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RESEARCH PAPER

Antagonistic bacterial strains isolated from cabbage rhizosphere release antimicrobial volatile organic compounds against *Pythium aphanidermatum*

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سلالات بكتيرية معادية معزولة من جذور الملفوف تطلق مركبات عضوية متطايرة مضادة لفطر البيثيوم افانيديرماتوم *Pythium aphanidermatum*

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Abstract. In a previous study, we isolated four antagonistic bacterial strains viz., *Pseudomonas aeruginosa* B1-SQU, *Pseudomonas indica* B2-SQU, *Serratia marcescens* B3-SQU and *Pseudomonas brenneri* B4-SQU from the rhizosphere of cabbage which suppressed damping-off in cabbage caused by *Pythium aphanidermatum*. In this study, potential of these bacterial isolates to produce antimicrobial volatile organic compounds (VOCs) against *P. aphanidermatum* was tested. The results of the two-sealed-base-plates assay revealed that all four bacterial strains produced VOCs against *P. aphanidermatum* with the maximum inhibition with *P. brenneri* B4-SQU followed by *S. marcescens* B3-SQU, *P. aeruginosa* B1-SQU and *P. indica* B2-SQU. Solid-phase microextraction coupled with gas chromatography-mass spectrometry was used to profile the VOCs of bacteria. A total of 20 VOCs were detected in *P. aeruginosa* B1-SQU and the major compounds identified were Carbon dioxide, 1-Butanol, 3-methyl- and Disulfide, dimethyl. The main volatile compounds detected in *P. indica* B2-SQU were 1-Butanol, 3-methyl-, Disulfide, dimethyl and 1,2-Propanediamine. Disulfide, dimethyl and 1,2-Propanediamine were the predominant compounds identified in *S. marcescens* B3-SQU among others. The major compounds detected in *P. brenneri* B4-SQU were 1-Butanol, 3-methyl-, 1,2-Propanediamine and Disulfide, dimethyl. Dimethyl disulfide, a well-known antimicrobial compound, was detected in the volatile profiles of all four antagonistic bacterial isolates. These results suggest that VOCs of antagonistic bacteria may be involved in the suppression of *P. aphanidermatum* and these antagonistic bacterial strains may be used as biofumigants for controlling damping-off of cucumber.

Keywords: Volatile organic compounds, *Pseudomonas aeruginosa, Pseudomonas indica, Serratia marcescens, Pseudomonas brenneri*, anti-oomycete activity

الملخص: في دراسة سابقة، قمنا بعزل أربع سلالات بكتيرية معادية، وهي *Pseudomonas aeruginosa* B1-SQU، *Pseudomonas indica* B2-SQU، *Serratia marcescens* B3-SQU و *Pseudomonas brenneri* B4-SQU من جذور الملفوف التي تسببت في تثبيط مرض موت البادرات في الملفوف الذي يسببه فطر *Pythium aphanidermatum*. في هذه الدراسة تم اختبار قدرة هذه العزلات البكتيرية على إنتاج مركبات عضوية متطايرة مضادة للميكروبات (VOC) ضد *P. aphanidermatum*. أظهرت نتائج اختبار صفيحتين قاعدتين أن جميع السلالات البكتيرية الأربعة أنتجت مركبات عضوية متطايرة ضد *P. aphanidermatum* بأقصى قدر من التثبيط مع *P. brenneri* B4-SQU يليها *S. marcescens* B3-SQU ثم *P. aeruginosa* B1-SQU ثم *P. indica* B2-SQU. تم استخدام الاستخراج المجهري في المرحلة الصلبة إلى جانب قياس الطيف الكتلي اللوني للغاز لتوصيف المركبات العضوية المتطايرة للبكتيريا. تم اكتشاف ٢٠ من المركبات العضوية المتطايرة في *P. aeruginosa* B1-SQU والمركبات الرئيسية التي تم تحديدها هي ثاني أكسيد الكربون، ١-بيوتانول، ٣-ميثيل- وثنائي كبريتيد، ثنائي ميثيل. كانت المركبات المتطايرة الرئيسية المكتشفة في *Pseudomonas indica* B2-SQU هي ١-بوتانول، ٣-ميثيل-، ثنائي كبريتيد، ثنائي ميثيل و١،٢-بروبانديامين. كانت مركبات ثنائي كبريتيد وثنائي ميثيل و١،٢-بروبانديامين هي المركبات السائدة التي تم تحديدها في *S. marcescens* B3-SQU من بين مركبات أخرى. كانت المركبات الرئيسية التي تم اكتشافها في *P. brenneri* B4-SQU هي ١-بوتانول، ٣-ميثيل، ١،٢-بروبانديامين وثنائي كبريتيد، ثنائي ميثيل. تم اكتشاف ثنائي ميثيل ثاني كبريتيد، وهو مركب معروف كمضاد للميكروبات في الملامح المتطايرة لجميع العزلات البكتيرية المعادية الأربعة. تشير هذه النتائج إلى أن المركبات العضوية المتطايرة للبكتيريا المضادة قد تكون ساهمت في قمع *P. aphanidermatum* ويمكن استخدام هذه السلالات البكتيرية المضادة كمواد تبخير حيوية للتحكم في مرض موت البادرات في الخيار.

