BIODIVERSITAS
Volume 21, Number 6, June 2020
Pages: 2727-2734

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d210648

Potential antagonistic rhizobacteria to control *Colletotrichum scovillei*, the cause of anthracnose disease in chili pepper

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Manuscript received: 5 April 2020. Revision accepted: 24 May 2020.

Abstract. *Darmadi AAK, Suprapta DN, Khalimi K. 2020. Potential antagonistic rhizobacteria to control* Colletotrichum scovillei, *the cause of anthracnose disease in chili pepper. Biodiversitas 21: 2727-2734.* Six species of *Colletotrichum* were identified as the cause of anthracnose disease on Bali Island, Indonesia in 2018. These species were *C. scovillei*, *C. acutatum*, *C. nymphaeae*, *C. gloesporioides*, *C. truncatum*, and *C. fructicola*. Among them, *C. scovillei* was the most prevalent at 55% of all samples tested. This study was conducted to find potential antagonistic rhizobacteria isolated from various rhizospheres of plants grown in Bali. A total of 1,040 rhizobacteria isolates were tested for their antagonistic activity against the growth of *C. scovillei* on potato dextrose agar. Results showed that 10 isolates inhibited the growth of *C. scovillei* by more than 80%. Among these isolates C1 and C7B possessed inhibitory activity at 94.9% and 94.3%, respectively. Molecular identification based on analysis of 16S rRNA gene showed that isolate C1 belonged to the species *Paenibacillus polymyxa*, whereas isolate C7B was identified as *Bacillus siamensis*. According to scanning electron serious damage on mycelia of *C. scovillei* was observed. Wrinkles were observed on mycelia of *C. scovillei* grown jointly with rhizobacterial isolate C1, whereas no wrinkle was observed on *C. scovillei* grown solely. Three compounds were detected in the hexane phase of cell-free filtrate *P. polymyxa* C1, namely, 3-hydroxy-2-butanone and 2,3-butanediol. These compounds may be responsible for antifungal activity against *C. scovillei*.

Keywords: Antagonistic rhizobacteria, anthracnose disease, *Colletotrichum scovillei*

INTRODUCTION

Anthracnose disease caused by *Colletotrichum* spp. was reported to be a serious problem in chili cultivation on Bali Island, Indonesia, with disease incidence of 84 % and disease intensity in the range of 22%-78% (Khalimi et al. 2019). Six species of *Colletotrichum*, namely *C. scovillei*, *C. acutatum*, *C. nymphaeae*, *C. gloesporioides*, *C. truncatum*, and *C. fructicola* were identified as the cause of the disease. Among them, *C. scovillei* was the most prevalent at55% (Khalimi et al. 2019). The disease potentially causes 10%-80% of yield losses depending on cultivation area (Asare-Bediako et al. 2015; Diao et al. 2017; Poonpolgul and Kumphai 2007).

Development of resistant chili cultivar is expected to effectively control the disease, but the resistance against *Colletotrichum* spp. is often broken down under field conditions (Park 2007). Synthetic fungicides are generally used by farmers to control anthracnose disease, but this measure is not always effective to reduce the disease incidence. Furthermore, the use of synthetic pesticides may have adverse effects on the environment and on the development of resistance against fungicide. This phenomenon was reported by Soesanto (2008), who found out that disease incidence caused by *Pythium*, *Fusarium*, and *Phytophthora* occurred as a result of the use of pentachlorobenzene.

Owing to the increased preference of consumers for organic agricultural products in Indonesia, the organic farming system has become popular among farmers and consumers (Shiotsu et al. 2015). In organic farming bio-control agents are necessary to manage plant fungal diseases. The use of bio-agents may reduce the use of synthetic chemical fungicides, thereby minimizing adverse effects on the environment. Several bio-agents have been found and reported to possess inhibitory activities against plant pathogens such as *Piriformospora indica*, *Trichoderma viride*, *Acremonium lolii*, and *C. lindemuthianum* against leaf rust of wheat caused by *Puccinia recondite* (Anwaar et al. 2019). *Bacillus cereus* SSB1 effectively controlled *Pectobacterium* infection in potato (Sarfraz et al. 2019), *B. amyloliquesfaciens* BA-16-8 inhibited the growth of *Penicillium expansum*, the cause of postharvest decay in apple (Fu et al. 2020).

