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Efficacy of antagonistic bacteria for controlling fungal rice (*Oryza sativa* L.) pathogens

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Abstract A total of 59 bacterial isolates were isolated from the rhizosphere soil of rice in Tha-rang sub-district, Ban-Lham district, Phetchaburi province. The preliminary study was conducted using a dual culture assay to investigate the efficacy of antagonistic bacteria to control rice fungal diseases, which include *Curvularia* spp., *Fusarium* spp. and *Rhizoctonia* spp. The result showed that 5 isolates: BL-44, BL-48, BL-55, BL-56 and BL-59 have an efficiency to control the fungal pathogens. The BL-59 isolate revealed a maximal percentage of mycelial growth inhibition against *Curvularia* spp. (65.67%) and *Fusarium* spp. (54.74%) and BL-44 isolate showed a maximal mycelial growth inhibition percentage (PIRG) against *Rhizoctonia* spp. (74.29%) ($P<0.05$) using PDA medium. Whereas, the inhibitory activity of BL-59, which performed on TSA medium had highly PIRG values of 93.33, 82.84 and 31.03% against *Curvularia* spp., *Fusarium* spp. and *Rhizoctonia* spp. tested on TSA medium, respectively. The volatile assay revealed that BL-44 isolate showed the highest antifungal efficacy against *Curvularia* spp. (82.26%) and *Fusarium* spp. (67.86%), whereas BL-48 and BL-56 isolates showed the highest antifungal efficacy against *Rhizoctonia* spp. by 73.33 and 76.67%, respectively ($P<0.05$). Microscopic observation of the hyphal morphology of fungal diseases revealed the severely damaged hyphae, including deformation, loss of apical growth, and lysis. Furthermore, these bacterial isolates produced volatile compounds that inhibited mycelial growth and reduced pigment production. In addition, BL-44 and BL-56 isolates demonstrated temperature endurance from 20 to 50ºC. BL-48 and BL-59 demonstrated tolerate salinity levels ranging from 4 to 7% NaCl. BL-48, BL-56 and BL-59 isolates were identified as *Enterobacter roggenkampii*, *Enterobacter cloacae* and *Bacillus subtilis* subsp. *spizizenii* based on 16S rRNA analysis. As a result, the antagonistic bacteria isolated from this study can be used as an alternative choice to control rice diseases caused by fungal pathogens.

Keywords: Rice disease, Biological control, Rhizospheric bacteria, Volatile compounds

Introduction

Rice (*Oryza sativa* L.) is the most important food crop in the world. Thailand is one of the leading rice producers and exporters in the top 12 countries

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of the global rice market (Muthayya *et al*., 2014). However, serious diseases affect rice cultivation globally, resulting in yield losses and seed quality (Nalley *et al*., 2016). Major rice diseases that often cause economic losses are rice blast (*Magnaporthe grisea*), sheath blight (*Rhizoctonia solani*), dirty panicle disease (*Alternaria padwickii*, *Curvularia lunata*, *Fusarium moniliforme*, *Bipolaris oryzae*), bacterial blight (*Xanthomonas oryzae*) and tungro virus disease (Arshad *et al*., 2009; Balgude *et al*., 2016; Goswami and Thind, 2018; Prabhu *et al.*, 2012).

Fungicide application was the primary method for controlling rice diseases. The farmers continuous use of fungicides that lead to fungicide resistance in pathogens and has side effects to farmers, consumers and environments (Riangwong *et al*., 2023). Biological control, the most current approach to plant disease management, is an alternative strategy for environmentally friendly methods to reduce chemical fungicides. Angonistic bacteria have been promoted as biological control agents due to their efficacy in antimicrobial activity such as antimicrobial metabolites, the production of volatile compounds, induced systemic resistance (ISR) and plant growth promotion, especially plant growth promoting rhizobacteria (PGPR) (Gulzar *et al*., 2023).