الكلمات المفتاحية: المركبات العضوية المتطايرة، *Pseudomonas aeruginosa*، *Pseudomonas indica*، *Serratia marcescens*، *Pseudomonas brenneri*، النشاط المضاد للأوميسيت

Introduction

Plant disease management using microbial biocontrol agents (MBCAs) is becoming a popular practice among farmers because of its low cost, environmentally-friendly nature, high efficacy against multiple phytopathogens, relatively ease of application and minimum labor requirements (Bonaterra et al., 2022; Lahlali et al., 2022; Palmieri et al., 2022). In "augmentative biological control", highly efficient antagonistic microorganisms against plant pathogens are selected, multiplied on artificial media in large scale and applied to crop plants/soil to control plant diseases (Eilenberg et al., 2001; van Lenteren et al., 2018; Kohl et al., 2019).

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A large number of endophytic and rhizospheric bacteria that are effective against important soilborne fungal pathogens have been reported (Al-Hussini et al., 2019; Al-Shibli et al. 2019; Al-Daghari et al., 2020a,b; Al-Ghafri et al., 2020; Al-Rashdi et al., 2022; Al-Rahbi et al., 2023). Over 100 MBCAs have been registered for plant disease management (van Lenteren et al., 2018). Several commercial biocontrol products such as Polyversum (based on *Pythium oligandrum* strain M1; Biopreparaty, spol. s.r.o., Czech Republic), Mycostop (based on *Streptomyces griseoviridis* strain K61; Verdera, Finland), Xedavir (based on *Trichoderma asperellum* TV1; Xeda Italia Srl, Italy), Companion and Kodiak (based on *Bacillus subtilis* GB03; Growth Products, USA), AtEze (based on *Pseudomonas chlororaphis* strain 63-28; EcoSoil Systems, San Diego, CA, USA), Bio-Ject, Spot Less (based on *Pseudomonas aureofaciens* strain TX-1; EcoSoil system, Canyon Lake, TX, USA), RootShield® and PlantShield® (based on *Trichoderma harzianum* T22; Bio works, Victor, NY, USA) are available worldwide (Lahlali et al., 2022). The biocontrol agents act on the plant pathogens by: (i) directly suppressing the pathogens through antibiosis, competition for space and nutrients and parasitism; (ii) interfering with the pathogenesis mechanisms of pathogens; and (iii) induction of defense mechanisms in host plants (Bardin et al., 2015). The emission of volatile organic compounds (VOCs) is considered to be one of the important mechanisms of action of MBCAs (Zhao et al., 2022). The VOCs are low-molecular weight compounds (<300 Da) with high vapor pressure and low polarity (Vespermann et al., 2007). VOCs of antagonistic microorganisms are known to cause fungal cell membrane damage by hydrolyzing the cell wall that results in the leakage of cellular contents and finally cell death (Giorgio et al., 2015; Hutchings et al., 2017; Choinska et al., 2020; Tyagi et al., 2020; Zhang et al., 2020). The production of VOCs by antagonistic microorganisms depends on several factors, including types of culture media, growing conditions, and their population density (Choinska et al., 2020). Furthermore, VOCs emitted by some bacterial strains influence the growth of other rhizosphere bacterial strains. For example, a study performed by Garbeva et al. (2014) revealed that the VOCs released by *Serratia plymuthica* and *Collimonas pratensis* stimulated the growth of *Pseudomonas fluorescens* Pf0-1. In addition, these VOCs triggered the production of antibacterial secondary metabolites and expression of genes involved in the motility in *P. fluorescens* Pf0-1. In the course of isolation of beneficial bacterial strains from the rhizosphere of cabbage (*Brassica oleracea var. capitata* L.), we observed that the bacterial strains *Pseudomonas aeruginosa* B1-SQU, *Pseudomonas indica* B2-SQU, *Serratia marcescens* B3-SQU and *Pseudomonas brenneri* B4-SQU could suppress the growth of *Pythium aphanidermatum* (Edson) Fitzpatrick, the causative agent of damping-off in cucumber (Dhuha et al. Unpublished). The objectives of this study were to assess the potential of these bacterial strains to release antimicrobial VOCs against *P. aphanidermatum* and to profile the VOCs of each bacterial strains.