Among bio-agents that can potentially be used for plant fungal disease control are rhizobacteria, which can colonize plant roots and provide beneficial effects through growth promotion (Saharan and Nehra 2011). *Enterobacter cloacae* KtB3 isolated from the rhizosphere of groundnut was proven to effectively control damping-off disease on soybean caused by *Sclerotium rolfsii* (Mahartha and Suprapta 2018). Rhizobacteria are able to produce phytohormones, such as indole acetic acid, ACC deaminase, to fix atmospheric nitrogen, act as antagonistic microbes against plant pathogen through the production of siderophores, β-1,3-glucanase, chitinase, cellulase, and antibiotics, and are able to dissolve phosphate and other nutrients in the soil (Guo et al. 2015; Saleem et al. 2015;

Soesanto 2008). For example, *Pseudomonas fluorescens* produced 2,4-*diacetyl phloroglucinol* that effectively suppresses plant fungal pathogens (Nowak-Thompson et al. 1994). *P. stutzeri* produced extracellular chitinase enzyme and laminarinase that can decompose the mycelia of *Fusarium solani* (Mauch et al. 1988).

Based on the fore mentioned considerations, this study was conducted to find potential antagonistic rhizobacteria that can be used as bio-control agents against anthracnose disease on chili pepper in Bali, Indonesia.

MATERIALS AND METHODS

Isolation of rhizobacteria

Isolation of rhizobacteria was conducted from rhizospheres of plants of the *Solanaceae*, *Graminae*, and *Leguminosae* families. First, 10 g of soil and root of plants from rhizospheres were obtained and diluted in 100 mL phosphate-buffered saline (PBS). Second, a series of dilutions (until 10^{-4}) was performed using PBS buffer. Cultural medium used for isolation was nutrient agar (NA) medium containing 0.3% beef extract, 0.5% peptone, 1.5% Bacto agar, and distilled water to make 1 L. The medium was added with Nystatin (20 mg l^{-1}) to suppress the growth of fungi. The rhizobacteria colony that appeared from the medium was placed in the NA medium using a wire loop to obtain a single colony. This single colony was then transferred onto slanted NA medium before it was used for further tests.

Selection of rhizobacteria with antifungal activity

All 540 isolates of rhizobacteria were tested for their antifungal activity against *Colletotrichum* spp. A colony of rhizobacteria isolates and *Colletotrichum* was grown side by side at a distance of 2 cm on potato dextrose agar (PDA) medium in a Petri dish (Pracahyo et al. 2014). All cultures were incubated in the dark for 72 h under room temperature. In that distance, the *Colletotrichum* has grown abnormally or inhibition zone was formed, indicating that the rhizobacterial isolate possessed antifungal activity. By contrast, the absence of an inhibition zone indicated no antifungal activity.

Test of antifungal activity level

Ten isolates of rhizobacteria that showed obvious antifungal activity against *C. scovillei* were tested for the level of their antifungal activity against *C. scovillei.* A method developed by Parwati et al. (2014) was applied in this test. A mycelial plug of *C. scovillei* was placed in the center of the PDA medium on a Petri dish, and then each isolate of rhizobacteria was inoculated on four sides of the fungal colony at 2 cm distance. For control, a fungal colony without rhizobacteria inoculation was prepared. Ten Petri dishes were prepared for each isolate and control. The cultures were incubated in the dark at a temperature of 28 ± 2ºC. The size of the fungal colony was measured on the third day of incubation using millimeter block paper. Antifungal activity was calculated according to the following formula:

$$AI = \frac{C-T}{C} \times 100\%,$$

Where:
AI : antifungal activity (%)
C : size of fungal colony on control
T : size of fungal colony with rhizobacteria treatment

Evaluation of cultural filtrate for antifungal activity

The antifungal activity of cultural filtrate of isolate C1 and C7B against *C. scovillei* was evaluated based on a method developed by Parwati et al. (2014) and Ambaradewi (2012). Each isolate of C1 and C7B was cultured in potato dextrose broth (PDB) medium. A 300 mL PDB medium was placed in a 500-mL Erlenmeyer flask and sterilized in an autoclave for 20 min. The 1 mL each of either C1 and C7B suspension (containing 10^7 CFU mL^{-1}) was inoculated into the PDB medium and incubated in an orbital shaker at 200 rpm for a month. This culture was then subjected to centrifugation at 1000 xg for 15 min. The pellet was discarded and the supernatant was collected and passed through a Millipore membrane with pore size of 0.45 µm (Yonezawa Ltd., Japan).

