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Full Length Article

Characterization of Plant Growth Promoting Bacteria (PGPB) as Biocontrol Agents Against Plant Pathogenic Fungi *In Vitro*

Haliatur Rahma[1*], Trizelia[1], Martinius[1], Yenny Liswarni[1], Rusdi Rusli[1], Nalwida Rozen[2], Leni Armelia[1], Amelia Putri[1] and Fitrah Qalbina[1]

[1]*Department of Plant Protection, Faculty of Agriculture, Andalas University, Campus Andalas University Limau Manis Padang, West Sumatera, 25163, Indonesia*

[2]*Department of Agrotechnology, Faculty of Agriculture Andalas University, Campus Andalas University Limau Manis Padang, West Sumatera, 25163, Indonesia*

[*]For correspondence author: haliaturrahma@agr.unand.ac.id

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Abstract

Plant Growth-Promoting Bacterial Endophytes (PGPBE) are organisms that live within plant tissues and are not harmful to the host plant. The organisms have the capacity to inhibit the growth of plant pathogens directly and indirectly. The purpose of this study was to find PGPBE isolates capable of controlling the fungus *Curvularia lunata* and *Rhizoctonia solani* Kühn *in vitro*. The experiment was carried out utilizing a Completely Randomized Design (CRD), with 24 treatments obtained from rice plant roots, leaves, and stems. Two species of pathogenic fungi, *C. lunata* and *R. solani*, were compared in dual culture on PDA media, with fungi lacking PGPBE serving as control agents. The experiment was replicated four times, with factors monitored such as the isolates' ability to reduce *in vitro* growth of the two harmful fungi. The findings revealed that seven PGPBE isolates, including LMA5 and LMA6 from rice roots, LMB35, LMB1, LMB4, and LMB21 from rice stems, and LMD13 from rice leaves, could suppress the growth of *C. lunata* and *R. solani*. These isolates could produce antifungal secondary metabolites such as hydrogen cyanide, siderophores, protease enzymes, and chitinase enzymes. The 16S rRNA gene identified LMA5, LMB21, LMB4, and LMD13 as *Lysinibacillus fusiformis* strains IRHB1-68, *Bacillus cereus* strain BS1, *L. fusiformis* strain S10510, and *Bacillus thuringiensis* strain Bt 35. All of these endophytic isolates demonstrated the potential to act as biocontrol agents for pathogenic fungus in rice.

Keywords: Antibiosis; Biocontrol agents; PGPBE sheath blight; *R. solani*

Introduction

Microorganisms are living entities that significantly influence various aspects of ecosystems, particularly in agriculture. The beneficial interaction of microorganisms with plant has garnered increasing study attention due to their essential role in supporting plant growth and preventing diseases in the soil environment. In a complex symbiotic collaboration, the living entities such as bacteria, fungi, actinomycetes, protozoa and algae form alliances with plant, creating a microbial ecosystem crucial to the success of modern agriculture.

Plant Growth Promoting Bacterial Endophytic (PGPBE) is a group of microorganisms that naturally inhabit the internal tissues of plants (endophytic) and have properties that improve plant growth and health (Santoyo *et al.* 2016). As an endophytic bacterium, PGPBE lives in plant tissue without showing disease symptoms (Olanrewaju and Babalola 2019). They can be found in various parts of plants, such as roots, stems, and leaves. Endophytic bacteria enter plant tissue through natural holes or lateral and primary gaps and various wounds due to plant growth. Wounds in plant tissue will cause leakage of plant metabolites and become an attractive place for these bacteria (Santoyo *et al.* 2016). Endophytic bacteria enter plant tissue through seed colonization and passively enter the tissue chemotaxis through wounds during root formation (Ali *et al.* 2014). Liu *et al.* (2017) reported that when endophytic bacteria colonize plant tissue, they experience less intense competition for nutrients than those outside the roots and less biotic stress than rhizosphere bacteria (Hardoim *et al.* 2015). According to Droby and Wisniewski (2018), once certain endophytic bacteria reach the inside of the plant, they can spread systemically and reach the flowers, fruit, and seeds. Some endophytes use the xylem tissue of their host roots to get to the meristem tissue. They accomplish this by utilizing flagella and plant

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transpiration currents. In addition, bacteria that become endophytes do not only come from plants; they can enter through several sites such as the caulosphere (stem), phyllosphere (leaf surface) (Zhou *et al.* 2024), anosphere (flowers), spermatosphere (seeds) and carposphere (fruit) (Frank *et al.* 2017). Researchers have isolated endophytic bacteria from nearly all plant parts, including roots, stems, leaves, bark, flower organs, and seeds (Hazarika *et al.* 2021). Endophytic bacteria can colonize numerous plant organs, including leaves, roots, stems, and fruit (Firdous *et al.* 2019). Moreover, researchers have identified multiple species of endophytic bacteria within a single plant (Gaiero *et al.* 2013).