Materials and Methods

Microbial Strains

Pseudomonas aeruginosa B1-SQU (GenBank accession number ON738574, *Pseudomonas indica* B2-SQU (acc. no ON738576), *Serratia marcescens* B3-SQU (acc.no OP837487) and *Pseudomonas brenneri* B4-SQU (acc. no ON738575) isolated from the rhizosphere of cabbage plants (un-published) were used in this study. These bacterial strains were grown on nutrient agar (NA) medium (Oxoid Ltd., UK) at 30°C. A virulent strain of *Pythium aphanidermatum* Sala1 (acc.no ON113866), originally isolated from a damping-off infected cucumber seedling, was provided by the Department of Plant Sciences, Sultan Qaboos University. The culture was maintained on potato dextrose agar (PDA) medium (Oxoid Ltd., UK) and stored at 4°C.

Antimicrobial Assay

The two-sealed-base-plates assay was used to test the production of antimicrobial VOCs by the bacterial strains (Al-Rashdi et al., 2022). Briefly, a mycelial disc of *P. aphanidermatum* (6-mm diameter) was taken from an actively growing PDA culture by using a sterile cork borer and placed aseptically at the center of a 1/5th strength PDA plate. The cell suspension of each test bacterial strain (100 µl) prepared from an overnight culture (10^8 cfu/ml) was applied on another NA plate and spread uniformly with a sterile glass spreader. The lids of both culture plates were removed and the base-plate with *P. aphanidermatum* was over laid with NA base-plate inoculated with the bacterium, aligned perfectly and sealed with two layers of parafilm. The sealed base-plates were incubated at 27°C until *P. aphanidermatum* mycelial growth covered the entire plate in the control. An un-inoculated NA base-plate paired with a PDA base-plate inoculated with *P. aphanidermatum* in the same manner served as control. At the end of incubation, the growth of *P. aphanidermatum* mycelium was measured using a ruler and % inhibition was calculated. The assay was conducted in triplicate.

Analysis of Volatile Compounds

Each bacterium was grown in 25 ml of nutrient broth (Oxoid Ltd., UK) in solid-phase micro extraction glass vials under aseptic conditions in an incubator shaker at 30°C and 170 rpm for 72h. The volatile compounds emitted by each bacterial strain were collected using headspace-solid phase microextraction (HS-SPME) technique. The SPME syringe containing fiber Carboxen/ Polydimethylsiloxane (SUPELCO, USA) was insert-

ed into the head space of vial and left for 45 min to trap the volatile compounds. The fiber containing the volatiles was injected into a gas chromatography-mass spectrometry (GC-MS) system. The analysis of volatile compounds was performed on a Shimadzu GC-2010 Plus, fitted with a Rtx-5MS capillary column (30 m × 0.25 mm; 0.25 µm; maximum temperature 350°C) and attached to a GCMS-QP2010 ULTRA MS. The carrier gas Helium (99.999% purity) was used at a constant flow of 1.0 ml/min. The mass spectrum libraries Wiley 9th edition and NIST (National Institute of Standards and Technology) 2011 v.2.3 were used for identification of compounds.

Statistical Analysis

A completely randomized design was used for the *in vitro* anti-oomycete effect of VOCs experiment. Analysis of the data was by one-way ANOVA using SAS v8 program (SAS Institute, NC, USA). Duncan's multiple range test (DMRT; $P < 0.05$) was used for comparison of means.

