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Contributed Paper

Nematicidal Activities of Bacterial Volatiles from *Pseudoduganella violaceinigra* G5-3 and *Novosphingobium pokkalii* G8-2 against the Pine Wood Nematode *Bursaphelenchus xylophilus*

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ABSTRACT

Naturally-occurring volatile compounds with nematicidal activities have significant economic importance in agriculture and forestry. We identified two Gram-negative bacteria *Pseudoduganella violaceinigra* G5-3 and *Novosphingobium pokkalii* G8-2 that produced volatile compounds with potent nematicidal activities. Mortalities for the supernatants of *P. violaceinigra* G5-3 and *N. pokkalii* G8-2 to *Bursaphelenchus xylophilus* were 98.26% and 93.10% in 24 h, respectively. Volatiles of the two strains were analyzed by solid-phase microextraction coupled with gas chromatography/mass spectrometry. The compounds 2,5-dimethyl pyrazine, 4-dimethylaminopyridine, benzyl acetate, phenethyl butyrate, phenylethyl alcohol and acetophenone possessed nematicidal activity against *B. xylophilus* with LC_{90} values of 33.72, 1.83, 0.92, 0.20, 13.22 and 10.11 mM as assayed using the pure commercial compounds, respectively. This study provides a theoretical basis for the utilization of microorganisms to generate compounds with nematicidal activity.

Keywords: bacteria, volatiles, *Bursaphelenchus xylophilus*, nematicidal activity

1. INTRODUCTION

Pine wilt disease caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus,* is a devastating disease of pine trees and has caused huge losses in Japan, China, South Korea [1] and Western Europe [2]. During the last decade, pine trees in at least 14 provinces in China have been infected with *B. xylophilus*. This has resulted in the loss of more than 300,000 hectares of pine forests and more than 500 million pine trees [3].

Pine wilt disease is transmitted in forest by pine sawyer beetle carrying *B. xylophilus* [4] and physical and chemical methods are commonly used for disease control. For example, felling of diseased pine trees, restricting new plantings away from affected areas and fumigation of insect vectors are commonly used measures [5]. Insecticidal agents such as thiacloprid, avermectin and carbaryl are often used to prevent and control

this disease [6]. However, chemical pesticides are highly toxic and have long residual half-lives that have led to serious environmental problems. For these reasons, the application of chemical agents is being gradually restricted [7] and eco-friendly natural pesticides are being developed.

Compared with these traditional chemical pesticide controls, there is increasing data demonstrating the effectiveness of biological control [8-9]. Microorganisms such as nematode predatory fungi [10], endophytic and toxigenic fungi [11-12], actinomycetes [13] and nematicidal bacteria have been utilized for their potential in control of pine wilt disease. Bacteria used for biocontrol are especially attractive for this purpose due to diversity in species and the active components they produce. Most of these bacteria-derived active substances are non-volatile such as quinines, alkaloids and terpenoids [14]. Bacteria producing volatile organic compounds (VOC) that inhibit or even kill the pine wood nematode have also been developed [15]. The greatest advantage of volatile nematicides over non-volatile nematicidal toxins is their better diffusivity and penetration. For example, the VOC produced by the rhizobacterium *Bacillus megaterium* were lethal for the southern root-knot nematode *Meloidogyne incognita* [16]. Two marine bacteria that produced nematicidal dimethyl trisulfide, benzaldehyde, dimethyl disulfide and tert-butylamine have also been identified [17]. In this study, we screened and identified bacteria with nematicidal activity from environmental samples, and studied the bacterial VOC which might be useful in prevention of pine wilt disease.

2. MATERIALS AND METHODS

2.1 Pine Wood Nematode

The pine wood nematode, *B. xylophilus,* was originally isolated from dead Japanese black pines (*Pinus thunbergii*) using the Baermann funnel method [17]. The nematodes were washed three times with sterile water and inoculated on a *Botrytis cinerea* culture growing on potato dextrose agar plates [18]. The nematodes were cultured at 25°C for 7 days and collected from the plates by rinsing with sterile water.

