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Antifungal activity of *Bacillus subtilis* subsp. *spizizenii* BL-59 to control some important postharvest diseases of mango fruits (*Mangifera indica* L.)

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Abstract The antifungal activity of antagonistic bacteria isolated from the rhizosphere soil of rice in Ban-Laem district, Phetchaburi province, Thailand was investigated. The fungal pathogens, *Colletotrichum* sp. and *Pestalotiopsis* sp. were isolated from the infected fruit, causing postharvest diseases in mango. The preliminary study was conducted using a dual culture assay to determine the antifungal activity of the BL-59 isolate. The dual culture assay showed that the antagonistic bacteria inhibited the mycelial growth of *Colletotrichum* sp. and *Pestalotiopsis* sp. by 49.31% and 42.55%, respectively. Furthermore, this isolate BL-59 produced volatile organic compounds (VOCs), which inhibited the mycelial growth of *Colletotrichum* sp. by 60.00%. Microscopic observation of the hyphal morphology of *Colletotrichum* sp. revealed the presence of abnormal hyphal structure. Morphological and biochemical studies of antagonistic bacteria BL-59 demonstrated that this isolate was classified as gram-positive, rod-shaped, and endospore-forming, and it showed survival growth under salinity stress and high temperature (45 ℃). Moreover, this strain produced catalase and oxidase enzymes. BL-59 was identified as closely related to *Bacillus subtilis* subsp. *spizizenii* (99.79%) using molecular identification based on the 16S rRNA gene. This study revealed that antagonistic bacteria can be used as an alternative choice to control anthracnose disease by reducing the chemical residues in agricultural production.

Keywords: Antifungal activity, Biological control, Volatile compounds, Biochemical test, 16S rRNA

Introduction

Mango (*Mangifera indica* L.) is an important tropical and subtropical crop (Mukherjee, 1953). Mango production in many countries has serious postharvest diseases that cause economic losses due to damage to fruits after harvesting (Prusky *et al*., 2002). The fungal pathogens have been recorded as major pests that cause anthracnose disease and stem end rot disease (Agrios, 1997, Karunanayake and Adikaram, 2020). The fungus *Colletotrichum* sp. is

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known as a causal agent of anthracnose disease, which appears as irregular-shaped brown to black necrotic spots on the ripened fruit (Arauz, 2000, Dodd *et al*., 1991). The stem end rots (SER) disease is caused by various fungi such as *Neofusicoccum parvum* (formerly known as *Dothiorella domanicana*), *N. mangiferae* (formerly known as *Dothiorella mangiferae*), *Lasiodiplodia theobromae* (Syn. *Botryodiplodia theobromae*), *Phomopsis mangiferae*, *Pestalotiopsis mangiferae*, and *Cytosphaera mangiferae* (Hong *et al*., 2012, Johnson *et al.,* 1992, Muller and Burt, 1989). However, many varieties of tropical and sub-tropical fruits are affected by SER disease, such as mango (*Mangifera indica* L.), avocado (*Persea americana*), papaya (*Carica papaya*), citrus (*Citrus sinensis*), mangosteen (*Garcinia mangostana*), and rambutan (*Nephelium lappaceum* L.) (Karunanayake and Adikaram, 2020). To control diseases, chemical fungicides are primarily used to prevent and control outbreaks of pathogens. However, the extensive improper use also led to the development of fungicide-resistant isolates (Chung *et al.,* 2006; Valero *et al.* 2010). Moreover, the chemical fungicide residuals are a serious concern for human and animal health, and environmental contamination (Nicolopoulou-Stamati *et al*., 2016). Thus, pesticide-free agricultural products are currently considered as alternative choices for new customer trends. So, farmers are recognized for their agricultural management to escape this harmful situation and move to biological control (Alabouvette *et al*., 2009).

Biological control using microorganisms such as bacteria has become very popular in recent years all around the world. Plant-growth-promoting rhizobacteria (PGPR), which live in the rhizosphere, are significant habitation zones for plant beneficial bacteria (Schroth and Hancock, 1982). These bacteria have been reported as biocontrol agents that can perform antagonistic activities such as production of antibiotics, synthesis of hydrolytic enzymes, and competition for nutrients to colonize the root surface (Beattie, 2006, Kamilova *et al*., 2005, Maksimov *et al*., 2011). Among bacterial genera, *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Streptomyces* have been identified as predominant biocontrol agents (Podile and Kishore, 2006).