Thereafter, 10 mL of melted PDA medium was placed in a Petri dish and mixed with 0.5 mL of spore's suspension of *C. scovillei* (containing 10^6 spores/mL). After the culture solidified, two diffusion wells were made for every Petri dish using a cork borer (with diameter of 5 mm). Into each well, 20 µl cell-free filtrate was added; one for cell-free filtrate of isolate C1 and another for isolate C7B. Sterile PDB broth at the same volume was added into the control wells. The 10 Petri dishes were prepared each for cell-free filtrate treatment and control. The cultures were incubated in the dark at room temperature (28± 2 ºC). The diameter of inhibition zone around the diffusion well was measured.

Identification of rhizobacteria isolates based on 16S rRNA analysis

Rhizobacteria isolates were grown in Erlenmeyer flasks containing Tryptic Soy broth media (17 g of Tryptone, 3 g of Phytone, 5 g of NaCl, 2.5 g of K2HPO4, and 2.5 g of glucose, filled up to 1,000 mL), incubated for 16 h, and shaken at a speed of 5 x g at room temperature. Then the rhizobacterial cells were placed in a 2 mL Eppendorf tube and centrifuged at a speed of 5.000 x g for 10 min. The supernatant was discarded. Rhizobacteria cells were re-suspended with 180 µl digestion solution, added with 20 µl proteinase solution, and mixed until homogeneous by raising the pipette or vortex and incubating at 56º C until the tissue was completely lysed (clear liquid was formed). Thereafter, 20 mL of RNase A solution was added, then vortexed and incubated for 10 min at room temperature. This mixture was added with 200 µl lysis solution, and vortexed for 15 s until the mixture became homogeneous. This mixture was added with 400 µl of 50% ethanol and vortexed. Then, the mixture was transferred to the genomic DNA purification column, which was placed on the collection tube. The column was centrifuged for a minute at 6,000 x g. The collection tube containing the solution was discarded, and the column was placed in the new collection tube and added with 500 µl wash buffer I (into which

ethanol had been added). This mixture was centrifuged for a minute at 8,000 g, the liquid was disposed into the collection tube, and the column was placed back into the collection tube. The mixture was added with 500 µl wash buffer II (into which ethanol had been added) into the column and centrifuged for 3 min at 12,000 x g. If solution residue was left in the column, the collection tube was emptied and the column was centrifuged for a minute at 12,000 x g. The collection tube containing the solution and column was moved to a new 1.5 mL sterile microtube. Then, 200 µl of the elution buffer was added into the middle of the column to dissolve the DNA. This column was incubated for 2 min at room temperature and centrifuged for 1 min at 8,000 x g. Then, the column was discarded. Pure DNA was stored at -20 ºC until it was used for further analysis.

Amplification of DNA by PCR

The 16S rRNA gene was amplified by PCR using 16S primary pairs (63F 5'-CAG GCC TAA CAC ATG CAA GTC-3 'and 1387R (5'-GGG CGG WGT GTA CAA GGC-3'). The reaction was conducted by using a SensoQuest Labcycler with 2x Kapa PCR Ready Mix (Kapa Biosystems, USA), in conditions of 94°C for min, followed by 30 consecutive cycles at 94°C for 30 s, 55°C for 45 s, 72°C for 2 min, and 72°C for 10 min.