2.2 Isolation and Screening of Nematicidal Bacteria

Bacteria were isolated from water, soil and biological samples that were collected in July 2017 on the Qingdao University campus. Soybean-sized samples were added to 5 mL sterile water and incubated at 25°C for 30 min. The aqueous phase was diluted 10 to 10^{-4} fold with sterilized water, and 100 µL diluted sample was spread evenly over nutrient agar plates which were then incubated at 28°C for 3 days, each dilution has three repetitions, single colonies were screened by streak plate method. Bacterial strains were selected according to their colony morphologies and stored at -80°C in nutrient broth liquid media containing 20% (v / v) glycerol.

Bacterial strains were cultivated in nutrient broth at 28°C for 1 day and the seed was transferred into a 150 mL Erlenmeyer flask containing 50 mL of nutrient broth and shaken at 28°C, 160 rpm for 3 days. Bacterial culture supernatants were obtained by centrifugation of 1 mL culture at 12000 rpm for 10 min.

The culture supernatants were tested for their nematicidal activity against PWN. In a 24-well plate, 450 µL culture supernatant was added to each well containing 50 µL nematode suspension to give approximately 100 nematodes per well. The nutrient broth medium was used as the negative control. Nematodes were observed under a

stereomicroscope after incubated at 25°C for 24 h. The nematode was considered dead if it was stiff when touched by a needle. Nematode mortality was determined as previously described using the equation CM = (d-c)/(1-c) × 100% [19] where CM represents the nematode corrected mortality (%), d represents mortality of nematodes in the treatment group (%), c represents mortality of nematode in the control group (%). Each treatment was repeated three times.

2.3 Identification of Bacteria

Two bacterial isolates of G5-3 and G8-2 with the most obvious nematicidal activity were selected for further identification according to their morphological characteristics and 16S rDNA sequence analysis. For studying morphological characteristics, the characteristics of colonies, cells and Gram staining were observed. Genomic DNA was extracted as previously described [20]. 16S rDNA was amplified by PCR using primers 5′-AGAGTTTGAT CCTGGCTCAG-3′ and 5′-GGTTACCTT GTTACGACTT-3′ and genomic DNA as template. The PCR program was carried out as follows: initial denaturation at 94°C for 5 min, followed by 32 cycles of 94°C for 30s, 56°C for 30s and 72°C for 1.5 min with a final extension at 72°C for 10 min. The amplicons were purified from an agarose gel and cloned into plasmid vector pMD-18T and the insert was sequenced by Shanghai Sangon (China). The 16S rDNA gene sequence was analyzed using the BLAST algorithm from the National Center for Biotechnology Information (NCBI) and the EzTaxon-e database [20]. Clustal X 1.83 and MEGA 7 software were used for alignments and phylogenic trees were generated using the neighbor-joining (N-J) method [21].

2.4 Effect of Culture Conditions on Nematicidal Activity of Bacteria

To investigate the effect of pH values on nematicidal activity, single colony was cultured for 12 h and inoculated into 50 mL nutrient broth with initial pH value of 5.0, 6.0, 7.0, 8.0 and 9.0, respectively, and incubated in a shaker at 28°C at 160 rpm for 3 days. To study the effect of temperature on nematicidal activity, the culture temperature was reset to 15, 20, 25, 30, 35 and 40°C. Other variables including the inoculum age (9, 15, 21, 27 and 33 h) and culture time (1.5, 3, 4.5, 6 and 7.5 d) were also investigated.

2.5 Stability of Nematicidal Compounds

Thermal stability of culture supernatants was tested at 20, 40, 60, 80 and 100°C for 2 h, respectively. The pH stability of culture supernatants was determined by adjusting culture supernatant to pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, respectively, and then incubating them at room temperature for 12 h followed by neutralizing them to pH 7.0. The nematicidal activities of these supernatants were assayed using nutrient broth medium as the control. All of the experiments were repeated 3 times.

2.6 Extraction and Identification of Volatile Nematicides

Bacteria in nutrient broth were cultured at 30°C, 160 rpm for 3 days. The cells were pelleted by centrifugation at 12000 rpm for 10 min. Supernatants were distilled under decompression at 100°C and the distillate was collected for the measurement of nematicidal activity and solid phase microextraction (SPME). Nutrient broth was used as a blank control. A 75 μm CAR/PDMS fiber head (Supelco, Bellefonte, PA, USA) for SPME was activated for 15 min. A 9 mL test sample was added to a 15 mL SPME extraction flask

and heated at 50°C for 1 h while stirring [22]. Three replications were performed.