Many *Bacillus* species have been reported to be potential microorganisms for phytopathogenic disease control and produce plant growth-promoting substances (Turner and Backman, 1991). Reva *et al*. (2004) investigated the ability of *B. amyloliquefaciens* and *B. subtilis* to colonize plant roots, which release antibiotics and chemical signals against phytopathogens. Fungal antagonistic activity against postharvest pathogens of *Bacillus* sp. has been reported. For example, *Bacillus* sp. MB61 and *Bacillus* sp. LB72 isolated from mango leaves showed more than 60% mycelial inhibition against *C. gloeosporioides* (Rungjindamai, 2016). *Bacillus siamensis* S3 and *B. tequilensis*

S5 showed high fungal growth inhibition against *Pestalotiopsis versicolor* XJ27 isolated from bayberry (Ali *et al*., 2020). Moreover, *B. subtilis* CF-3 was recently reported to produce volatile organic compounds (VOCs) to control *C. gloeosporioides* (Wang *et al*., 2021). The exploration of the new fungal antagonist is still ongoing research which provides a chance to find new promising effective postharvest biocontrol agents for controlling mango fungal diseases.

Therefore, the present study was carried out to isolate the antagonistic rhizobacteria strains from paddy fields and investigated the potential antifungal activity against mango fungal pathogens, *Colletotrichum* sp. and *Pestalotiopsis* sp., causal agents of postharvest diseases on mango fruits. The mycelial growth inhibition activity was evaluated by a dual culture assay and a volatile organic compound assay. The results hence the production of active VOCs with promising antifungal activity by this bacterial isolate.

Materials and methods

Isolation and identification of fungal pathogens from mango fruits

The infected mango fruits showing anthracnose and stem end rot symptoms were collected from Huahin market, Huahin district, Prachuap Khiri Khan province, Thailand. The fruit samples were cleaned on the surface using 70% ethanol. The infected tissues were cut into small pieces of 5×5 mm and sterilized using 1% Sodium Hypochlorite (NaOCl) for 30 seconds. Small pieces of tissues were rinsed in distilled water for 2-3 times and placed on sterile paper. Dried tissues were placed on Potato Dextrose Agar (PDA) and incubated at room temperature (25-27℃) for 3-5 days. The mecelial growth on PDA medium was transferred to a new PDA plate for purification. The fungi, *Colletotrichum* sp. and *Pestalotiopsis* sp. were identified based on cultural and microscopic characteristics according to descriptions of Alexopoulos *et al*. (2002) and Barnett and Hunter (1986).

Rhizospheric bacterial isolation

Rice rhizosphere soil samples were collected from a field under a rice plantation, which is located in Ban Laem District, Phetchaburi Province, Thailand. The fine-grained soils were oven-dried at 45℃ for 24 hrs. after drying at room temperature. Soils were resuspended in 0.85% Sodium Chloride (NaCl), which was prepared in deionized water (w/v). The bacterial isolation was carried out using the serial dilution technique (Ko *et al*., 2009). The

suspensions were serially diluted and 100 µl of dilutions were spread onto a nutrient agar (NA) plate. The plates were incubated at 37℃ for 24 hr. Rhizobacteria were re-isolated to obtain the pure single colonies and conserved on NA medium for further study (Urrea *et al*., 2011).

Screening for antagonistic activity against Colletotrichum sp. and Pestalotiopsis sp.

In previous studies, three bacterial isolates (BL-44, BL-48 and BL-59) had the highest inhibitory activity against rice fungal pathogens, which are *Curvularia* sp., *Fusarium* sp. and *Rhizoctonia* sp. (data not shown). Hence, antagonist bacteria isolates (BL-44, BL-48 and BL-59) were selected to investigate the antagonistic potential against *Colletotrichum* sp. and *Pestalotiopsis* sp. using a dual culture assay (Zhao *et al*., 2018). The antifungal activity of bacteria was monitored on Potato Dextrose Agar (PDA) plate by inoculating bacteria on one side of plate and placing a 5 mm agar plug of fungal pathogens apart from bacteria in 4 cm. The PDA plate without inoculated bacteria was used as a control. All plates were incubated at 27±2 ℃ for 7 days. The inhibition zone was measured to calculate the percentage of radial growth inhibition using the following formula (Sivakumar *et al*., 2000):

Percentage inhibition (%) = [R1 – R2]/R1 × 100

Where R1 is a radial growth measurement of the pathogen in control and R2 is a radial growth measurement of the pathogen in treatment.