According to Chaiharn *et al*. (2009), the siderophore producing rhizobacteria showed a strong antagonistic effect against rice fungal pathogens: *Alternaria* sp., *Fusarium oxysporum*, *Pyricularia oryzae* and *Sclerotium* sp. The highly effective isolates were identified as Genus *Bacillus*, *Pseudomonas* and *Kocuria*. *Bacillus altitudinis* CH05 and *Bacillus tropicus* CH13, both isolates showed inhibitory activity on mycelial growth based on volatile organic compounds (VOCs) production against phytopathogenic fungi (Espinosa Bernal *et al*., 2024). Moreover, two endophytic *Bacillus subtilis* isolates (NIIST B616 and NIIST B627) had efficacy to control sheath blight disease caused by the fungus *Rhizoctonia solani* with significant declines of 50% yield losses. And the antifungal metabolites of two isolates were analyzed and identified as cyclo-(Pro-Leu) and cyclo-(Pro-Phe) that can induce ISR in plants (Krishnan *et al*., 2024). In addition, three of the 513 bacterial strains isolated from peat swamp forests had a highly fungal growth inhibitory effect on the fungal pathogens including *Curvularia lunata*, *Bipolaris oryzae* and *Fusarium incarnatum*. The three strains were identified as *Bacillus* and *Brevibacillus* based on 16S rRNA gene analysis (Unartngam *et al*., 2021).

The aims of the present study were to explore the rhizobacteria isolated from rhizospheric soil of rice paddy and investigate the efficacy of antifungal activity against rice fungal pathogens: *Curvularia* spp., *Fusarium* spp. and *Rhizoctonia* spp.

Materials and methods

Collection and Isolation of fungal rice pathogens

Rice fields in Tha-rang sub-district, Ban-Lham district, Phetchaburi province, Thailand, served as the collection site for rice diseases. The symptomatic rices observed on the seed and sheath were isolated fungal pathogens using the blotter method (Butt *et al*., 2011; Khan *et al*., 2023) and tissue transplanting method (Waller, 2002). All fungal isolates were cultured on slants of potato dextrose agar (PDA) at 4ºC for further study and subcultured onto PDA plates for experiment. The fungal isolates were identified according to morphological characteristics at the genus level (Barnett and Hunter, 1972).

Isolation and identification of angonistic bacteria

The rhizospheric soil of rice was used to be a source for bacterial isolation. The soil in the root zone, which is located in the same location as above, was randomly collected. The soil samples were air dried by heating at 45ºC for 24 hr. The serial tenfold dilutions were performed to isolate bacteria. Saline solution 0.85% was used as a diluent. The suspensions were spun in a vortex. 100 µl of dilution was spread onto a nutrient agar (NA) plate and incubated at 37ºC for 24 hr. The pure cultures of rhizobacteria were streaked on NA for further study. The bacterial isolates that revealed the high potential of antagonistic activity were selected and identified by molecular analysis.

Screening of potential bacterial isolates against fungal pathogens

The bacterial isolates were determined the antagonistic efficacy using the dual culture technique (Nysanth *et al*., 2022). Bacterial isolates were streaked and inculated with the fungal pathogens in the same PDA plates 3 cm apart. The PDA plate without inoculated bacteria was used as a control. The fungal growth inhibitory (%inhibition) was examined and calculated using the formula: $(R_1-R_2)/R_1 \times 100$

Where R_1 was radial colony of fungal growth in control treatment and R_2 was radial colony in treatment.

In vitro evaluation of antagonistic activity of rhizospheric bacteria

The high potential inhibitory activity of antagonistic bacteria was screened and used to examine the antagonistic activity against fungal pathogens using the

dual culture assay on tryptic soy agar (TSA) and PDA media. The fungal pathogens were cultured for 7 days on PDA medium. The fungal culture disc was conducted 0.6 mm in diameter and transferred to new agar plates and then streaked the antagonistic bacteria in the same plate 7 cm apart. The radial growth measurement of the fungal pathogen was carried out to calculate the percentage (%) of growth inhibition as the formula above.

Determination of antifungal volatile compounds (VOCs)

The tryptic soy agar (TSA) plate containing colonies of antagonistic bacteria was prepared and covered with another PDA plate containing a 6 mm-diameter culture disc of fungal pathogens. Both plates were then sealed with parafilm and incubated at 27ºC for 10 days. The effect of VOCs to inhibit the hyphal growth was determined based on colony diameter (Gao *et al*., 2022). Additionally, the morphological characteristics were investigated under a light microscope. The percentage (%) of hyphal growth inhibition was calculated using the formula below:

% of inhibition = $(D_1-D_2)/D_1 \times 100$

Where D_1 was colony diameter on control treatment and D_2 was colony diameter on treatments.