Results and Discussion

It is evident from the results that all four rhizosphere bacterial strains evaluated in this study produced VOCs that suppressed *P. aphanidermatum* mycelial growth in the *in vitro* assay. *P. brenneri* B4-SQU showed the maximum inhibition (29.3%) followed by *S. marcescens* B3-SQU (24.4%), *P. aeruginosa* B1-SQU (23.2%) and *P. indica* B2-SQU (18.3%) (Table 1). The inhibition of mycelial growth of *P. aphanidermatum* upon exposure to VOCs of *P. brenneri* B4-SQU is shown in Figure 1. The production of VOCs is one of the modes of action of many antagonistic fungi (Wheatley et al., 1997; Zhang et al., 2014; Choinska et al., 2020; Intana et al., 2021; Khruengsai et al., 2021; Rajani et al., 2021; Ruangwong et al., 2021; Kong et al., 2022) and antagonistic bacteria (Chaurasia et al., 2005; Chaves-Lopez et al., 2015; Gotor-Vila et al., 2017; Lim et al., 2017; Chen et al., 2020; Delgado et al., 2021; Al-Rashdi et al., 2022) to suppress phytopathogenic fungi. VOCs of bacteria are known to cause several abnormalities in the fungal structures including cytoplasmic cavitation and vacuolation, coagulation of cytoplasmic contents and degradation of fungal hyphal membrane (Toral et al., 2021). Chaurasia et al. (2005) while studying the antimicrobial effect of diffusible and volatile compounds produced by *Bacillus subtilis* on phytopathogenic fungi *Alternaria alternata*, *Cladosporium oxysporum, Fusarium oxysporum* and *Paecilomyces lilacinus* reported that the inhibitory effect caused by volatile compounds was greater than that by diffusible compounds. Das et al. (2022) reported the loss of plasma membrane integrity and oxidative stress in *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Juxtiphoma eupyrena*, and *Neurospora crassa* when exposed to volatiles of *Serratia plymuthica*. Al-Toubi et al. (2022) demonstrated that VOCs released by *Hypomyces perniciosus* and *Cladobotryum mycophilum* suppressed the growth of *Agaricus bisporus*. In this study, HS-SPME-GC-MS was used to profile the volatile compounds from the bacterial strains. A total of 20 VOCs were detected in *P. aeruginosa* B1-SQU. Among them, carbon dioxide had the highest peak area (26.04%), followed by 1-Butanol, 3-methyl- (syn: Isopentanol/ Isoamyl alcohol) (18.18%) and Disulfide, dimethyl (Dimethyl disulfide) (10.23%) (Table 2; Figure 2). In *P. indica* B2-SQU, 1-Butanol, 3-methyl- showed the highest peak area (36.94%), followed by Disulfide, dimethyl (25.35%) and 1,2-Propanediamine (9.72%) (Table 3; Fig. 3). Disulfide, dimethyl (28.96%) and 1,2-Propanediamine (syn: Propylenediamine/ 1,2-Diaminopropane/ 1,2-Propylenediamine) (19.72%) were the predominant compounds identified in *S. marcescens* B3-SQU among others (Table 4; Figure 4). The major compounds detected in *Pseudomonas brenneri* B4-SQU were 1-Butanol, 3-methyl- (34.70%), 1,2-Propanediamine (19.49%) and Disulfide, dimethyl (8.13%) (Table 5; Figure 5). Dimethyl disulfide (DMDS), a sulphur-containing compound was identified as one of the major components in common in the VOCs of all four antagonistic bacteria tested. Several reports describe the inhibitory activity of DMDS against plant pathogenic fungi (Groenhagen et al., 2013; Tyagi et al., 2020; Lin et al., 2021). Many antagonistic bacterial strains including *Pseudomonas aeruginosa* PC5 (Al-Rashdi et al., 2022) and *Burkholderia gladioli* BBB-01 (Lin et al., 2021) have been demonstrated to produce DMDS. Inhibition of the oomycete pathogen *Phytophthora infestans* by DMDS produced by *Pseudomonas* sp. has been reported (De Vrieze et al., 2015; Guevara-Avendano et al., 2019). DMDS is known to damage the cell membrane of *Sclerotinia minor* and interfere with its growth and pathogenicity (Tyagi et al., 2020). In addition,

Table 1. Inhibition of mycelial growth of *Pythium aphanidermatum* by volatile compounds released by antagonistic bacterial strains isolated from cabbage rhizosphere

Bacterial strain	Diameter growth of *P. aphanidermatum* (cm)	% inhibition
Pseudomonas aeruginosa B1-SQU	6.3 c	23.2
Pseudomonas indica B2-SQU	6.7 b	18.3
Serratia marcescens B3-SQU	6.2 c	24.4
Pseudomonas brenneri B4-SQU	5.8 d	29.3
Control	8.2 a	-

Data are means of 6 replications
Means followed by the same letter in a column are not significantly different from each other at $P < 0.05$ (DMRT)

