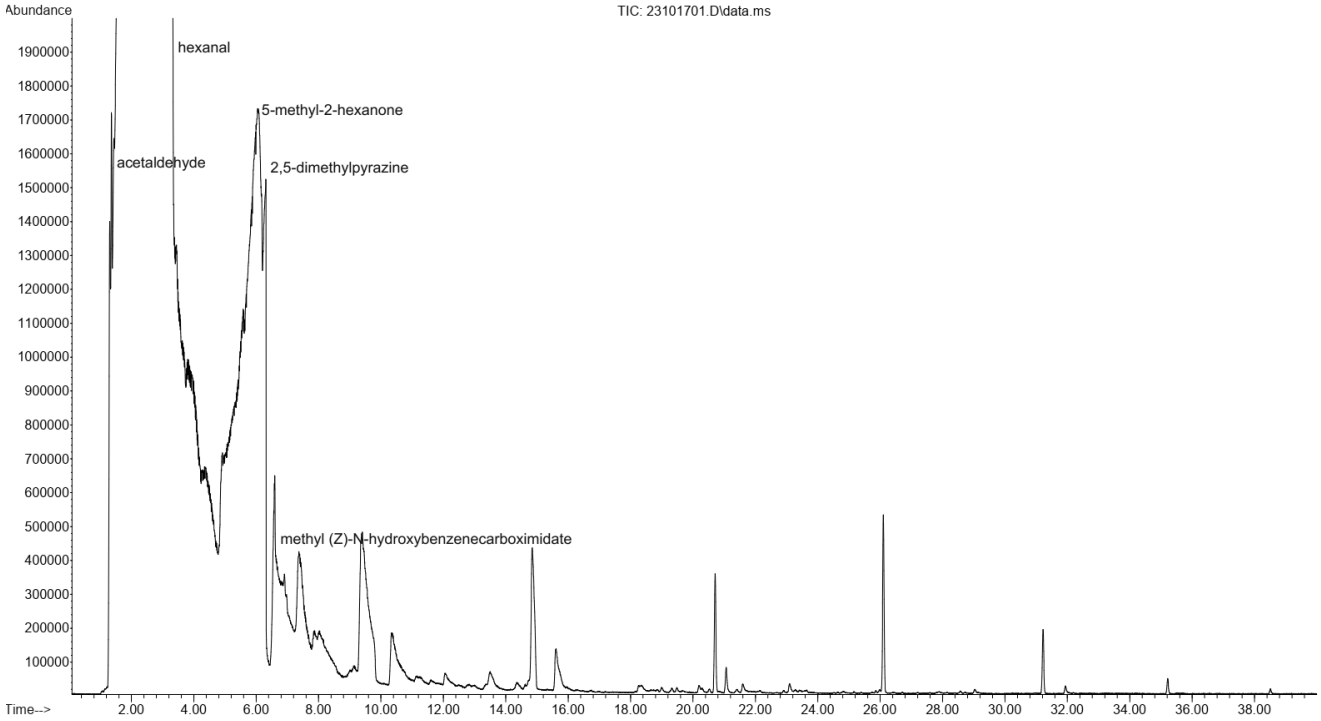

Figure 4. Chromatograms of volatile organic compounds produced by *Bacillus subtilis* BE6, showing major components including acetaldehyde, hexanal, 5-methyl-2-hexanone, 2,5-dimethylpyrazine, and methyl (Z)-N-hydroxybenzenecarboximidate