In vitro screening of bacterial antagonists for abiotic stress tolerance

The high potential of antagonistic bacterial isolates was determined the tolerance of stress conditions: temperature tolerance and salinity tolerance. NA medium and incubated at 20, 30, 37, 40, 45 and 50ºC overnight in all treatments. For the salinity tolerance test, the sterile NA broth medium supplemented with 1 to 7% NaCl was prepared in tubes. The bacterial suspension was incubated in NA broth tubes at 37 ºC and investigated the bacterial growth after 24 hr.

Molecular analysis based on 16S rRNA

The effective antagonistic bacterial isolates were conducted to molecular characterization based on 16S rRNA gene sequencing. The universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used for PCR amplification (James, 2010). The amplified fragments were sequenced and confirmed using NCBI database by high similarity percentage. The gene sequences were aligned and analyzed using the software MEGA version 10.0. The accession numbers were obtained by gene sequence deposition to the NCBI GenBank.

Statistical analysis

The data of all experiments were conducted in Completely Randomized Design (CRD) and compared with Duncan's multiple range test (DMRT). Each experiment was carried out with 3 replications. All analyses were performed with R statistical software. Statistical significance was accepted with p-value <0.05.

Results

Isolation and characterization of fungal rice pathogens

The infected seed and sheath on rice were visible symptoms shown in Figure 1A and Figure 3A. The rice seeds showed the symptoms as brown to dark brown spots and discoloration. The fungal colony on PDA plate had suede-like, gray to dark brown mycelium (Figure 1B). Conidial characteristics are ellipsoidal with lunate rounded at the end, pale brown to dark brown, 1-3 septa. The septate hyphae showed as dermatiaceous fungi (Figure 2A). The symptoms and microscopic study were identified as *Curvularia* spp. Furthermore, another fungal isolate showed colony appearance as fluffy white mycelium with pink pigment (Figure 1C). In addition to conidium formation, macroconidia were two to several-celled, hyaline (Figure 2B). The fungi were identified as *Fusarium* spp. Both *Curvularia* spp. and *Fusarium* spp. are the casual agents of rice dirty panicle disease. Symptoms appeared at the sheath and presented irregular lesions with brown to black above the water line (Figure 3A). The macroscopic and microscopic characteristics had white mycelium (Figure 3B) and produced hyaline septate hyphae that grow at right angle to the main hyphae (Figure 3C). As presented in Figure 3, the fungi were identified as *Rhizoctonia* spp.

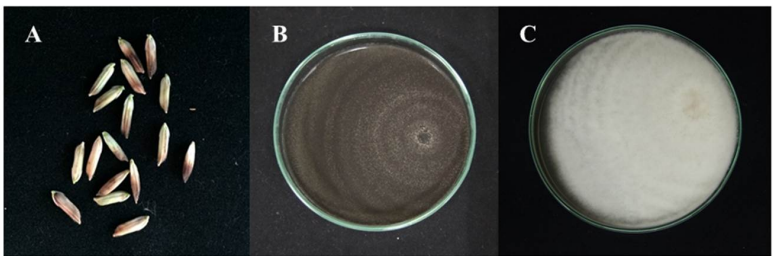

Figure 1. Morphological characteristics of fungal pathogens, causal agent of rice dirty panicle disease: (A) symptom on infected seeds (B) and (C) mycelial hyphae cultured on PDA medium of *Curvularia* spp. and *Fusarium* spp., respectively

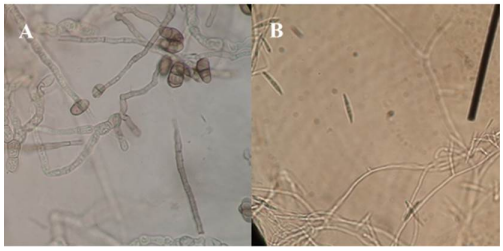

Figure 2. Microscopic characteristics of fungal pathogens causing rice dirty panicle disease: (A) *Curvularia* spp. and (B) *Fusarium* spp. observed under microscope (40X)