All treatments were carried out with 4 replications and designed experiments in Completely Randomized Design (CRD). Treatment means were compared with Duncan's multiple range test (DMRT). The statistical analysis was performed by using R statistical software.

Determination of volatile organic compounds (VOCs) against mycelial growth of fungal pathogens

Antagonistic bacteria BL-59 was cultured in Luria-Bertani (LB) broth and incubated at 30 ℃. Cell suspension of BL-59 was adjusted to 10^8 CFU/ml using the 0.5 McFarland standard (Balouiri *et al*., 2016) to be ready for testing. Then, 100 µl of BL-59 bacterial culture was spread on the LB plate. A 5-mm mycelium plug of the fungal pathogen that was cultured on a PDA plate for 7 days was taken from the margin colony and placed in the center of another PDA agar plate. The plates containing the mycelial plugs were inverted over the bacterial plate and sealed together face to face, followed by incubation at 27±2 ℃. The diameter of fungal mycelium was measured every day. The

fungal plate with the cover replaced by LB plate containing no bacterial culture was used as a control. The rate of inhibition of mycelial growth was calculated using the formula: $[C - T]/C \times 100\%$

Where C is the colony diameter measurement on the control plate and T is the colony diameter measurement on the treated plate.

All treatments were carried out with 4 replications and designed by using a factorial experiment in Completely Randomized Design (CRD). Treatment means were compared with Duncan's multiple range test (DMRT). The statistical analysis was performed by using R statistical software.

Identification of effective antagonistic bacteria

Phenotypic characterization of bacteria

The BL-59 colonies were identified based on morphological, biochemical and physiological characterization (Schaad *et al*., 1992). The morphological studies examined the BL-59 colonies, which grow on Nutrient agar (NA) plates. In gram staining (Katznelson *et al*., 1964), endospore forming, production of catalase and oxidase were classified based on their biochemical characteristics (Rahman *et al*., 2010). For further physiological characteristic identification, bacteria were cultured on NA agar plates and incubated at 20, 30, 37, 40, 45, and 50 ℃. Moreover, the salt concentration of Sodium Chloride (NaCl) was varied from 1-7% to investigate their salt endurance range (Li *et al*., 2014).

Molecular phylogenetic analysis of 16S rRNA

The identification of BL-59 isolate was confirmed by molecular analysis based on 16S rRNA gene sequencing. The universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used for PCR amplification (de Lillo *et al*., 2006). 16S rRNA gene sequences were analysed using the software MEGA version 10.0, and aligned for sequence similarity using the BLAST program of the GenBank database (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were constructed by the Maximum Likelihood (ML) method with 1000 bootstrap replications, and BL-59 gene sequences have been submitted to the NCBI GenBank database for accession numbers (Table 1).

Table 1. Bacterial species and GenBank accession numbers of 16S rRNA sequences were used as reference for phylogenetic tree construction in this study

Species	Strains	Accession numbers in GenBank
		16S rRNA
Bacillus amyloliquefaciens* subsp. *amyloliquefaciens	CCMMB996	KF879306
Ba* subsp. *Amyloliquefaciens	CCMMB997	KF879307
B. atrophaeus	NBRC16183	AB681057
B. licheniformis	BFR-5	LT599743
B. megaterium	21:1	FR715572
B. mojavensis	AT1RS16	LT221166
B. pumilus	HPS1	HF937217
B. subtilis	-	AB192294
B. subtilis	ST51	LC506466
Bacillus subtilis* subsp. *spizizenii	6	AB999946
Bs* subsp. *spizizenii	MDL1	MN493770
Bs* subsp. *Spizizenii	BL-59	MZ577211[1/]
Bs* subsp. *Subtilis	AZFS3	LC599401
Bs* subsp. *Subtilis	133-1	AB999941
B. vallismortis	SR1-57	LT838175