16S rRNA gene sequencing and DNA sequence analysis

Nucleotide sequences were determined using an ABI Prism 3100 Avant Genetic Analyzer. Then DNA sequences of the results were trimmed and assembled by using ChromasPro version 1.5. The assembled data were then processed BLAST with data registered with the National Center for Biotechnology Information (NCNI) through the website http://www.ncbi.nlm.nih.gov/BLAST. Some of the BLAST homologous sequence data, which are the closest species, were taken from the GenBank data of NCBI. Then, the data were analyzed again by aligning the sequence using MEGA version 6.0. Furthermore, the data were examined with PAUP 4.0b using maximum parsimony method with 1,000 replication bootstrap. Then, at phylogenetic tree was designed using Tree Graph 2.0 (Stover and Muller 2010).

Ultra-structural responses of *C. scovillei*

Ultra-structural responses of *C. scovillei* against treatment of cell-free filtrate of *Paenibacillus polymyxa* C1 were examined through scanning electron microscopy (SEM) according to a method developed by Kawuri et al. (2018) with slight modification. Then, 1 mL cell-free filtrate of *P. polymyxa* C1 was placed in Petri dish and then added with 9 mL melted PDA medium and shaken horizontally to mix the filtrate evenly with PDA. A mycelial plug taken from the edge of *C. scovillei* colony (with diameter of 5 mm) was placed in the middle of a Petri dish and then incubated in the dark at 25 ºC for 3 d. Fungal colony on the colony edge was cut into 3 mm and 1 mm pieces, followed by the sample preparation process for SEM (JSM-6701F, JEOL, Japan) using an acceleration voltage of 5 kV.

GC-MS analysis

Identification of active compounds in the cell-free filtrate of *P. polymyxa* C1 was performed using gas chromatography-mass spectroscopy (GC-MS) according to a method developed by Khalimi (2017) with modification. A 50-mL cell-free cultural filtrate of *P. polymyxa* C1 was added with 50 mL hexane (PA grade), mixed thoroughly, and placed in a separating funnel. The water and hexane phases were collected separately and the hexane phase was evaporated in a vacuum rotary evaporator (Iwaki, Japan) until the volume was approximately 10 mL. This hexane phase proceeded for GC-MS analysis (QP 2010 Shimadzu, Japan). Liquid nitrogen was used as elution solvent with flow rate of 1 mL/min on a Wakosil ODS/5c18-200 column under temperature of 250 ºC. Then, UV light at a length of 254 nm was used for detection and matched with identified compounds in the GC-MS library.

RESULTS AND DISCUSSION

Isolates with antifungal activity against *C. scovillei*

A total of 540 isolates of rhizobacteria were obtained in this study and tested for their antifungal activity against *C. scovillei*. Then, 10 isolates of rhizobacteria showed obvious antifungal activity against *C. scovillei* as shown in Table 1. All 10 isolates of rhizobacteria showed over 80% inhibitory activity and among them, the C1 and C7B isolates exhibited antifungal activity at 94.9% and 94.3%, respectively. Based on this result, these two isolates were used for further tests.

Antifungal activity of cell-free filtrate

Clear zones (inhibition zone) around the wells filled with cell-free filtrates of isolates C1 and C7B were formed as shown in Fig. 1. The average diameter of the inhibition zones was 18.7 $\pm$ 1.1 mm and 17.4 $\pm$ 1.3 mm, respectively, for isolates C1 and C7B as presented in Table 2. This result revealed that both isolates produced antifungal substances that inhibited the growth of *C. scovillei*.

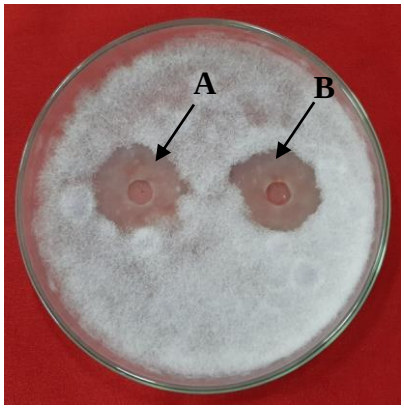

Figure 1. Inhibition zones formed around the wells filled with cell-free filtrates of (A) isolate C1 and (B) C7B on PDA medium inoculated with *Colletotrichum scovillei.* Arrows indicate the inhibition zones

Species of rhizobacteria

Based on analysis of the genes of 16S rRNA, the sequence of isolate C1 is as follows:

CGGCGGCTGGCTCCTTGCGGTTACCTCACCGACTTCGGGTGTTGTAAACTCTCGTGGTGT
GACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTA
CTAGCAATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGACCGGCTT
TTCTAGGATTGGCTCCACATCGCGGCTTCGCTTCCCGTTGTACCGGCCATTGTAGTACGT
GTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTT
GTCACCGGCAGTCTGCTTAGAGTGCCCAGCTTGACCTGCTGGCAACTAAGCATAAGGGTT
GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACC
ACCTGTCTCCTCTGTCCCGAAGGAAAGGYCTATCTCTAGACCGGTCAGAGGGATGTCAAG
ACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATACTCCACTGCTTGTGCGGG
TCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAATGCTTAA
TGTGTTAACTTCGGCACCAAGGGTATCGAAACCCCTAACACCTAGCATTCATCGTTTACG
GCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCA
GTTACAGCCCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCAC
CGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGCTCCCCAGTTTCCAGTGCGA
CCCGAAGTTGAGCCTCGGGATTAAACACCAGACTTAAAGAGCCGCCTGCGCGCGCTTTAC
GCCCAATAATTCCGGACAACGCTTGCCCCCTACGTATTACCGCGGCTGCTGGCACGTAGT
TAGCCGGGGCTTTCTTCTCAGGTACCGTCACTCCTATAGCAGTTACTCTACAAGACGTTC
TTCCCTGGCAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCG
TCAGGCTTTCGCCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCG
TGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGT
AGGCCTTTACCCCACCAACTAGCTAATGCGCCGCAGGCCCATCCACAAGTGACAGATTGC
TCCGCCTTTCCTCCTTCTCCCATGCAGGAAAAGGATGTATCGGGTATTAGCTACCGTTTC
CGGTAGTTATCCCTGTCTTGTGGGCAGGTTGCCTACGTGTTACTCACCCGTCCGCCGCTA
GGTTAATTAGAAGCAAGCTTC

Analytical result of 16S rRNA gene of isolate C7B is as follows:

GGCGGCTGGCTCCATAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGT
GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTA
CTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATT
TGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTG
TGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTG
TCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCG
CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACC
TGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACC
TGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC
CCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGC
GTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCG
TGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTT
ACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGC
TACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTC
CCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGC
CCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTA
GCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCGCCCTATTTGAACGGCACTTGTTCT
TCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGT
CAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGT
GTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTG
AGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAG
CCACCTTTTATGTCTGAACCATGCGGTTCAGACAACCATCCGGTATTAGCCCCGGTTTCC
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CATCAGGGAGCAAGCTCCCATCTGTCCGCTCGA

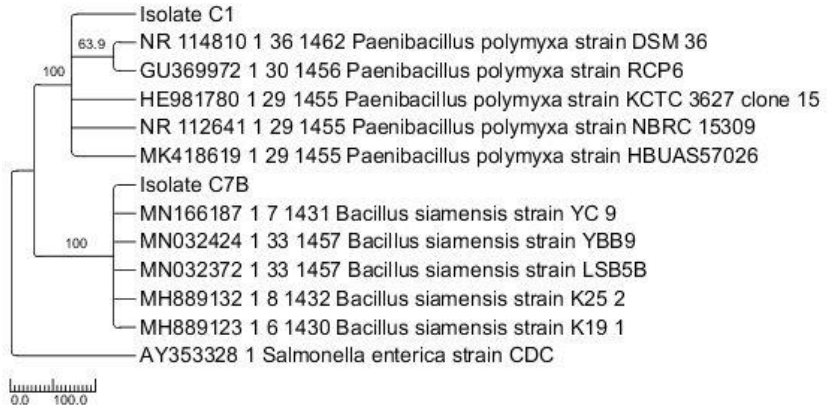

Figure 2. Phylogenetic relationship tree of isolates C1 and C7B with 14 isolates of bacteria based on 16S rRNA gene using maximum parsimony method

Table 1. Level of antifungal activity of 10 isolates of rhizobacteria against *Colletotrichum scovillei*