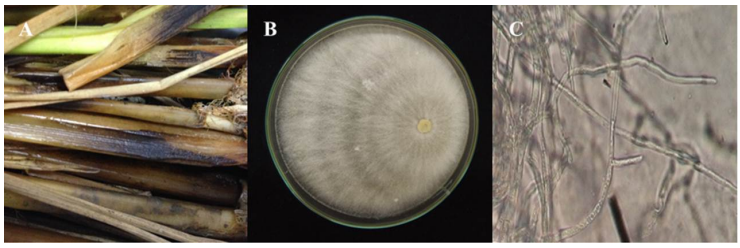

Figure 3. The causal agents of sheath blight disease: (A) symptoms (B) mycelial growth on PDA medium and (C) microscopic characteristics observed under microscope (40X)

Screening of potential bacterial isolates against fungal pathogens

A total of 59 rhizospheric bacteria isolates were screened for antagonistic activity against the mycelial growth of fungal rice pathogens. Five bacterial isolates (BL-44, BL-48, BL-55, BL-56 and BL-59) showed the maximum inhibition rate (%) in radial growth of *Curvularia* spp. (40-50%), *Fusarium* spp. (40-70%) and *Rhizoctonia* spp. (40-70%).

In vitro evaluation of antagonistic activity of rhizosheric bacteria

The efficacy of antagonistic activity of five bacterial isolates (BL-44, BL-48, BL-55, BL-56 and BL-59) was carried out against three fungal pathogens (*Curvularia* spp., *Fusarium* spp. and *Rhizoctonia* spp.) (Figure 4, 5 and 6). The percentage inhibition of radial growth (PIRG) values ranged from 28.36 to 65.67%, 31.39 to 54.74% and 0.00 to 74.29% against *Curvularia* spp., *Fusarium* spp. and *Rhizoctonia* spp. using PDA medium, respectively. Using TSA medium, PIRG values ranged from 28.89 to 93.33%, 0.00 to 82.84% and 0.00 to 31.03% against *Curvularia* spp., *Fusarium* spp. and *Rhizoctonia* spp., respectively (Table

1). Of the effective bacteria isolates evaluated on PDA medium, BL-59 had the highest PIRG values (65.67%) against *Curvularia* spp. whereas 54.74% against *Fusarium* spp. Furthermore, BL-59 showed the greatest PIRG values of 93.33, 82.84 and 31.03% against *Curvularia* spp., *Fusarium* spp. and *Rhizoctonia* spp. tested on TSA medium, respectively (Table 1).

Table 1. The percentage inhibition of radial growth (PIRG) of rhizospheric bacteria against fungal rice pathogens by dual culture method

The percentage inhibition of radial growth (%PIRG)[1/]						
Isolates	**PIRG on PDA**			**PIRG on TSA**		
	***Curvularia* spp.**	***Fusarium* spp.**	***Rhizoctonia* spp.**	***Curvularia* spp.**	***Fusarium* spp.**	***Rhizoctonia* spp**
BL-44	28.36^{e}	43.07^{bc}	74.29^{a}	28.89^{e}	0^{d}	0^{b}
BL-48	61.19^{b}	51.82^{ab}	36.43^{c}	50.00^{b}	57.46^{b}	0^{b}
BL-55	37.31^{d}	31.39^{d}	0^{e}	31.11^{d}	32.09^{c}	0^{b}
BL-56	40.30^{c}	37.96^{cd}	4.29^{d}	33.33^{c}	31.34^{c}	0^{b}
BL-59	65.67^{a}	54.74^{a}	41.43^{b}	93.33^{a}	82.84^{a}	31.03^{a}
P-value	7.46e-07***	0.0079**	9.2e-0.5***	2.25e-0.8***	0.00128***	0.276*
CV	8.76	2.04	2.96	1.26	9.658	30.48

[1]/The letters indicate significant differences by DMRT within the same column (P<0.05)