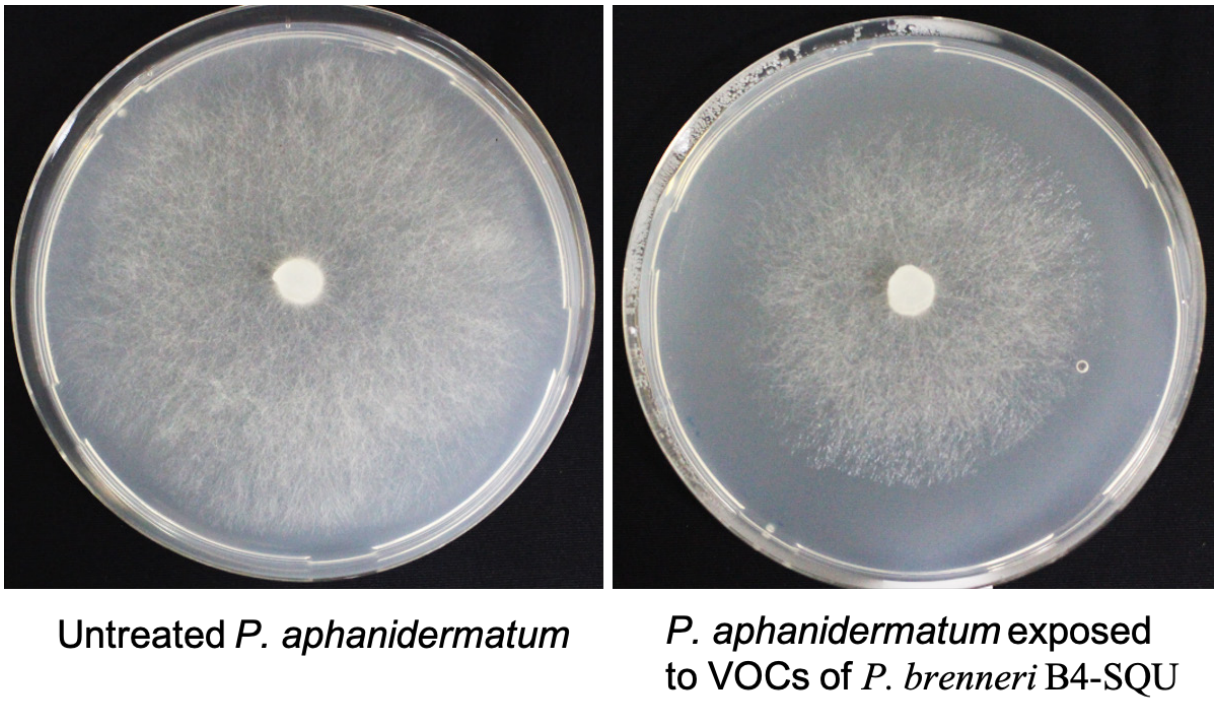

Figure 1. Inhibition of mycelial growth of *Pythium aphanidermatum* upon exposure to VOCs of *Pseudomonas brenneri* B4-SQU as assessed by the two-sealed-base-plates assay

plant growth promotion effect and induction of systemic resistance in plants have been reported by treatment with DMDS (Meldau et al., 2013; Tyagi et al., 2020). Similarly, antifungal and antibacterial activities of isoamyl alcohol have been reported (Ando et al., 2015). Rodriguez Lopez et al. (2019) reported prevention of hyphal formation in

Table 2. Volatile organic compounds released by *Pseudomonas aeruginosa* B1-SQU isolated from cabbage rhizosphere

Compound	Retention time (min)	Area %
Carbon dioxide	1.272	26.04
Methanethiol	1.396	4.64
Dimethylamine	1.538	6.05
Ethylene oxide	1.58	4.65
Methane, oxybis[chloro-	1.676	4.20
Hydroxyurea	1.737	4.07
1,2-Ethanediamine, N,N'-dimethyl-	1.825	0.82
L-Alanine-4-nitroanilide	1.85	1.19
Pentane, 2,4-dimethyl-	1.945	4.14
Unidentified	2.0	1.34
Unidentified	2.061	2.02
Acetamide, 2,2-dichloro-	2.095	1.56
Pentanal	2.38	3.90
1-Butanol	2.466	2.19
D-Alanine	2.63	1.30
2,2-Difluoroethanol, TMS derivative	2.733	2.08
Unidentified	2.84	0.65
Unidentified	3.235	0.66
1-Butanol, 3-methyl-	3.348	18.18
Disulfide, dimethyl	3.554	10.23