Discussion

The present study suggests that all of the antagonistic bacteria, i.e., four isolates of *Bacillus* (*B. subtilis* BE6, *B. cereus* BE29, *B. amyloliquefaciens* P7, *B. cepacia* SS19.7), three isolates of *Brucella* (*B. intermedia* BE60, *B. intermedia* T2, *B. intermedia* S018), and *S. surfactantfaciens* S108, produced unique VOCs that differentially affected the growth of pathogenic fungal colonies of *S. rolfsii*. Antifungal compounds produced by the antagonistic bacteria offer significant potential for controlling plant pathogens due to their environmental safety and minimal risk to human health. This study found that the inhibitory efficacy of genera *Bacillus* and *Serratia* against *S. rolfsii* ranged from 40-60%, representing significant potential to support sustainable crop protection. Bacterial genera such as *Bacillus* and *Serratia* are well-known biological control agents due to their diverse antagonistic mechanisms, including the production of inhibitory VOCs. For instance, *B. amyloliquefaciens* T-5 has been documented to suppress the soil-borne pathogen *Ralstonia solanacearum* in tomatoes (Raza et al. 2016). *B. amyloliquefaciens* P7 and *S. surfactantfaciens* S108 demonstrated significant antifungal potential; their VOCs are known to induce morphological changes and inhibit the mycelial growth of *Rigidoporus microporus* by 60-100% (Amaria et al. 2024). Furthermore, VOCs emitted by various *B. subtilis* strains have been reported to inhibit a broad spectrum of pathogens, including *Fusarium arcuatisporum*, *Alternaria iridiaustralis*, *Colletotrichum fioriniae*, *S. rolfsii*, and *Aspergillus flavus* (Ling et al. 2021; Ling et al. 2022; Cheng et al. 2025).

Several reports indicate that VOCs produced by *Bacillus* species exhibit antifungal properties. Gao et al. (2017) stated that *B. subtilis* CF-3 releases VOCs such as alcohol, ketones, aldehydes, and pyrazines, which effectively inhibit the mycelial growth of *Monilinia fracticola* and *Colletotrichum gloeosporioides*. Another study reported that *Bacillus halotolerans* NYG5 produced ketone, carboxylic acid, and alcohol, which suppressed the growth of several phytopathogenic fungi (Rana et al. 2024). Meanwhile, *B. subtilis* BS-01produced VOCs identified as hexane, dichloromethane, and ethyl acetate and significantly inhibited *Alternaria solani* (Awan et al. 2023).

The high inhibitory potential observed in this study (up to 60%) highlights the specific potency of Indonesian indigenous isolates, such as *B. subtilis* BE6. Unlike previously documented strains, these isolates exhibit a unique VOC profile dominated by a synergistic blend of cis-2,3-epoxybutane and specific pyrazine derivatives, providing a distinct biological resource for regional sustainable agriculture. However, literature regarding the antifungal properties of epoxybutane compounds remains limited, indicating a significant gap in current research that this study begins to address. To date, the only documented report pertains to 3,3-dimethyl-1,2-epoxybutane produced by *Staphylococcus saprophyticus* L-38, which was shown to induce severe structural damage to *A. flavus* cells. This specific derivative effectively inhibits mycelial growth, spore germination, and conidiophore development, while also suppressing aflatoxin production (Gong et al. 2020). The identification of cis-2,3-Epoxybutane in *B. subtilis* BE6, therefore, provides significant new insights into the antifungal repertoire of this chemical group.

Several pyrazine derivatives were also detected in VOCs produced by *B. subtilis* BE6, including 2,5-dimethylpyrazine, 3-ethyl-2,5-dimethylpyrazine, 3-(3-methylbutyl)-2,5-dimethylpyrazine, and 1,4-dimethylpiperazine. Pyrazine compounds are to play a crucial role in microbial antagonism by interfering with fungal signaling and enzyme function. This mechanism is required for the inhibition of antagonistic bacteria agents against pathogenic fungi. According to Prasad et al. (2021), pyrazine derivatives possess strong binding affinities to fungal target proteins, leading to enzymatic inhibition and suppressed pathogen growth. Similarly, Janssens et al. (2019) also reported that pyrazines induce cell wall stress and disrupt peptidoglycan turnover, weakening fungal cell wall integrity. Supporting these findings, *Bacillus* sp. WG4 and the newly characterized *Bacillus* strain JS6 both produce pyrazine-based volatiles with potent antifungal properties that significantly inhibited *Pythium myriotylum* and other plant pathogens (Jimtha et al. 2016; Elamin et al. 2021). Furthermore, *B. subtilis* strains producing pyrazine derivatives, along with carbonyl compounds, alcohols, and alkanes, have demonstrated potent antagonistic activity against *Fusarium oxysporum* (Stocki et al. 2025).