PGPBE colonization of plant tissue has an impact on stimulating plant growth, which occurs through direct and indirect mechanisms PGPBE have a role as biocontrol agents and also increase plant growth (Morales-Cedeño *et al.* 2021). PGPBE directly facilitates the acquisition of nutrients for plants, such as nitrogen fixation and mobilization of phosphorus and iron through the production of organic acids and siderophores production (Glick 2012), and phytohormone production (auxin, cytokinin, gibberellin and ethylene) (Hu and Xu 2020). Meanwhile, PGPBE indirectly influences plant growth by suppressing phytopathogenic activity through various strategies, such as competition for space and nutrients, achieved through antibiotic production (Aeron *et al.* 2011), lytic enzyme production (Cao *et al.* 2018; Sumardi *et al.* 2018; Dermiyati *et al.* 2023), toxin inhibition (Cipriano *et al.* 2021), cyanide acid production, and *Induced Systemic Resistance (ISR)* (Compant *et al.* 2005; Salwan *et al.* 2023).

Köberl *et al.* (2013) found that bacteria can be employed as biocontrol agents by selecting a single bacterial strain that is opposed to various plant pathogens *in vitro*. Further analysis was carried out by controlling plant diseases *in vivo*. Laboratory conditions differ from field conditions, with various pathogens often attacking plants simultaneously. Therefore, selecting antagonistic PGPBE strains becomes essential to control various pathogens *in vitro*. According to (Dermiyati *et al.* 2023), three species of Bacillus from oil palm empty fruit bunches can suppress the growth of *Ganoderma boninense in vitro*. Safdarpour and Khodakaramian (2019) discovered that endophytic bacteria from tomato plants produce antifungal substances that can inhibit the growth of *Verticillium dahlia in vitro*. Chlebek *et al.* (2022) discovered that the *Serratia quinivorans* strain KP32 have biological activity against many pathogenic fungus, including *Rhizoctonia solani*, *Colletotrichum dematium*, *Fusarium avenaceum*, and *Sclerotinia sclerotiorum*. This strain produces a variety of biological control substances, including amylase, chitinases, and proteases, siderophores, volatile organic and inorganic chemicals, salicylic acid, and N-acyl-homoserine lactones. This study aimed to obtain PGPBE isolates that could potentially control fungi pathogens, including *R. solani* and *C. lunata*, causing sheath blight and black grain disease in rice, respectively.

Materials and Methods

Experimental details and treatments

This research was carried out from April to October 2023 at the Microbiology Laboratory, Faculty of Agriculture, Andalas University, Padang. The materials used in this research were 24 isolates of PGPBE and the fungi pathogens *R. solani* and *C. lunata*. In this study, an experiment was used to test the antimicrobial activity of 21 PGPBE isolates against the fungi *R. solani* and *C. lunata in vitro* using dual culture method. Research observation data is presented in the form of tables and Figures. Next, the data was analyzed using variance. If it is significantly different, continue with a further LSD test at a level of 5%.

Pure culture of pathogenic fungi

R. solani and *C. lunata* were sourced from the Microbiology Laboratory, Department of Plant Protection, Faculty of Agriculture, Universitas Andalas. These fungi were cultured on Potato Dextrose Agar (PDA) plates and allowed to incubated at room temperature and dark conditions for 4–7 days. The growing mycelium is transferred to a new PDA medium to get pure culture. Observations were made macroscopically and microscopically for obtained isolates of *R. solani* (Debbarma and Dutta 2015; Kusai *et al.* 2016).