After extraction, the extraction tip was inserted into a gas chromatograph/mass spectrometer (GC/MS) (QP2010 SE, Shimadzu). The fiber head was pushed out into the gasification chamber and treated at 210°C for 1 min. An Rxi-1MScapillary column (30 m×0.25 mm×0.25 μm) (Model QP2010, Shimadzu) was used for GC/MS and the sample was loaded using split carrier injection with a gas flow of 1 mL/min. The program temperature was raised to 50°C and stayed for 2 min and the temperature was increased to 180°C at a rate of 8°C/min and then to 240°C at a rate of 10°C/min and kept at this level for 6 minutes. The temperatures of the transfer line and ion trap were 250°C and 300°C, respectively. VOC were analyzed and compared using the NIST library of standard GC/MS data (https://chemdata.nist.gov/). Compounds with similarities (SI) > 90 were selected for further analysis.

2.7 Nematicidal Activity of VOC *in vitro*

To investigate the candidate VOC with nematicidal activity against PWN, 6 commercial compounds found in VOC from bacterial isolates of G5-3 and G8-2 were selected to study their activity *in vitro*. These analytical reagents included 2, 5-dimethyl pyrazine, benzyl acetate, 4-dimethylaminopyridine, phenethyl butyrate (Macklin, Shanghai, China), phenylethyl alcohol and acetophenone (Sinopharm Chemical, Shanghai, China). The compounds were dissolved in 2% Triton X-100 at different concentrations and the corrected mortality was tested using the method described above. Triton X-100 (2%) was used as the control. Four replicates were used for each treatment.

2.8 Statistical Analysis

The data were analyzed using analysis of variance (ANOVA), and the means were compared by the least significant differences (LSD) at P = 0.05 using SPSS l7.0 software and LC_{90} was calculated according to the probit model [23].

3. RESULTS AND DISCUSSION

3.1 Isolation and Nematicidal Activity of Bacterial Strains

A total of 200 bacterial isolates were obtained from different samples collected In Qingdao campus, among which 20 isolates demonstrated nematicidal activity and 16 isolates showed a corrected mortality greater than 50% within 24 h (Table 1). Two isolates designated G5-3 and G8-2 with a corrected mortality of 98.26% and 93.10%, respectively, were selected for further study.

The two bacterial isolates were grown on nutrient agar medium for 3 days at 28°C. G5-3 colonies were milky white, round and opaque with thick and bulging surfaces and irregular edges. The cells of G5-3 were short rods (1.0~3.5 × 1.0~2.5 μm). G8-2 colonies were circular, transparent with light yellow sticky surfaces and regular colony edges. The cells of G5-3 were curved rods (0.5~1.0 × 1.0~3.5 μm). Both bacterial isolates were Gram-negative and motile with flagella. The 16S rDNA fragment amplified from isolates G5-3 and G8-2 was approximately 1400 bp in length. Phylogenetic tree was constructed using the neighbor-joining method based on the 16S rDNA sequences of the two isolates and those of their closest relatives which had similarities of more than 95% obtained from the EzTaxon-e database (Figure 1). Sequences blast results indicated that strains G5-3 and G8-2 showed a pairwise similarity of 98.79% and 99.27% with *Pseudoduganella violaceinigra* BT HNGU 29 and *Novosphingobium pokkalii* L3E4,

Table 1. List of the 20 bacterial isolates tested and their nematicidal activities against *B. xylophilus*. Controls were nutrient broth. Data were means ± SD of three replicates. Means in the column followed by the same letter did not differ significantly at $P < 0.05$ (method of multiple comparisons).