Name of isolate	Sampling sites	Antifungal activity against *C. scovillei* (%) ± deviation standard
ORPg7	Pegok, Denpasar	83.2 ± 3.7
GbBd35	Ungasan, Badung	83.2 ± 2.9
GbBd68	Ungasan, Badung	81.2 ± 4.3
SuBl45	Suter, Bangli	80.6 ± 4.7
Cl	Kedisan, Bangli	94.9 ± 3.8*
GgSg13	Gerokgak, Buleleng	80.2 ± 3.5
KsSg12	Kalisada, Buleleng	84.6 ± 5.7
MjSg42	Menjangan, Buleleng	81.2 ± 4.8
C7B	TNBB, Jembrana	94.3 ± 4.2*
TNJb36	TNBB, Jembrana	80.6 ± 3.2

Note: * These isolates were processed for identification

Table 2. Average size of inhibition zones formed around the wells filled with cell-free filtrates of isolate C1 and C7B.

Isolate	Diameter of inhibition zones (mm) ± standard deviation
C1	18.7 ± 1.1*
C7B	17.4 ± 1.3

Note: * Average of 10 replicates

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Based on the alignment of 16S rRNA gene with database in GenBank using BlastN program, isolate Cl belongs to the *P. polymyxa* group because this isolate is homologous with bacteria in the GenBank data-base with the maximum percentage of similarity by 99.71%, as shown in Table 3 and Fig. 2. For isolate C7B which belongs to the *Bacillus siamensis* group because this isolate is homologous with bacteria in the GenBank database with 100% maximum percentage of similarity, as shown in Table 4 and Fig. 2.

Ultra-structural response of *S. scovillei*

Scanning electron microscopy showed that serious damage on the mycelia of *C. scovillei* was observed. Wrinkles were observed on the mycelia of *C. scovillei* grown side by side with *P. polymyxa* C1, whereas no such wrinkle was observed on *C. scovillei* grown solely, as exhibited in Fig. 3.

Compounds in cell-free filtrate of *P. polymyxa*

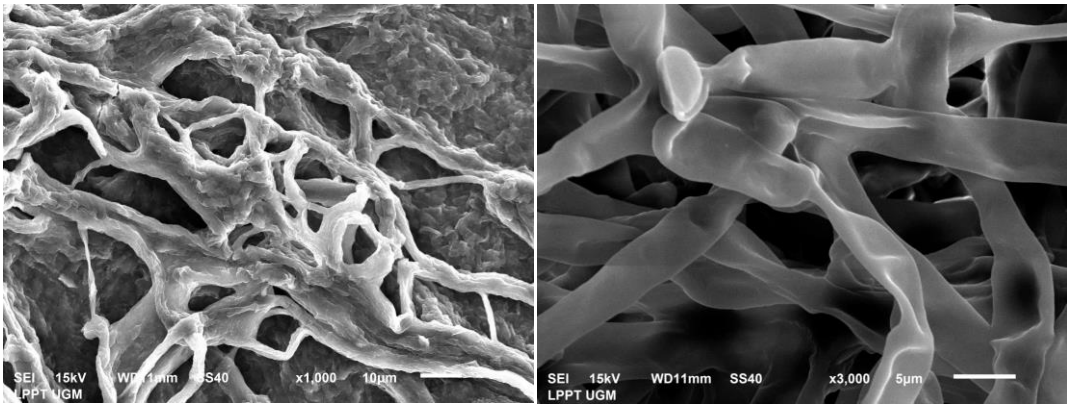
Based on analysis using gas chromatography-mass spectroscopy (GC-MS), three peaks were detected in the cell-free filtrate of *P. polymyxa* C1 with retention times of 2.260, 2.925, and 3.077, respectively for peaks 1, 2, and 3 (Fig. 4). These compounds were identified as butanediol with peak area of 3.44% (peak 3), as presented in Table 5.