Among the VOCs identified (Table 2), the aldehyde derivatives were identified as the most dominant of VOCs produced by *B. subtilis* BE6, including hexanal, acetaldehyde, furan-2-carbaldehyde (furfural), furan-3-carbaldehyde, and 2-phenylacetaldehyde. Aldehydes are well known for their antimicrobial activity, primarily through disruption of microbial cell metabolism. Hexanal has been demonstrated to reduce intracellular ATP levels, increase membrane permeability, and alter the morphology and ultrastructure of *Vibrio parahaemolyticus* cells (Fan et al. 2023). In addition to aldehydes, several ketones were detected in this study, such as 5-methyl-2-hexanone, 3,6-dimethyl-5-octen-2-one, octane-2,3-dione, 1-phenylthanone, and (1R,4S)-1,7,7-Trimethylbicyclo [2.2.1] heptan-2-one. Ketones are also known to have antimicrobial properties, particularly disrupting fungal cell structure and metabolism. Zhang et al. (2020) reported that *B. subtilis* ZD01 produced ketones as the most abundant volatiles, which affect the mycelial growth, hyphae penetration, and sporulation by decreasing the expression of genes *slt2* and *sod*. Previous study has demonstrated that ketone and aldehyde ligands are capable of forming hydrogen bonds with fungal hyphal wall proteins, thereby disrupting cell wall structure and compromising membrane integrity (Rajasekharan and Shemesh 2022; Ouyang et al. 2023). Aldehydes and ketones emitted by *Bacillus velezensis* have also been recognized as potent VOCs against postharvest fungal pathogens, with minimum inhibitory concentrations as low as 0.005 mL L^{-1} (Calvo et al. 2020).

The volatile-mediated antagonism observed in this study highlights a multi-targeted inhibitory mechanism. The potent mycelial disintegration induced by *B. subtilis* BE6 VOCs, characterized by the presence of unique VOCs (aldehyde, epoxides, oximes, ketone, and pyrazine), suggests that these metabolites act to disrupt fungal structural integrity. This conceptual framework supports the potential application of these endophytic isolates as effective bioagents for the sustainable management of *S. rolfsii.*

Despite the significance of the findings, this study has several limitations. The antifungal activity was evaluated exclusively under in vitro conditions, which may not fully represent the complex interactions in natural soil environments. In addition, the GC-MS analysis was semi-quantitative, relying on relative peak area percentages; therefore, the exact concentrations of individual volatile compounds required to elicit the observed inhibitory effects remain to be determined.

To address these limitations, future research should focus on evaluating individual compounds (e.g., pyrazine and hexanal) and determining the Minimum Inhibitory Concentration (MIC). Furthermore, prioritize the optimization of VOC-based biocontrol strategies under field conditions. One promising approach involves developing efficient delivery systems that enable the gradual and stable release of VOCs into the soil. Considering that activated charcoal and biochar possess strong adsorption and retention capacities for volatile compounds, biochar enriched with antagonistic bacteria like *B. subtilis* BE6 could serve as an effective carrier for VOC delivery. Biochar derived from various organic residues not only enhances soil structure, nutrient availability, and microbial activity but also contributes to long-term carbon sequestration and environmental sustainability (Jagnade et al. 2023). Moreover, its porous structure and large surface area can help stabilize and slowly release bacterial VOCs, thereby improving their persistence and biocontrol efficacy against soil-borne pathogens.

In conclusion, this study confirms the hypothesis that VOCs emitted by antagonistic bacteria in this study serve as potent inhibitors of *S. rolfsii*. We identified *B. subtilis* BE6 as the most effective isolate, achieving a 57.2±10.5% inhibition rate, which was significantly higher than that of *B. amyloliquefaciens* P7 (43.5±7.3%) and *S. surfactantfaciens* S108 (42.7±10.9%). Scanning electron microscopy revealed that VOCs produced by *B. subtilis* BE6 induced severe ultrastructural damage to *S. rolfsii* hyphae, including wrinkling, shrinkage, deformation, and lysis, indicating irreversible disruption of fungal cell integrity. Chemical profiling using HS-SPME/GC-MS identified 27 VOCs, with dominant compounds including cis-2,3-epoxybutane (27.34%), methyl (Z)-N-hydroxybenzenecarboximidate (17.19%), 5-methyl-2-hexanone (14.10%), hexanal (12.46%), and 2,5-dimethylpyrazine (2.89%). These compounds belong mainly to aldehyde, epoxide, oxime, ketone, and pyrazine groups, which are widely associated with antimicrobial activity.