Isolate	Source	Mean corrected mortality (%) ±SD
Control	Nutrient broth	1.35 ± 1.12a
N1-2	Lawn root soil	83.63 ± 4.58h
N1-3	Lawn root soil	35.67 ± 4.04c
G1-3	Lawn root soil	55.63 ± 0.75e
N2-2	Silt	84.90 ± 1.97h
N2-3	Silt	78.83 ± 2.05g
G2-2	Silt	60.00 ± 5.32d
N3-2	Water moss	58.27 ± 6.27d
N3-4	Water moss	21.00 ± 5.00b
N4-3	Lichens	83.67 ± 2.52h
N4-5	Lichens	53.58 ± 1.63e
G5-1	Ginkgo root soil	77.45 ± 3.83g
G5-3	Ginkgo root soil	98.26 ± 3.14i
G6-5	Bathing hall walls	33.07 ± 4.69c
G8-1	Pond water	64.80 ± 1.55e
G8-2	Pond water	93.10 ± 2.79i
G8-3	Pond water	89.93 ± 1.68i
N10-1	Reed root	79.23 ± 4.67g
N10-3	Reed root	41.62 ± 3.46d
N10-5	Reed root	69.72 ± 1.55f
G10-1	Reed root	82.93 ± 5.25h

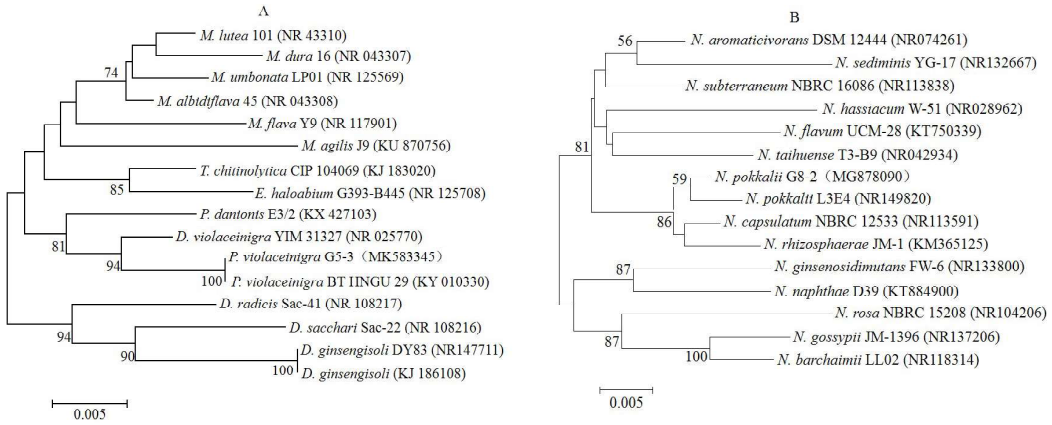

Figure 1. Neighbor-joining phylogenetic trees based on 16S rDNA sequences of (A) *P. violaceinigra* G5-3 and (B) *N. pokkalii* G8-2. Numbers at the nodes indicated the bootstrap values on neighbor-joining analysis (Bar = 0.05).

respectively. Therefore, isolates G5-3 and G8-2 were identified as *P. violaceinigra* G5-3 and *N. pokkalii* G8-2, respectively.

3.2 Effect of Culture Conditions on Nematicidal Activity

Nematicidal activities of culture supernatants of *P. violaceinigra* G5-3 and *N. pokkalii* G8-2 were measured under different initial pH values of medium, inoculum ages and incubation times and culture temperatures. Results showed that culture conditions had different effects on nematicidal activities of the culture supernatants. The optimal culture temperatures and initial pH values of the medium were 30°C and 7.0