Table 3. Similarity of 16S rRNA gene of isolate C1 with sequences of DNA in GenBank using BlastN program

Species of bacteria in GenBank	Percentage of similarity	Accession number
P. polymyxa strain JSa-9 16S ribosomal gene partial sequence	99.71	EU882855.1
P.polymyxa partial 16S rRNA gene strain KCTC3627	99.64	HE981790.1
P.polymyxa isolate TN76 16S rRNA gene partial sequence	99.64	EU362604.1
P. polymyxa strain DSM 36 16S rRNA partial gene sequence	99.64	NR 114810.1
P. polymyxa strain RCP6 16S rRNA gene partial sequence	99.57	GU369972.1
P. polymyxa strain NRBC 15309 16S rRNA gene partial sequence	99.50	NR 112641.1
P. polymyxa strain IAM 13419 16 S rRNA gene partial sequence	99.50	NR 112117.1

Table 4. Similarity of 16S rRNA gene of isolate C7B with sequences of DNA in GenBank using BlastN program

Species of bacteria in GenBank	Percentage of similarity	Accession number
B. siamensis strain YC-9 16S ribosomal RNA gene partial sequence	100	MN166187.1
B. siamensis strain YBB9 16S ribosomal RNA gene partial sequence	100	MN042424.1
B. siamensis strain LSB5B 16S ribosomal RNA gene partial sequence	100	MN032372.1
B. siamensis strain LSB5A 16S ribosomal RNA gene partial sequence	100	MN032371.1
B. siamensis strain NSB5 16S ribosomal RNA gene partial sequence	100	MN032365.1



A **B**

Figure 3. Scanning electron micrographs of mycelia of *Colletotrichum scovillei* treated with *Paenibacillus polymyxa* C1 (A) and control (B)

Table 5. Compounds detected in cell-free filtrate of *Paenibacillus polymyxa* C1

Peak number	Retention time (minutes)	Name of compound	Peak area (%)
1	2.260	3-hydroxy-2-butanone	5.62
2	2.925	2,3-butanediol	23.51
3	3.077	2,3-butanediol	3.44

Discussion

In our present study, we found two isolates of local rhizobacteria, namely, *P. polymyxa* C1 and *B. siamensis* C7B, which showed strong antifungal activity against *C. scovillei*, the prominent cause of anthracnose disease on chili pepper in Bali, Indonesia. Both of these isolates inhibited the growth of *C. scovillei* on the PDA medium.

Several species of microbes have been tested and showed antifungal activities against plant fungal pathogens, and some of them have been successfully controlled plant fungal diseases of important agricultural crops (Adame-Garcia et al. 2016; Guo et al. 2015; Hudge 2015; Parwati et al. 2014; Suprapta 2012; Suprapta et al. 2014a; Widnyana et al. 2013).

Antagonism between rhizobacteria and plant fungal pathogens may have happened through antibiosis, parasitism, predation, competition, production of extracellular enzymes, and induced resistance (Zhang 2004). *E. cloacae* EcCT -501 was reported to have effectively suppressed the damping-off disease on cucumber through the production of siderophore hydroxamate, aerobactin, and catechol (Costa and Loper 1993). *E. cloacae* subsp. *cloacae* ENHKU01 was also reported to possess antagonistic activity against *Colletotrichum capsici*, *Sclerotinia sclerotiorum*, *Alternaria* sp., *Didymella bryoniae*, and *Fusarium oxysporum* under *in vitro* condition by producing chitinase enzyme, siderophore aerobactin, and enterobactin (Liu et al. 2013). *Vibrio* sp. R-10 produced siderophore amphibactin that acts as an antifungal substance (Martinez et al. 2003). *Burkholderia* sp. strain MSSP produced hydroxymethyl-chroman-4-one that acts as an antifungal against *Pythium ultimum*, *Phytophthora capsici*, and *Sclerotinia sclerotiorum* (Kang et al. 2004).

Another study showed that *E. agglomerans* Al7Kla effectively reduced the intensity of blast disease on rice caused by *Pyricularia oryzae* (Suprapta et al. 2014a), whereas *E. cloacae* Gg14D was reported to be plant-growth-promoting rhizobacteria, without antifungal activity (Suprapta et al. 2014b).