[1]/Accession number obtained in this study

Results

Isolation of Colletotrichum sp. and Pestalotiopsis sp. from mango

The infected mango fruits appeared with the symptoms as irregular black lesions on the fruit surface (Figure 1, A). The severe injury lesion was visible as the orange conidial masses on the fruit skin. The colony on PDA medium has a light gray to dark gray mycelium with an irregular margin (Figure 1, B). The vegetative hyphae were smooth-walled, hyaline and septate. Conidia were hyaline, cylindrical shapes with both ends rounded, smooth-walled (Figure 1, C). In a morphological study, this pathogen was identified as *Colletotrichum* sp., the casual agent of anthracnose disease. In addition, the ripened fruit showed a dark rot from the stem end area and spread into the whole fruit (Figure 1, D). Colony characteristic had visible white zonate colonies, aerial smooth mycelium, and crenated margin (Figure 1, E). Conidia are five-celled, with hyaline apical and basal cells. But, the three median cells reveal brown to dark brown color. Smooth-walled, hyaline and septate vegetative hyphae were found (Figure 1, F). The fungi were identified as *Pestalotiopsis* sp. based on conidial morphology.

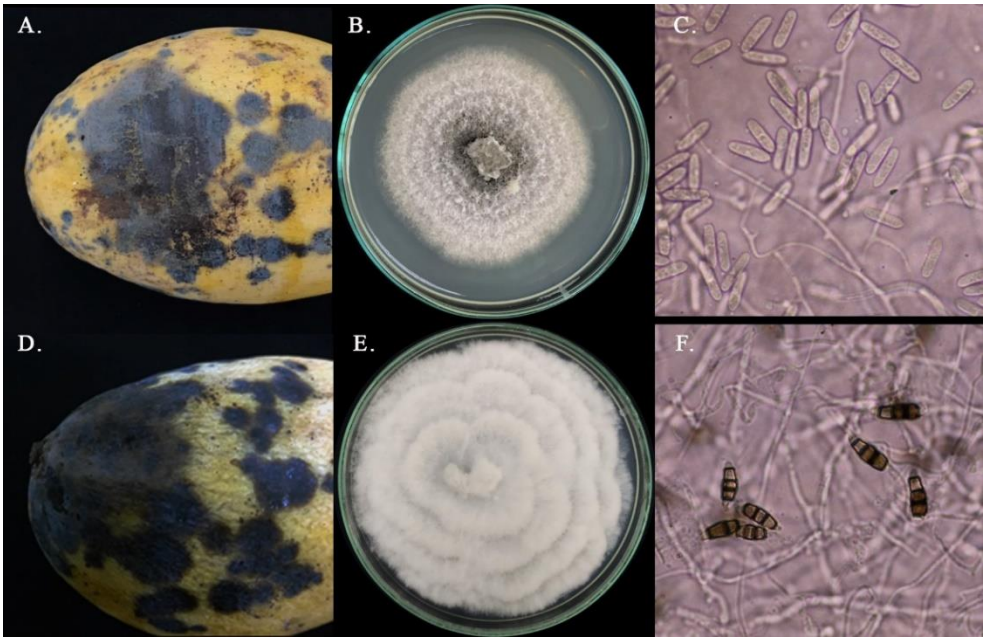

Figure 1. Fungal pathogens isolated from mango fruits. A. Mango fruit with anthracnose disease B. Mycelium growth of *Colletotrichum* sp. on PDA medium C. Hyaline conidia of *Colletotrichum* sp. D. Stem end rot (SER) in mango fruit E. Mycelium growth of *Pestalotiopsis* sp. on PDA medium and F. Typical conidia of *Pestalotiopsis* sp. observed under microscope (40X)

Antifungal activities of antagonistic bacteria against mango fungal pathogens, Colletotrichum sp. and Pestalotiopsis sp. using dual culture assay

A total of 59 bacterial isolates were isolated from soil in paddy fields in the Ban Laem District, Phetchaburi, Thailand. Among the 59 rhizobacterial isolates, 3 isolates (BL-44, BL-48, and BL-59) had potential antifungal activity against some rice fungal pathogens (data not shown), which were selected to investigate the antifungal test with *Colletotrichum* sp. and *Pestalotiopsis* sp. using a dual culture assay. The results showed that isolate BL-59 had the most potential antagonistic activities with an inhibition percentage of 49.31% and 42.55% against *Colletotrichum* sp., and *Pestalotiopsis* sp., respectively. Besides BL-59, BL-44 and BL-48 isolates showed poor antifungal activity in the range of 12-16% inhibition (Figure 2 and Table 2).