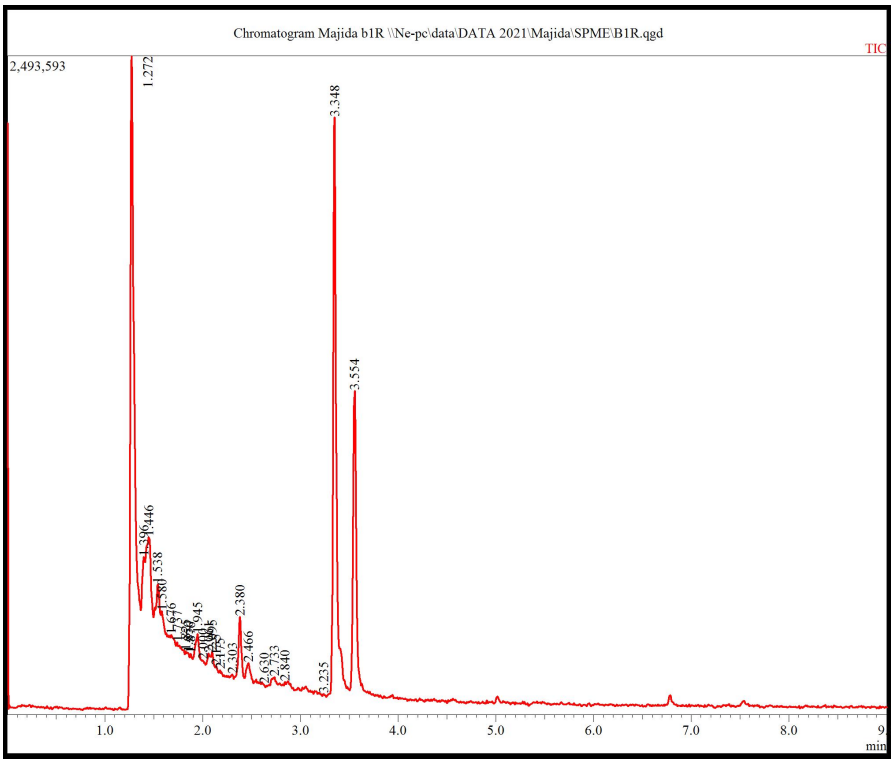

Figure 2. Chromatogram of volatile organic compounds released by *Pseudomonas aeruginosa* B1-SQU isolated from cabbage rhizosphere

Candida albicans by isoamyl alcohol. Toral et al. (2021) reported the production of isopentanol as the principal volatile compounds by extremophilic bacteria viz., *Peribacillus* sp. N3, *Pseudomonas segetis* P6, *Psychrobacillus vulpis* Z8 and *Staphylococcus equorum* subsp. *equorum* EN21. However, the mechanism of action of isoamyl alcohol on fungi has not been fully elucidated.

Conclusion

Through this study we demonstrated that cabbage rhizosphere bacterial strains suppressed the growth of *P. aphanidermatum* up to 29% through the release of VOCs. Disulfide, dimethyl, 1-Butanol, 3-methyl- and 1,2-Propanediamine were the major components in

Table 3. Volatile organic compounds released by *Pseudomonas indica* B2-SQU isolated from cabbage rhizosphere

Compound	Retention time (min)	Area %
1,2-Propanediamine	1.397	9.72
Unidentified	1.47	1.21
Acetone	1.652	5.15
1,3-Pentadiene	1.695	4.17
Unidentified	1.79	3.87
2-Butanone	2.059	1.95
Trichloromethane	2.2	1.81
Butanal, 3-methyl-	2.491	3.57
Unidentified	2.569	2.14
2-Pentanone	2.824	0.52
1-Butanol, 3-methyl-	3.444	36.94
Disulfide, dimethyl	3.649	25.35
2-Heptanone	6.84	0.74
Dimethyl trisulfide	9.074	1.21
Benzene, 1-methoxy-4-methyl-	10.567	0.85
3-Decen-1-ol, acetate, (Z)-	22.899	0.72