PGPBE used in this study

All 24 PGPBE isolates used in this study came from the Microbiology Laboratory in the Department of Plant Protection. They are LMA2, LMA3, LMA5, LMA6, LMD11, LMD13, LMD14, LMD15, LMD16, LMB1, LMB2, LMB4, LMB6, LMB7, LMB8, LM12, LMB16, LMB19, LMB20, LMB21, LMB22, LMB27, LMB33 and LMB35 (Table 1). The isolates were revitalized on nutrient agar (NA) using the quadrant streaking method and incubated for 2 x 24 h. Purification involved streaking a single bacteria colony onto a brand new NA medium petri dish using an inoculation needle and then incubating it for 2 x 24 h. Subsequently, PGPBE isolates were confirmed through the hypersensitive reaction and Gram tests using 3% KOH.

Potential of PGPBE to suppress growth of pathogenic fungi *in vitro*

To assess the antibiosis of PGPBE against *R. solani* and *C. Lunata*, the dual culture method was adopted. The test incorporated simultaneous growth of each pathogenic fungi and PGPBE in a petri dish containing a 50% NA and 50% PDA media mixture. Fungi colonies were extracted using a cork borer (Ø 5 mm) and transferred to the center of a petri dish containing the solid NA and PDA mixture. PGPBE isolates were streaked at a distance of 3 cm from pathogenic

fungi. Control agents consisted of fungi grown without PGPBE, followed by an incubation period of 7 x 24 h. The percentage of PGPBE inhibition against fungi was measured after 7 days of incubation in the NA and PDA media mixture. The inhibition index was calculated using the formula below (Safdarpour and Khodakaramian 2019):

$$I = [(C – T)/C)] \times 100$$

Where I: Percentage of inhibition index
C: Radius of pathogenic fungi colony in the control
T: Radius of pathogenic fungi colony in PGPBE treatment

Characterization of PGPBE as biocontrol agents

Hydrogen cyanide (HCN) production: Detection of HCN production followed the method by (Lotfi *et al.* 2022), and single colonies of PGPBE were cultured on an NA medium. Whatman filter paper no. 1 was immersed in cyanide detection solution (CDS) (2 g picric acid and 8 g sodium carbonate in 200 mL sterile distilled water) and air-dried for 5 min. Placing the filter paper on the lid of a petri dish, a control disc without PGPBE was prepared in the same manner and incubated at room temperature for 4 x 24 h.

Siderophore production: Hu and Xu (2020) state that siderophore production uses the Chrome Azurol Sulfonate (CAS) agar medium. Each liter of CAS agar medium had four solutions with the following compositions: The Fe-CAS indicator, Solution (1), was made by mixing 10 mL of $FeCl_3.6H_2O$ (1 m*M*) with 10 m*M* HCL, 50 mL of CAS (1.21 mg/mL), and 40 mL of hexadecyl-trimethylammonium bromide (1.82 mg/mL). 30.40 g of piperazine-N, N-bis[2-ethanesulfolfonic acid] (PIPES) were dissolved in 750 mL of salt solution (3 g KH_2PO_4, 5 g NaCl, 10 g NH_4Cl, 20 m*M* $MgSO_4$, 1 m*M* $CaCl_2$). This Solution (2), a buffer, was then made. Distilled water was added to make the solution volume 800 mL, and the pH was measured and corrected to 6.8 using 50% KOH. Furthermore, 20 g of agar-agar was added to the Solution before sterilization. There were 493 mg of MgSO4.7H2O, 11 mg of $MnSO_4.H_2O$, 1.4 mg of H_3BO_3, 0.04 mg of $CuSO_4.5H_2O$, 1.2 mg of $ZnSO_4.7H_2O$ and 1 mg of $NaMoO_4.2H_2O$ in Solution 3. All components were dissolved in 70 mL of distilled water. Finally, Solution (4) included 30 mL of 10% (w/v) cassamino acid sterilized with a 0.45 μm membrane filter. After sterilization, solutions 2 and 4 were combined to make the CAS medium, which was heated to 50°C. Solutions 3 and 1 were slowly introduced and the mixture was homogenized with a magnetic stir bar. The CAS medium was dark green, and siderophore production was measured by streaking two 24-h-old PGPBEs. An orange tint around the bacteria colonies detected isolates capable of producing siderophores.