Table 2. Percentage inhibition of radial growth (PIRG) (mean ± S.D., n=4) of bacterial isolates against *Colletotrichum* sp. and *Pestalotiopsis* sp. using dual culture assay

Isolates	*Colletotrichum* sp.	*Pestalotiopsis* sp.
	%PIRG	
BL-44	15.97 ± 4.17[b]	12.06 ± 4.01[b]
BL-48	15.28 ± 8.33[b]	13.48 ± 8.82[b]
BL-59	49.31 ± 4.74[a]	42.55 ± 12.77[a]

[1]/Similar letters are not different at $p \leq 0.05$

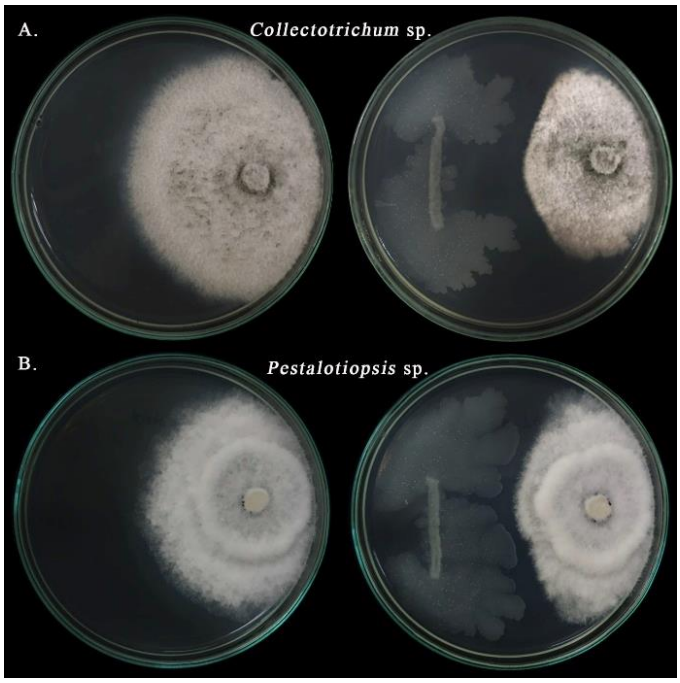

Figure 2. Dual culture assay on potato dextrose agar plate of antagonistic bacteria BL-59 against *Colletotrichum* sp. (A) and *Pestalotiopsis* sp. (B) compared with control

Volatile organic compounds (VOCs) assay for antifungal activity of BL-59 against Colletotrichum sp. and Pestalotiopsis sp.

The effect of BL-59 VOCs on *Colletotrichum* sp. and *Pestalotiopsis* sp. revealed that VOCs had the maximum potential to inhibit mycelial growth of *Colletotrichum* sp. and *Pestalotiopsis* sp. by 60.0% and 56.3%, respectively. The antifungal activity of VOCs had the most efficiency to control mycelial

growth of *Colletotrichum* sp. on day 3 and day 4, where as *Pestalotiopsis* sp. highly inhibited mycelial growth on day 4 (Figure 3 and 4).

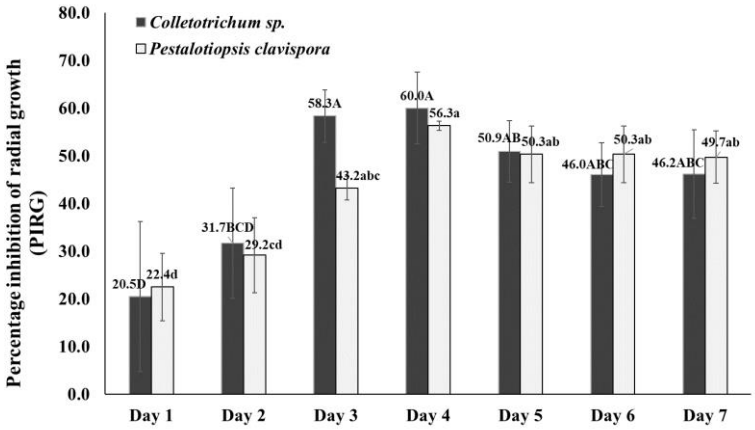

Figure 3. Antifungal activity of BL-59 against *Colletotrichum* sp. and *Pestalotiopsis* sp. by volatile organic compounds (VOCs) assay