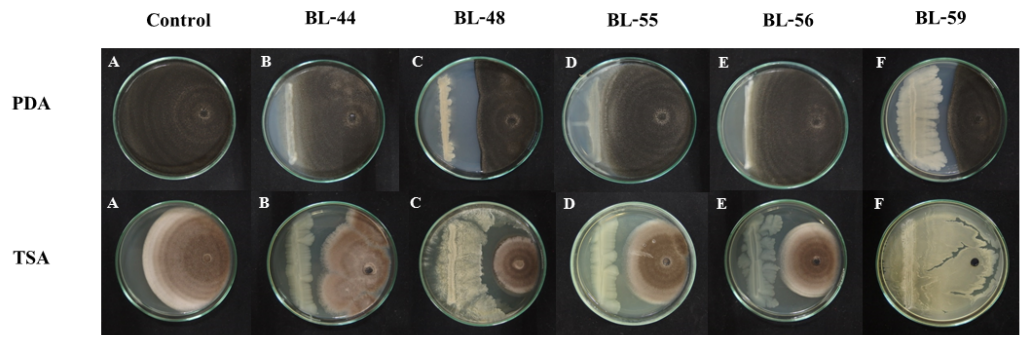

Figure 4. Antagonism of bacterial isolates against *Curvularia* spp. using dual culture (A) control (B) BL-44 (C) BL-48 (D) BL-55 (E) BL-56 and (F) BL-59
*Noted the upper row: PDA medium and the lower row: TSA medium

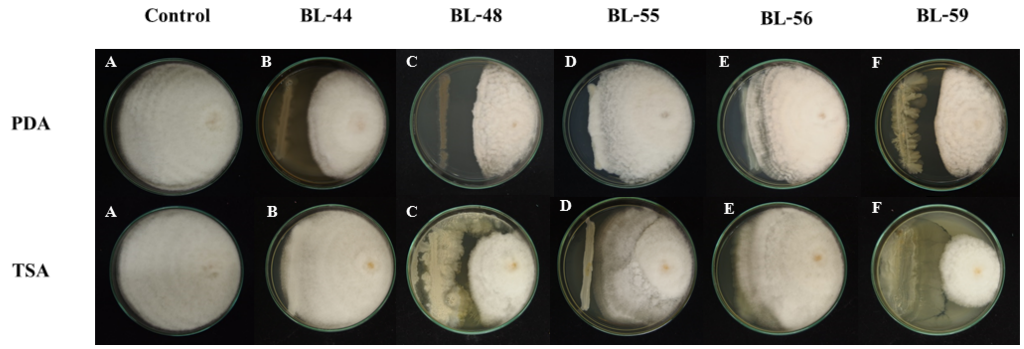

Figure 5. Dual culture plate assay for antifungal activity of bacterial isolates against *Fusarium* spp. (A) control (B) BL-44 (C) BL-48 (D) BL-55 (E) BL-56 and (F) BL-59
*Noted the upper row: PDA medium and the lower row: TSA medium

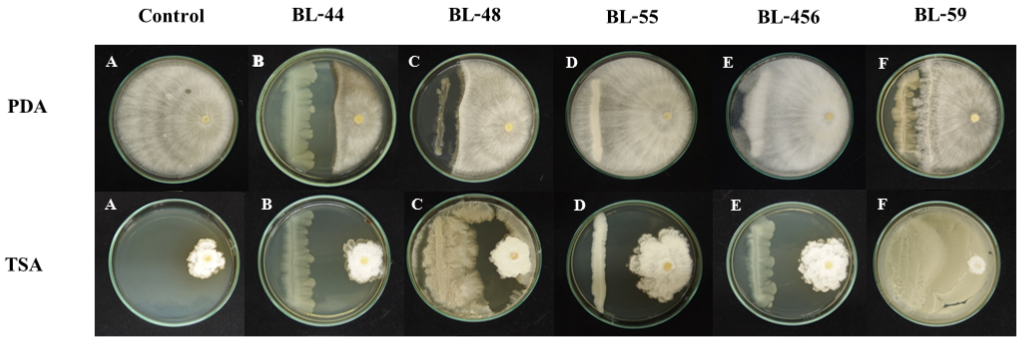

Figure 6. Antifungal dual plate assay of bacterial isolates against *Rhizoctonia* spp. using dual culture (A) control (B) BL-44 (C) BL-48 (D) BL-55 (E) BL-56 and (F) BL-59
*Noted the upper row: PDA medium and the lower row: TSA medium

The effect of volatile compounds (VOCs) against rice fungal pathogens

The effect of VOCs of the effective bacterial isolates on the mycelial growth of fungal pathogens was investigated by the percentage (%) of hyphal growth inhibition. As presented in Table 2, BL-44 showed the most efficiency in reducing the mycelial growth of *Curvularia* spp. and *Fusarium* spp. by inhibition of 82.26 and 67.86%, respectively. The VOCs of BL-48 and BL-56 had the significant highest efficacy of mycelial growth inhibition by 73.33 and 76.67%, respectively. Besides, the inhibitory effect of VOCs on mycelial growth demonstrated antifungal activity as shown in Figures 7 to 9. The results revealed

that VOCs caused damage to hyphal morphology by hyphae deformation and the pigmentation became lighter in *Curvularia* spp. (Figures 7, 8 and 9).