Protease production test: Detection of protease production was conducted in Skim Milk medium (20 g skim milk, 24 g agar per liter, 1000 mL distilled water) (Parmar 2017). Discs (diameter 6 mm) were immersed in PGPBE suspension (population density 10^8 cells/mL), air-dried for 5 min, and placed in the center of the skim milk medium. As control agents, discs (diameter 6 mm) were dipped in sterile distilled water and positioned on the medium, followed by incubation at room temperature for 2 x 24 h.

Chitinase enzyme production: For chitinase enzyme production, PGPBE cultures aged 24 h on NA were transferred using toothpicks to Petri dishes containing minimal agar medium composed of 0.5 g $MgSO_4.7H_2O$, 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.001 g $ZnSO_4$, 0.001 g $MnCl_2$, 1% colloidal chitin, and 1000 mL distilled water. Incubation of the cultures was carried out for 1–3 days at room temperature. Chitinase enzyme production was showed by the presence of a clearing zone around bacteria colonies (Mohammed 2020).

Identification of PGPBE based on the 16S rRNA gene

The 16S rRNA gene was used to identify PGPBE in rice. DNA was extracted using the Quick-DNA Fungi/Bacteria Miniprep Kit (Zymo Research D6005), and the manual protocol steps were followed. The amplification DNA of LMA5, LMD13, LMB4 and LMB21 isolates was carried out through the polymerase chain reaction (PCR) method with universal primers 27F (5' AGAGTTTGATCCTGGTCAG-3') and 1492R (5'-GGTTACCTTGTT ACGACTT-3') (Santos *et al.* 2019). A total of 50 L of dH2O, 1 L of each primer (20 M), 1 L of genomic DNA (200 ng), and 25 L of Taq HS Red Mix (Bioline, BIO-25048) were used in the PCR mixture. Using the GeneAmp PCR System 9700 (Applied Biosystems, USA), the PCR process started with DNA being denaturated at 95°C for 5 min. 30 cycles of denaturation at 95°C for 1 min, primer attachment at 55°C for 1 min, DNA elongation at 72°C for 1.5 min, and a final stage at 72°C for 5 min came after this. To separate the amplification results, a 1% agarose gel electrophoresis was used under an ultraviolet transilluminator. A DNA marker size of 1 kb was used as a standard. The size of the 16S rRNA gene sequence was ± 1500 bp. Moreover, the DNA sequence analysis was conducted using the Basic Local Alignment Search Tool (BLAST) through the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/) (Altschul *et al.* 1997).

Data analysis

Experimental data were subjected to statistical analysis using Analysis of Variance (ANOVA). Subsequently, data showing significant differences were subjected to the Least Significance Difference (LSD) test at the 5% level. A phylogenetic tree sequence was constructed using the neighbor-joining method (Saitou and Nei 1987) with the MEGA6 software (Kumar *et al.* 2004).

Results

The potential of PGPBE to suppress pathogenic fungi growth *in vitro*

A total of 24 isolates of PGPBE originating from rice plants, including four isolates from roots, five isolates from leaves, and 15 isolates from stems, were tested for their potential to suppress two pathogenic fungi, namely *C. lunata* and *R. solani*. All PGPBE isolates showed the potential to suppress the pathogenic fungi *C. Lunata* and *R. solani* growth. Isolates from rice roots could inhibit the growth of *C. Lunata* and *R. solani* by 15,667–44,763% and 12,333–47,417%, respectively. PGPBE from rice leaves also could inhibit the growth of *C. lunata* and *R. solani by* 27,980–60,520 and 21,223–60,617%, respectively. Meanwhile, isolates from rice stems showed the potential to suppress *C. lunata* and *R. solani* by 17,330–56,180 and 8,056–53,000%, respectively (Table 2). Two isolates from rice roots, namely LMA5 and LMA6, had the highest potential to suppress both pathogens compared to isolates from other roots. The LMD13 isolate from leaves was the best isolate to suppress both pathogens, while the LMB35 isolate from stems showed the highest potential to suppress both fungi compared to isolates from other stems.