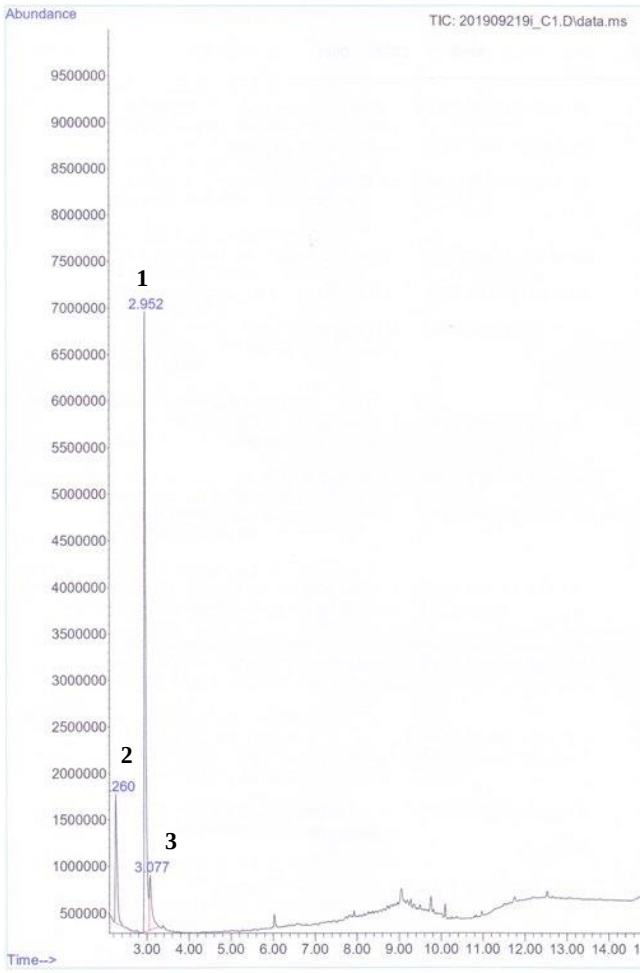


Figure 4. Chromatograms of compounds produced by *Paenibacillus polymyxa* C1 using GC-MS

In the present study, we detected three compounds in the cell-free filtrate of *P. polymixa* C1, namely, 3-hydroxy-2-butanone; 2,3-butanediol, and 2,3-butanediol. These compounds may be responsible for the antifungal activity of *P. polymyxa* C1 against *C. scovillei*. Total peak areas of 2,3-Butanediol (compound numbers 2 and 3 in Fig. 4 and Table 5) was 26.95%, whereas that for 3-hydroxy-2-butanone (compound 1) was only 5.62%. However, further study is needed to identify the compound that contributed dominantly to antifungal activity. Other researchers proved that 3-hydroxy-2-butanone is one of the volatile compounds with antifungal activity (Lim et al. 2017). Arrebola et al. (2010) also proved that 3-hydroxy-2-butanone is one of the volatile compounds with antifungal activity against *Penicillium crustosum*. A compound 2,3-butanediol extracted from *P. polymyxa* culture was proven to induce systemic acquired resistance against *Phytophthora parasitica* var. *nicotianae* (Park et al. 2018).

In conclusion, two species of local rhizobacteria, namely, *P. polymyxa* C1 and *B. siamensis* C7B, were first reported to have effectively inhibited the growth of *C. scovillei*, the cause of anthracnose disease on chili pepper in Bali. The cell-free cultural filtrates of both isolates inhibited the growth of *C. Scovillei. P. polymyxa* C1 caused wrinkles on mycelia of *C. scovillei*. Two types of compounds were detected in the cell-free filtrate of *P. polymyxa* C1 namely 3-hydroxy-2-butanone and 2,3-butanediol. These compounds may be responsible for the antifungal activity against *C. scovillei*. *P. polymyxa* C1 can be considered as a potential bio-agent to control anthracnose disease on chili pepper in Bali. Further study and field tests are necessary to develop a *P. polymyxa* C1 formula that can be used in a green-house.

ACKNOWLEDGEMENTS

The authors grateful to the Institute of Research and Community Services, Udayana University, for providing the research grant in fiscal year 2019 under the Penelitian Percepatan Guru Besar scheme. High appreciations are sent to Dr. Fumitaka Shiotsu of Meiji University, Japan, and Dr. Azwan Mat Lazim of Universiti Kebangsaan Malaysia for valuable comments and suggestions to improve this paper.

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