Overall, the findings confirm that VOCs produced by *B. subtilis* BE6 play a major role in inhibiting and lysing *S. rolfsii* mycelia, highlighting the strong potential of indigenous bacterial VOCs as eco-friendly alternatives to chemical fungicides for managing soil-borne plant diseases.

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Table S1. Statistical analysis of the effect of bacterial VOCs on the mycelial growth of *Sclerotium rolfsii*

Analysis stage	Statistical Test	Degrees of Freedom (df)	Test Statistic (W or F)	p-value	Conclusion
Assumptions Test	Shapiro-Wilk (Normality)	-	W=0.9654	0.8528	Normality assumed (P>0.05)
	Levene's Test (Homogeneity)	(2,6)	F=1.1491	0.3780	Homogeneity assumed (P>0.05)
Main Analysis	One-way ANOVA	(8, 27)	F=14.31	6.67E-08	Significant treatment effect

Table S2. Statistical analysis of the inhibition rate bacterial VOCs on the growth of *Sclerotium rolfsii*

Analysis stage	Statistical Test	Degrees of Freedom (df)	Test Statistic (W or F)	p-value	Conclusion
Assumptions Test	Shapiro-Wilk (Normality)	-	W=0.9675	0.4331	Normality assumed (P>0.05)
	Levene's Test (Homogeneity)	(7,24)	F=0.5988	0.7507	Homogeneity assumed (P>0.05)
Main Analysis	One-way ANOVA	(7, 24)	F=7.62	7.23E-05	Significant treatment effect

Table S3. Volatile organic compounds identified from *Bacillus subtilis* BE6 using HS-SPME GC-MS analysis

RT (min)	Compound	CAS	Area
1.6812	Acetaldehyde	75-07-0	288125026
2.1509	(Z)-2,3-Epoxybutane	1758-33-4	918279966
3.8931	Hexanal	66-25-1	418572944
5.1418	Furfural	98-01-1	229140551
5.5045	3-Furaldehyde	498-60-2	194298786
6.0753	2-Hexanone, 5-methyl-	110-12-3	473546282
6.5688	Lupetazine	106-58-1	553863539
6.8126	Pyrazine, 2,5-dimethyl-	123-32-0	97148687
7.5023	Oxime-, methoxy-phenyl-	1000222-86-6	577531366
8.1207	p-Toluic acid, 2-amino-	2305-36-4	52167047
9.2266	2-Amino-6-methylbenzoic acid	4389-50-8	9485133
10.3921	2-Ethylhexanol	104-76-7	13187677
10.564	2-Decyn-1-ol	4117-14-0	3962973
10.72	5-Octen-2-one, 3,6-dimethyl-	64165-21-5	1962456
10.865	Benzeneacetaldehyde	122-78-1	2131063
11.2126	γ-Caprolactone	695-06-7	2750113
11.339	2,3-Octanedione	585-25-1	1035607
11.6942	Acetophenone	98-86-2	790702
12.1164	Pyrazine, 3-ethyl-2,5-dimethyl-	13360-65-1	1822237
14.3818	(+)-2-Bornanone	464-49-3	2494548
14.6434	Octane, 2,4,6-trimethyl-	62016-37-9	1118917
15.6305	Naphthalene	91-20-3	3617273
18.2586	Benzene, m-di-tert-butyl-	1014-60-4	2580828
18.3359	Ethanol, 2-(hexyloxy)-	112-25-4	5074999
19.4894	Undecane, 4,6-dimethyl-	17312-82-2	477652
20.2148	Pyrazine, 3-isopentyl-2,5-dimethyl-	18433-98-2	1228100
21.071	Decane, 3,6-dimethyl-	17312-53-7	1312619