Table 2. The antifungal activity of VOCs produced by rhizospheric bacteria against *Curvularia* spp., *Fusarium* spp. and *Rhizoctonia* spp.

Isolates	Percent (%) inhibition of hyphal growth[1/]		
	***Curvularia* spp.**	***Fusarium* spp.**	***Rhizoctonia* spp.**
BL-44	82.26^{a}	67.86^{a}	47.22^{b}
BL-48	80.65^{ab}	42.86^{b}	73.33^{a}
BL-55	32.74^{d}	3.57^{c}	43.33^{b}
BL-56	74.19^{ab}	60.71^{ab}	76.67^{a}
BL-59	70.67^{c}	20.00^{bc}	46.67^{b}
P-value	5.78e-05***	0.0157**	0.000761***
CV	4.20	19.39	6.72

[1]/The letters indicate significant differences by DMRT within the same column (P<0.05)

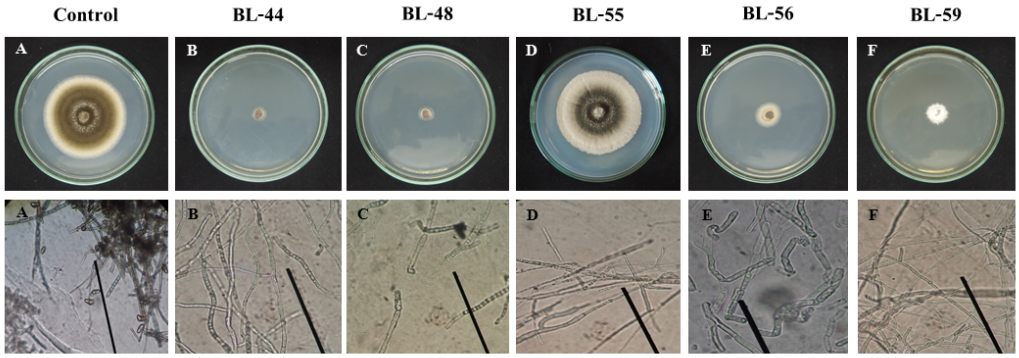

Figure 7. Antifungal activity of VOCs produced by antagonistic bacteria to control *Curvularia* spp. (A) *Curvularia* spp. growing for 10 days as control (B) *Curvularia* spp. growing treated by BL-44 (C) *Curvularia* spp. growing treated by BL-48 (D) *Curvularia* spp. growing treated by BL-55 (E) *Curvularia* spp. growing treated by BL-56 and (F) *Curvularia* spp. growing treated by BL-59
*Noted the upper row: double petri dish assay for antifungal activity investigation and the lower row: hyphal morphology under a light microscope (40X)