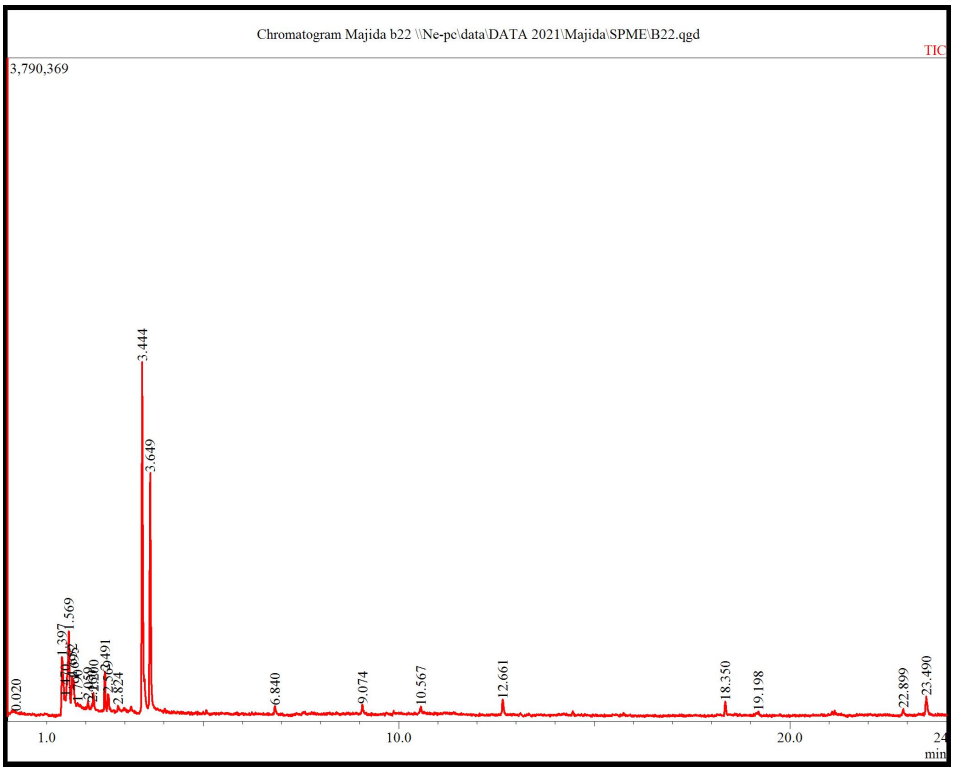

Figure 3. Chromatogram of volatile organic compounds released by *Pseudomonas indica* B2-SQU isolated from cabbage rhizosphere

the VOCs of antagonistic bacterial strains tested in this study. Antimicrobial activities of Disulfide, dimethyl and 1-Butanol, 3-methyl- have been well established in earlier studies. Several factors including the type of culture media, growing condition, physiological state and population of the bacterial isolates determine the quantity of

Table 4. Volatile organic compounds released by *Serratia marcescens* B3-SQU isolated from cabbage rhizosphere

Compound	Retention time (min)	Area %
1,2-Propanediamine	1.341	19.72
Isoprene	1.636	7.53
Dimethyl sulfide	1.675	2.73
Ethyl Acetate	2.11	2.05
Trichloromethane	2.145	2.64
Pentanal	2.438	1.49
1-Butanol	2.512	3.15
Unidentified	3.114	0.77
1-Butanol, 2-methyl-, (S)-	3.402	8.11
1-Butanol, 3-methyl-	3.462	2.46
Disulfide, dimethyl	3.601	28.96
Pyrrole	3.779	1.41
Butanoic acid, 2-methyl-, ethyl ester	5.768	0.91
Butanoic acid, 3-methyl-, ethyl ester	5.845	7.27
1-Nonene	6.796	1.31
Pentane, 1-(methylthio)-	7.519	0.83
Thiopivalic acid	8.178	3.76
2-Methyl-3-(methylthio) furan	8.501	1.25
2,6-Octadiene, 4,5-dimethyl-	21.143	2.37
2-Heptadecanone	23.504	1.19