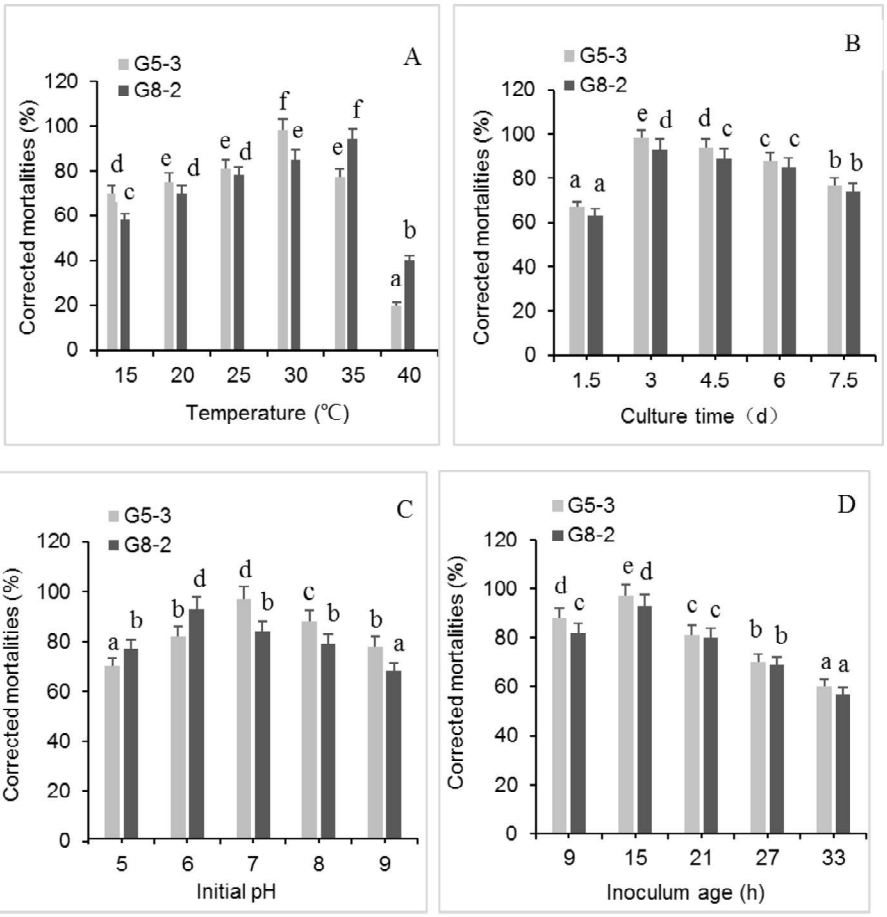

Figure 2. Effects of (A) temperature (B) , culture time (C), initial pH and (D) inoculum age on nematicidal activity (corrected mortality, %) of strains G5-3 and G8-2 against *B. xylophilus*. Corrected mortalities were reported as the means of three replicates. Data were given as mean ± SD. Means with the same superscript letters were not significantly different at $P < 0.05$.

for *P. violaceinigra* G5-3 and 35°C and 6.0 for *N. pokkalii* G8-2. The optimal inoculum age for these two strains was 15 h and optimal culture time was 3 days (Figure 2).

3.3 Stability of Nematicidal Compounds

The stability of the nematicidal compounds in culture supernatants of *P. violaceinigra* G5-3 and *N. pokkalii* G8-2 were tested under different pH values and temperatures. The nematicidal components from the two strains were relatively thermally stable and incubations at 20, 40, 60, 80 and 100°C for 2 h did not alter the nematicidal activities. However, the nematicidal components of both strains were highly

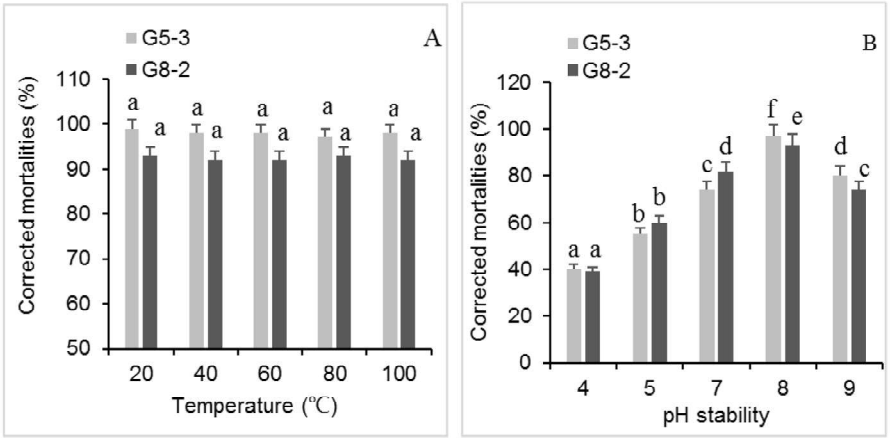

Figure 3. The (A) thermal and (B) pH stability of nematicidal components from strains G5-3 and G8-2. Corrected mortalities against *B. xylophilus* were given as mean ± SD with n=3. Means in the column followed by the same letter did not differ significantly at $P < 0.05$.

sensitive to pH values. The compounds with nematicidal activity were relatively stable at pH 8.0 while acidification or alkalization decreased their nematicidal activity (Figure 3).