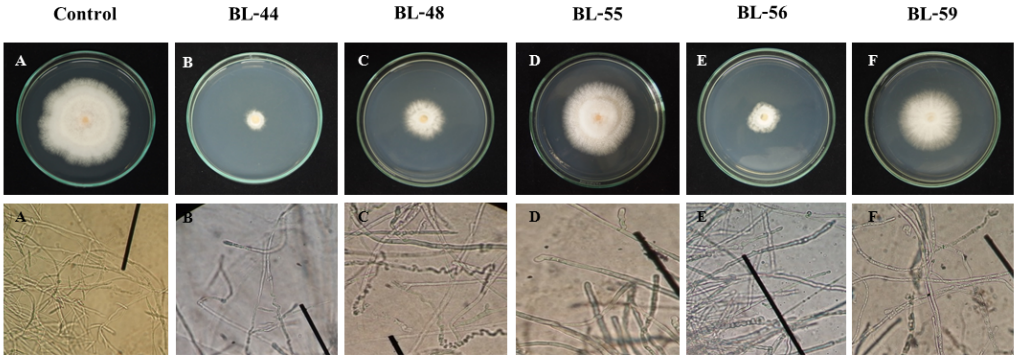

Figure 8. Antifungal activity of VOCs produced by antagonistic bacteria to control *Fusarium* spp. (A) *Fusarium* spp. growing for 10 days as control (B) *Fusarium* spp. growing treated by BL-44 (C) *Fusarium* spp. growing treated by BL-48 (D) *Fusarium* spp. growing treated by BL-55 (E) *Fusarium* spp. growing treated by BL-56 and (F) *Fusarium* spp. growing treated by BL-59
*Noted the upper row: double petri dish assay for antifungal activity investigation and the lower row: hyphal morphology under a light microscope (40X)

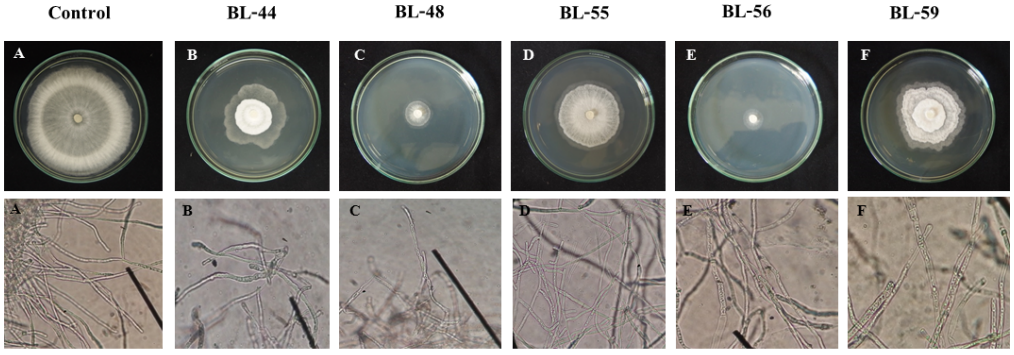

Figure 9. Antifungal activity of VOCs produced by antagonistic bacteria to control *Rhizoctonia* spp. (A) *Rhizoctonia* spp. growing for 10 days as control (B) *Rhizoctonia* spp. growing treated by BL-44 (C) *Rhizoctonia* spp. growing treated by BL-48 (D) *Rhizoctonia* spp. growing treated by BL-55 (E) *Rhizoctonia* spp. growing treated by BL-56 and (F) *Rhizoctonia* spp. growing treated by BL-59
*Noted the upper row: double petri dish assay for antifungal activity investigation and the lower row: hyphal morphology under a light microscope (40X)

In vitro screening of bacterial antagonists for abiotic stress tolerance

All five bacterial isolates were screened for ability to tolerate temperature and salinity *in vitro*. The results of their ability to abiotic stress tolerance were summarized in Table 3. Isolates BL-44 and BL-56 showed temperature tolerance in the range of 20 to 50ºC. Among the salinity tolerances, BL-48 and BL-59 revealed tolerate salinity levels of 4 to 7% NaCl.

Table 3. Five bacterial isolates showing tolerance to various abiotic stresses

Isolates	Temperature (˚C)						NaCl (%)			
	20	**30**	**37**	**40**	**45**	**50**	**4**	**5**	**6**	**7**
BL-44	+	+	+	+	+	+	+	+	+	-
BL-48	-	+	+	+	+	+	+	+	+	+
BL-55	+	+	+	+	+	-	+	+	-	-
BL-56	+	+	+	+	+	+	+	+	+	-
BL-59	+	+	+	+	+	-	+	+	+	+

Molecular analysis of antagonistic bacteria

The three (BL-48, BL-56 and BL-59) effective isolates were analyzed and identified using the molecular analysis based on 16S rRNA. The BL-48 belonged to *Enterobacter roggenkampii*, which showed the similarity percentage at 99.99%. And, gene sequence of BL-56 displayed 100% similarity with *Enterobacter cloacae*. The sequences were submitted in NCBI database (GenBank) with accession numbers PQ357576 and PQ357348, respectively (Table 4). In addition, the sequence of BL-59 was identified as *Bacillus subtilis* subsp. *spizizenii* and deposited in GenBank under accession number MZ577211 in the previously studied.