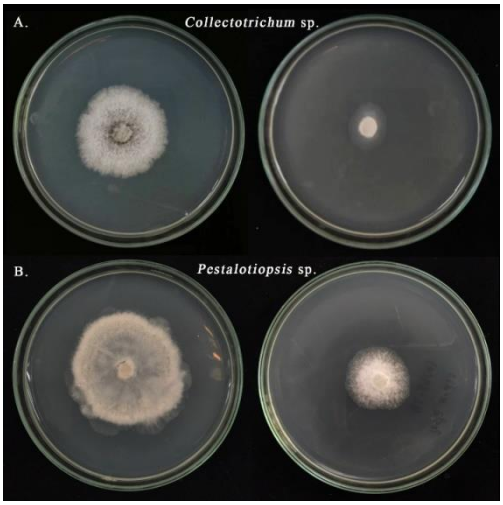

Figure 4. *In vitro* antifungal inhibitory of BL-59 VOCs against *Colletotrichum* sp. (A.) and *Pestalotiopsis* sp. (B.) after 7 days of incubation. The plate on the left-handed was a control, the plate on the right-handed was BL-59 treatment

Identification of antagonistic bacteria BL-59

The colonies BL-59 on NA medium plate is a cream color with wavy edges. The morphological studies under a microscope with a 100X objective is investigated that bacterial cells were classified as gram-positive, rod-shaped,

and produced endospores. Moreover, this isolate revealed a positive reaction to the catalase and oxidase enzyme tests. In addition, BL-59 colonies grew in various environmental conditions of temperature and salinity stress, as shown in Table 3.

Table 3. Physiological characteristics of bacteria BL-59 under extreme environmental conditions

Isolate	Temperature (℃)						NaCl (%)			
	20	30	37	40	45	50	4	5	6	7
BL-59	+	+	+	+	+	-	+	+	+	+

[1]/ + indicates bacteria growth was observed. – indicates no bacterial growth was observed.

The rhizobacteria BL-59 was identified which based on the 16s rRNA gene. The bacterial sequence showed BLAST results up to 99.79% similarity to *Bacillus subtilis* subsp. *spizizenii*. The sequence was deposited in GenBank with the accession number MZ577211. The phylogenetic tree was constructed, which revealed that BL-59 was grouped into the *Bs* subsp. *spizizenii* clade (Figure 5).

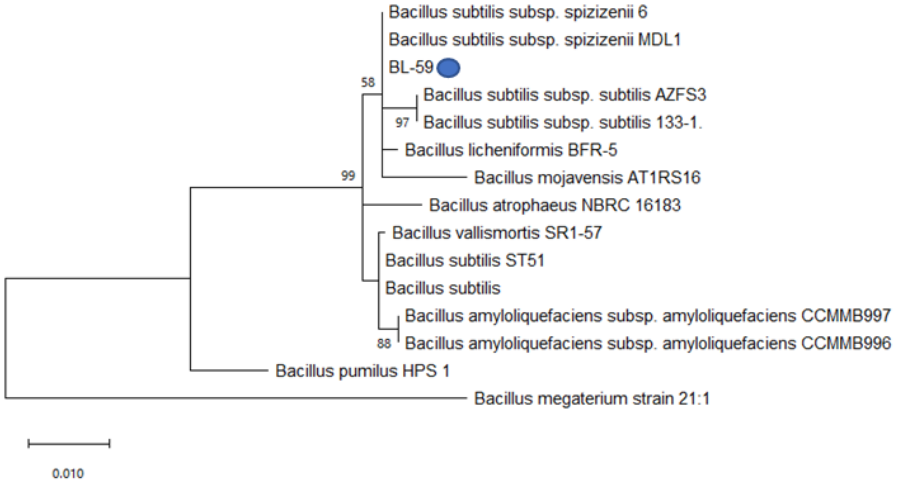

Figure 5. Phylogenetic tree constructed by the maximum likelihood method based upon the alignment of partial 16S rRNA gene sequences of antagonistic bacteria. Bootstrap analysis (1000 replicates) for node values more than 50% is shown. The scale bar indicates the number of substitutions per site