3.4 Extraction and Identification of VOCs with Nematicidal Activity

The culture supernatant from each strain grown under optimal conditions was distilled

Table 2. Nematicidal activity of cultures and volatile crude extracts from Strain G5-3 and G8-2 against *B. xylophilus*.
Data were means ± SD of three replicates.

Strain	Sample	Corrected mortality (%)
G5-3	Culture supernatant	98.26 ± 2.26
	Crude volatile extract	99.21 ± 2.48
G8-2	Culture supernatant	93.10 ± 3.91
	Crude volatile extract	99.06 ± 3.35
Control 1	Nutrient broth	1.22 ± 1.87
Control 2	Volatile extract of Nutrient broth	1.14 ± 1.53

and crude volatile extract was collected. Bioassay showed that the corrected mortality of crude volatile extract from both strains was greater than that of the corresponding culture supernatants, which indicated that there were active VOC in the crude volatile extracts (Table 2).

GC/MS analysis indicated that a total of 229 peaks were obtained from the crude volatile extracts of *P. violaceinigra* G5-3 and *N. pokkalii* G8-2 and nutrient broth medium, of which 57 peaks possessed a SI value greater than 90. The GC column produced 7 VOC and nutrient broth contributed to 26 VOC. The remaining 24 VOC were only found in the crude volatile extracts of culture supernantants, among which, 18 compounds were produced by *P. violaceinigra* G5-3 and 6 compounds were originated from *N. pokkalii* G8-2. These VOC included alkyls,

Table 3. Six bacterial VOC from crude volatile extracts. Samples M1 to M5 were from strain G5-2. Sample M6 was obtained from strain G8-2.

Sample	VOC	Total peak area (%)	Retention time (min)
M1	2,5-Dimethyl pyrazine	2.67	9.682
M2	4-Dimethylaminopyridine	1.45	15.192
M3	Benzyl acetate	6.31	16.397
M4	Phenethyl butyrate	2.28	18.209
M5	Phenethyl alcohol	13.74	18.955
M6	Acetophenone	4.06	15.048

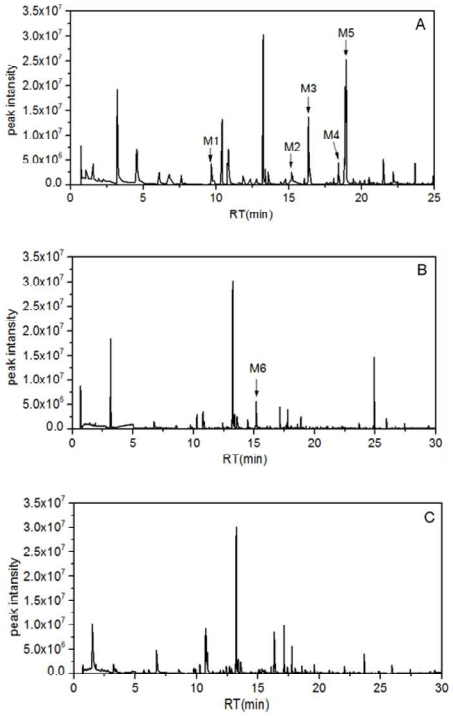

Figure 4. GC profiles of bacterial VOCs identified from crude volatile extracts. (A) G5-3 (B) G8-2 and (C) nutrient broth volatile extracts analyzed by gas chromatography. M1 to M6 labels were identified in Table 3.

alcohols, aldehydes, ketones, alkenes, alkynes, esters, acids, ethers and heterocyclics. Six VOC with a peak area greater than 1% was selected for further examination. These VOC included 2,5-dimethyl pyrazine, 4-dimethylaminopyridine, benzyl acetate, phenethyl butyrate and phenylethyl alcohol from *P. violaceinigra* G5-3 and acetophenone from *N. pokkalii* G8-2 (Table 3, Figure 4).

Table 4. LC_{90} values of commercial orthologs of VOCs for *B. xylophilus*. Corrected mortalities were reported as mean ± SD with n=4. $P < 0.05$ was defined as statistically significant.