Table 4. BLAST result 16S rRNA sequence identity between selected antagonistic bacteria and GenBank sequence

Isolates	Accession number	% identity	Highest similarity % (Genbank Accession No.)
BL-48	PQ357576	99.91	*Enterobacter roggenkampii* (CP138222)
BL-56	PQ357348	100	*Enterobacter cloacae* (KX262850)

Discussion

The rice diseases were surveyed on a rice paddy field and collected visible symptoms of seed discoloration and sheath blight. The symptoms showed the brown to dark brown spots on seeds and presented irregular lesions with brown to black on rice sheath. In the present study, these symptoms were reported as dirty panicle disease and sheath blight disease, respectively (Kongcharoen *et al*., 2020, Singh *et al*., 2019). The morphological study of fungal pathogens isolated from infected plant tissues was confirmed as *Curvularia* spp. and *Fusarium* spp., which are the casual agents of rice dirty panicle disease and *Rhizoctonia* spp. causing sheath blight disease (Alexopoulos *et al*., 2002, Barnett and Hunter, 1986).

Rhizobacteria are important microorganisms that colonized plant root zones for sustainable agriculture (Umer *et al*., 2021). In a recent research study, rhizobacteria are considered to be used as biocontrol agents due to their properties that can suppress the pathogenic microorganisms (Maloy and Lang, 2003) and induce the enhancement of plant growth (Van Loon, 2007). The mechanisms of biocontrol microbes can inhibit pathogens by producing antifungal metabolites, degrading enzymes, parasitism and competition for nutrients (Fravel, 1988). A total of 59 rhizobacteria were isolated and investigated for antagonistic activity against *Curvularia* spp., *Fusarium* spp. and *Rhizoctonia* spp. The results of the present study revealed that the effective bacteria isolates (BL-48, BL-56 and BL-59) had the greatest inhibition rate (%) in mycelial growth of fungal pathogens. The bacteria are identified commonly by morphological characteristics, including molecular analysis for accurate data. The 16S rRNA gene contains both highly conserved and variable regions that can be used to identify a wide variety of bacteria (Plongla and Miller, 2017). In the present study, the isolates BL-48 and BL-56 were identified as *Enterobacter roggenkampii* and *Enterobacter cloacae* based on 16S rRNA gene sequences. However, the BL-59 isolate was reported in the previously conducted research and identified as *Bacillus subtilis* subsp. *spizizenii*. This isolate had the efficacy to control fungal pathogens causing postharvest diseases in mango fruits (Duangkaew and Monkhung, 2021). *Enterobacter* bacteria have been examined for their potential as biological control agents and plant growth promoters. *Enterobacter roggenkampii* isolated from tomato leaves showed antagonistic activity against *Alternaria* fruit rot of tomato. Furthermore, this species can reduce the lesion size by 60% on tomato fruit rot disease (Al-Maawali *et al*., 2020). According to Ranawat *et al.* (2021), *E. hormaechei* increased the ability for nitrogen fixation in plant development, resulting in increased agricultural yield. In 2019, Macedo-Raygoza *et al*. demonstrated the endophytic bacteria:

Enterobacter cloacae had the potential to control black sigatoka disease on banana plants caused by *Pseudocercospora fijiensis* and promote banana plant growth by root colonization. The antimicrobial activity of volatile organic compounds (VOCs) are chemical compounds emitted as gases. Gram negative bacteria: *Enterobacter* produced volatile compounds, which were analyzed and identified as alcohols comprising 1-octanol, 1-decanol, and 1-dodecanol (Elgaali *et al*., 2002). Studies on abiotic tolerance in the potential of antimicrobial activity of rhizobacteria, *Bacillus* and *Pseudomonas* spp. showed both salinity and temperature tolerance that were survival and promise antagonistic activity. Bacteria strains are capable of enduring high temperatures (50°C), salinity (7% NaCl), and drought (−1.2 MPa) (Praveen Kumar *et al*., 2014).

Within the present study, rhizobacteria were evaluated for antifungal activity against rice fungal pathogens: *Curvularia* spp., *Fusarium* spp. and *Rhizoctonia* spp. The bioactive activity is presented as secondary metabolites in agar culture plates using dual culture assays and volatile metabolites. These results show that rhizobacteria have potential antifungal activity and can be developed as a biocontrol product. However, further studies under field conditions are required to manage plant diseases.

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