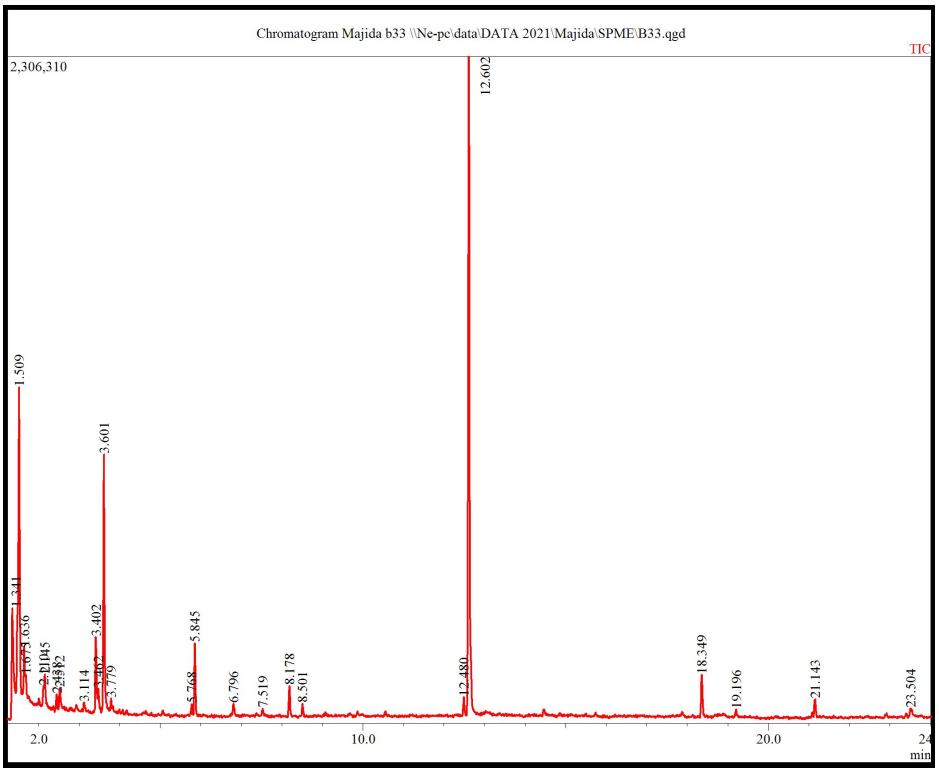

Figure 4. Chromatogram of volatile organic compounds released by *Serratia marcescens* B3-SQU isolated from cabbage rhizosphere

production and emission of VOCs by antagonistic bacterial isolates. In addition to VOCs, other mechanisms

Table 5. Volatile compounds released by *Pseudomonas brenneri* B4-SQU isolated from cabbage rhizosphere

Compound	Retention time (min)	Area %
1,2-Propanediamine	1.311	19.49
1-Nitro-2-propanone	1.589	6.17
2-Pentanamine	1.661	2.31
Silanol, trimethyl-	1.799	8.14
Unidentified	1.881	1.10
Unidentified	1.925	0.80
Unidentified	1.95	1.31
2-Butanone	1.999	1.82
Ethyl Acetate	2.108	3.00
Acetamide, 2,2-dichloro-	2.15	0.95
Pentanal	2.432	2.13
Unidentified	2.507	1.78
Octodrine	3.21	0.30
1-Butanol, 3-methyl-	3.395	34.70
Disulfide, dimethyl	3.601	8.13
2-Pentanone, 3-methyl-	3.714	1.18
Unidentified	4.111	0.38
Unidentified	5.052	0.66
Unidentified	6.025	0.54
Anisole	7.556	3.87
Dimethyl trisulfide	9.066	0.59
Undecane, 3-ethyl-	25.926	0.54

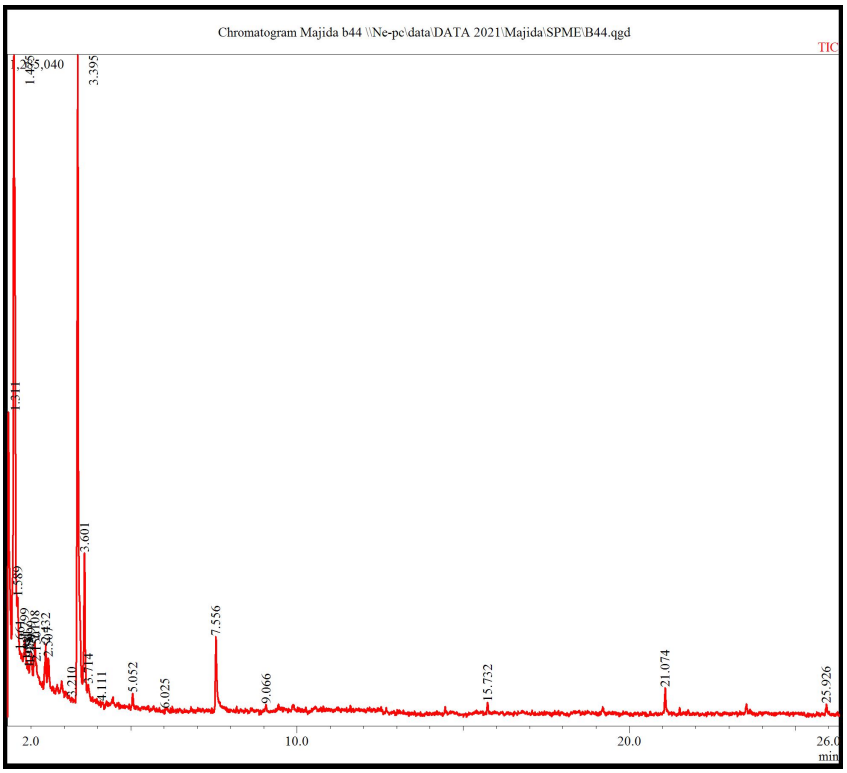

Figure 5. Chromatogram of volatile compounds released by *Pseudomonas brenneri* B4-SQU isolated from cabbage rhizosphere

such as production of cell wall lytic enzymes, siderophores and other antimicrobial secondary metabolites may also be involved in the antagonistic action of these bacterial isolates against *P. aphanidermatum*. Further studies are required to test the role of these VOCs in inhibition of *P. aphanidermatum* and other soilborne pathogens of cucumber and elucidate their mode of action

Acknowledgments

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