Discussion

Postharvest diseases of mango fruits can cause reduction in quality due to rot symptoms on fruits' skin. As a result of this study, anthracnose disease, caused by *Colletotrichum* sp., was identified based on microscopic and

macroscopic observations. *Colletotrichum* species infected mango have previously been reported in species such as *C. asianum*, *C. cliviicola*, *C. cordylinicola*, *C. endophytica*, *C. fructicola*, *C. gigasporum*, *C. gloeosporioides*, *C. karstii*, *C. liaoningense*, *C. musae*, *C. scovillei*, *C. siamense*, and *C. tropicale* (Li *et al*., 2019). However, *C. gloeosporioides* was referred to a fungal agent causing anthracnose disease on mango fruit (Đinh *et. al*., 2009, Prusky *et. al*., 2009, Reyes-Perez *et. al*., 2019). Another symptom showed stem end rot of the fruit, which is a severe symptom at the fruit ripening stage. The casual pathogen of this disease was determined as *Pestalotiopsis* sp. In 1997, Coates and Johnson reported that the stem end rot (SER) disease is caused by *Pestalotiopsis mangiferae*. Both diseases cause symptoms at the same time during fruit ripening and are considered important and common postharvest diseases (Karunanayake and Adikaram, 2020).

In recent years, bacteria had become a popular choice for biological control in plant disease management. In particular, rhizospheric bacteria were isolated from soils where the area is enriched with numerous nutrients from plant root exudates (Berg and Smalla, 2009, Wardle *et al*., 2004). For bacterial identification, morphological and molecular phylogenetic analysis were used to determine precise data. The 16S rRNA genes are conserved region to be useful for classification and identification of endospore forming bacteria (Reva *et al*., 2004). In the present study, the isolate BL-59 was identified as *Bacillus subtilis* subsp. *spizizenii* BL-59 by 16S rRNA gene analysis. The bacteria in the genus *Bacillus* were determined to have the potential antifungal activity and broad spectrum to control various plant diseases (Stein, 2005). *Bs* subsp. *spizizenii* has been shown to possess high antimicrobial activity against several gram-positive pathogens such as *Staphylococcus aureus* and *Enterococcus faecalis*. In the present study, *B. subtilis* subsp. *spizizenii* BL-59 demonstrated 40-50% inhibitory activity against the mycelial growth of *Colletotrichum* sp. and *Pestalotiopsis* sp. *in vitro*. However, the percentage of mycelial growth inhibition was less than *Bacillus* sp. LB72 against *C. gloeosporioides*, as reported by Rungjindamai (2016).

The antibiotic substance of *Bs* subsp. spizizenii has been identified as a subtilin-like lantibiotic, according to Fuchs *et al.*, 2011. In our study herein, we found that *Bs* subsp. *spizizenii* BL-59 can produce active VOCs with a potential antifungal activity to control *Colletotrichum* sp. and *Pestalotiopsis* sp. by 60.0% and 56.3%, respectively. VOCs are well known for antifungal activity against fungal pathogens (Dukare *et al*., 2019). The previous report showed that *B. thuringiensis* and *B. pumilus* produced VOCs which could suppress anthracnose infections in mango by 88.5% (Zheng *et al.*, 2013). Active VOCs, 2,4-*di*-*tert*-butylthiophenol, produced by *B. subtilis* CF-3 was

recently reported as an active control agent against *C. gloeosporioides* (Wang *et al*., 2021). VOCs activities against *C. gloeosporioides* were also demonstrated in some other bacteria, such as *Rahnella aquatilis* JZ-GX1 (Kong *et al*., 2020) and the marine bacterium *Stenotrophomonas rhizophila* (Reyes-Perez *et al*., 2019).

In conclusion, our present study demonstrated the isolation of antagonistic bacteria *B. subtilis* subsp. *spizizenii* BL-59 with potential activity against *Colletotrichum* sp. and *Pestalotiopsis* sp. fungal pathogens from mango fruits. VOCs assay revealed its ability to produce active VOCs as one of its mechanisms in the control of these important fungal pathogens of mango. Further study on its antifungal mechanisms and *in vivo* disease suppression could provide more information for commercial application in the future.

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