Compound	LC_{90}(mM)	Confidence limit (95%)	X^2 test
2,5-Dimethyl pyrazine	33.72	32.58-34.64	1.48
4-Dimethylaminopyridine	1.83	1.66-2.16	5.71
Benzyl acetate	0.92	0.85-1.01	2.77
Phenethyl butyrate	0.20	0.16-0.24	1.50
Phenethyl alcohol	13.22	12.23-14.01	3.19
Acetophenone	10.11	9.26-10.77	1.43

3.5 Nematicidal Activity Testing of Pure Compounds

Six commercial compounds corresponding to the selected VOC were used to determine the concentration required for 90% mortality of nematodes in 24 h. Bioassay indicated that the LC_{90} values of the 6 VOC ranged from 0.20 to 33.72 mM. The VOC varied in their nematicidal activities against PWN, of which phenethyl butyrate showed the best nematicidal activity with LC_{90} of 0.20 mM (Table 4).

4. CONCLUSIONS

In this study, two Gram-negative bacteria *P. violaceinigra* G5-3 and *N. pokkalii* G8-2 with nematicidal activity were isolated and identified. This is the first report of nematicidal activity associated with these bacteria. VOC from the culture supernatants of the two bacterial strains also showed nematicidal activity and were analyzed by GC/MS. Six VOC were bioassayed for their nematicidal activity using commercial products, and phenethyl butyrate from *P. violaceinigra* G5-3 was found to be the best nematicidal activity with LC_{90} of 0.20 mM.

The species of genera *Pseudoduganella* and *Novosphingobium* are common in the environment. *P. violaceinigra* was also sometimes named after *Duganella violaceinigra* [24], and this strain was found to have an antibacterial activity against multidrug resistant *Staphylococcus aureus* [25]. A strain of *N. pokkalii* was reported to have some plant growth-promoting functions [26]. Our results in this paper indicated that both *P. violaceinigra* G5-3 and *N. pokkalii* G8-2 produced nematicidal VOC, of which 4-dimethylaminopyridine, benzyl acetate and phenethyl butyrate possessed relatively strong nematicidal activity with LC_{90} less than 2.0 mM in 24 h. These compounds are potential candidates for a conventional resource to carry out forest testing for pine wilt disease control. Besides, we also found that 20 of 200 bacterial strains showed nematicidal activity, which indicated that bacteria might be a good biological resource to obtain nematicides.

At present, research on nematicidal substances from plants and microbial metabolites has focused on nonvolatile substances and there are few reports of nematicidal activity of VOC. However, volatiles play a vital role in the recognition between entomopathogenic nematodes and their hosts [15]. Nematode control would be more efficient using multiple compounds and resistance selection would be less likely than for a single compound [27]. Our bioassay results indicated that *P. violaceinigra* G5-3 could produce several violate compounds with nematicidal activity, which makes this strain a promising microorganism in the biocontrol of pine wilt disease. The investigation of active volatile substances derived from bacteria can provide a theoretical basis for developing new types of highly effective nematicides.

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REFERENCES

[1] Zhao B.G. and Li R.G., The Roles of Bacteria Associated with the Pine Wood Nematode in Pathogenicity and Toxin - Production related to Pine Wilt; in Futai K., Sutherland J.R. and Takeuchi Y., eds., *Pine Wilt Disease*, Tokyo, Japan, Springer, 2008: 250-259.

[2] Mota M.M., Braasch H., Penas A.C., Burgermeister W., Metge k., Sousa E. and Bravo M.A., *Nematology*, 1999; **1**: 727-734. DOI 10.1163/156854199508757.

[3] Meng Q.H., Shi X.X., Meng F., Feng X. and Sun J.H., *Biotechnol. Lett*., 2012; **34**: 1847. DOI 10.1007/s10529-012-0967-7.

[4] Abelleira A., Picoaga A., Mansilla J.P. and Aguin O., *Plant Dis*., 2011; **99**: 776-776. DOI 10.1094/PDIS-12-10-0902.

[5] Lee B.H., Yang J.O., Beckett S. and Ren Y., *Pest Manag. Sci*., 2017; **73**: 7. DOI 10.1002/ps.4476.

[6] Nadel R.L., Wingfield M.J., Scholes M.C., Lawson S.A. and Slippers B., *Ann. For. Sci*., 2012; **69**: 1-11. DOI 10.1007/s13595-012-0200-9.

[7] Kwon T.S., Shin J.H., Lim J.H., Kim Y.K. and Lee E.J., *For. Ecol. Manag*., 2011; **261**: 562-9. DOI 10.1016/j.foreco.2010.11.008.

[8] Tian B., Yang J. and Zhang K.Q., *FEMS Microbiol. Ecol*., 2007; **61**: 197-213. DOI 10.1111/j.1574-6941.2007.00349.x.

[9] Lee J.H., Ma K.C., Ko S.J., Kang B.R., Kim I.S. and Kim Y.C., *Curr. Microbiol*., 2011; **62**: 746. DOI 10.1007/s00284-010-9779-y.

[10] Ju Y., Wang X., Guan T., Peng D. and Li H., *Int. J. Parasitol*., 2016; **46**: 819-828. DOI 10.1016/j.ijpara.2016.08.001.

[11] Zhu Y.H., Dong J.Y., Le W., Wei Z., Lei L., He H. and Zhang K., *Ann. Microbiol*., 2008; **58**: 375-80. DOI 10.1007/BF03175531.

[12] Liu Y.J., Zhai C.Y., Yi. L. and Zhang K.Q., *J. Microbiol*., 2009; **47**: 248-52. DOI 10.1007/s12275-009-0012-2.

[13] Cao Y., Tian B., Ji X., Shang S., Lu C. and Zhang K., *J. Basic Microbiol*., 2015; **55**: 950-60. DOI 10.1002/jobm.201400816.

[14] Wang Z., Wang C.Y., Fang Z.M., Zhang D.L., Gu L.J., Mi R.L., Liu L., Chang K.S., Li Z. and Mo E.K., *Biocontrol*,

2011; **56**: 91-100. DOI 10.1007/s 00284-010-9731-1.

[15] Lewis E.E., Campbell J., Griffin C., Kaya H. and Peters A., *Biol. Control*, 2006; **38**: 66-79. DOI 10.1016/j.biocontrol. 2005.11.007.

[16] Huang Y., Xu C.K., Ma L., Zhang K.Q., Duan C.Q. and Mo M.H., *Eur. J. Plant Pathol.*, 2010; **126**: 417-22. DOI 10.1007/ s10658-009-9550-z.

[17] Yu J., Li R.G., Chen C.C., Du G.C., Li L., Zhou C.J., Guo D.S. and Li Z., *Nematology*, 2015; **17**: 9. DOI 10.1163/ 15685411-00002920.

[18] Viglierchio D.R. and Schmitt R.V., *J. Nematol.*, 1983; **15**: 438-44.

[19] Wang J.Y., Pan X., Yi H., Guo D.S., Guo Q.Q. and Li R.G., *Mar. Drugs*, 2012; **10**: 2729-40. DOI 10.3390/md 10122729.

[20] Kim O.S., Cho Y.J., Lee K., Yoon S.H., Kim M., Na H., Park S.C., Jeon Y.S., Lee J.H. and Yi H., *Int. J. Syst. Evol. Microbiol.*, 2012; **62**: 716. DOI 10.1099/ ijs.0.038075-0.

[21] Kumar S., Stecher G. and Tamura K., *Mol. Biol. Evol.*, 2016; **33**: 1870. DOI 10.1093/molbev/msw054.

[22] Zeng D., Chen B.S. and Ying J., *Forensic Sci. Int.*, 2006; **159**: 168. DOI 10.1016/ j.forsciint.2005.07.013.

[23] Finney D.J., *J. Royal Stat. Soc.*, 1971; **21**: 57-57.

[24] Kampfer P., Wellner S., Lohse K., Martin K. and Lodders N., *Syst. Appl. Microbiol.*, 2012; **35**: 19-23. DOI 10.1016/ j.syapm.2011.10.003.

[25] Choi S.Y., Kim S., Lyuck S., Kim S.B. and Mitchell R.J., *Sci. Rep.*, 2015; **5**: 155 98. DOI 10.1038/srep15598.

[26] Krishnan R., Menon R.R., Likhitha, Busse H.J., Tanaka N., Krishnamurthi S. and Rameshkumar N., *Res. Microbiol.*, 2016; **168**: 113-121. DOI 10.1016/ j.resmic.2016.09.001.

[27] Gu Y.Q., Mo M.H., Zhou J.P., Zou C.S. and Zhang K.Q., *Soil Biol. Biochem.*, 2007; **39**: 2567-75. DOI 10.1016/j.soilbio. 2007.05.011.