Characterization of PGPBE as biocontrol agents

The ability of PGPBE to inhibit the growth of *C. lunata* and *R. solani* fungi is correlated with the production of secondary metabolite compounds. In addition to antifungal metabolite compounds, PGPBE can make cyanide acid (HCN), protease enzymes, siderophores, and chitinase enzymes. These compounds, which are integral to the interactions between microorganisms and plants, directly suppress pathogen growth. Table 3 and Fig. 1 & 2 show data on PGPBE rice plants' ability to produce secondary metabolite compounds relevant to their biocontrol potential. Based on their characteristics, isolates LMA5, LMA6, LMB4, LMB21, and LMD13 are included in the Gram-positive bacteria category, while LMB1 and LMB35 were identified as Gram-negative bacteria.

Identification of PGPBE based on the 16S rRNA gene

Based on the 16S rRNA gene, DNA analysis showed that the four PGPBE isolates from rice were similarity with the *Bacillus* spp. group in the gene bank, ranging from 98.42 to 99.02%. LMA5 had a similarity of 98.76% with *L. fusiformis* strain IRHB1-68/OP364549.1, endophytes of soybean from China. LMB4 showed a similarity of 99.14% with *L. fusiformis* strain S10510 (KF956556) obtained from the rhizosphere of *Phaseolus vulgaris* in India. Furthermore, LMD13 had a similarity value of 99.86% with *B. thuringiensis* strain Bt 35 PGPBE derived from rice leaves in India. LMB21 shared a similarity of 98.76 with the *B. cereus* bacteria strain BS1 (KR063181) originating from the rhizosphere of kale plant in China (Table 4). According to the dendrogram in Fig. 3, there are four groups of bacteria,

Table 1: Isolates PGPBE used in this study

S. No	Name of the isolates	Isolation source	Location
1	LMA 2	rice roots	Padang
2	LMA 3	rice roots	Padang
3	LMA 5	rice roots	Padang
4	LMA 6	rice roots	Padang
5	LMD 11	rice leaves	Padang
6	LMD 13	rice leaves	Padang
7	LMD 14	rice leaves	Padang
8	LMD 15	rice leaves	Padang
9	LMD 16	rice leaves	Padang
10	LMB 1	Rice straw	Padang
11	LMB 2	Rice straw	Padang
12	LMB 4	Rice straw	Padang
13	LMB 6	Rice straw	Padang
14	LMB 7	Rice straw	Padang
15	LMB 8	Rice straw	Padang
16	LMB 12	Rice straw	Padang
17	LMB16	Rice straw	Padang
18	LMB 19	Rice straw	Padang
19	LMB 20	Rice straw	Padang
20	LMB 21	Rice straw	Padang
21	LMB 22	Rice straw	Padang
22	LMB 27	Rice straw	Padang
23	LMB 33	Rice straw	Padang
24	LMB 35	Rice straw	Padang

Table 2: The potential for PGPBE to suppress growth of pathogenic fungi and bacteria *in vitro*

Isolate	Inhibitory (%)	Isolate	Inhibitory (%)
C. lunata		*R. solani*	
Root endophytes			
LMA 5	44.763 a	LMA 5	47.417 a
LMA 6	42.593 a	LMA6	32.223 ab
LMA 3	26.667 b	LMA3	20.000 bc
LMA 2	15.667 c	LMA 2	12.333 cd
Control	0.0000 d	Control	0.0000 d
Leaf endophyte			
LMD 13	60.520 a	LMD13	60.617 a
LMD 14	43.003 b	LMD14	37.223 b
LMD 16	42.127 b	LMD 11	34.103 bc
LMD 11	33.137 c	LMD16	30.490 c
LMD 15	27.980 c	LMD15	21.223 d
Control	0.0000 d	Control	0.0000 e
Stem endophyte			
LMB 35	56.180 a	LmB1	53.000 a
LMB 1	52.137 ab	LMB 35	46.177 b
LMB 4	46.660 abc	LMB 21	43.113 bc
LMB 21	45.630 abc	LMB 6	42.535 bc
LMB 22	38.533 bcd	LMB 12	40.833 cd
LMB 27	38.287 bcd	LMB 27	37.083 de
LMB 8	36.700 bcd	LMB 20	36.767 de
LMB 7	34.160 cd	LMB 19	35.833 e
LMB 33	33.303 cd	LMB 4	35.157 e
LMB 6	29.333 de	LMB 8	33.543 e
LMB 19	28.333 de	LMB 33	33.183 e
LMB 20	28.003 de	LMB 22	20.863 f
LMB 12	26.667 de	LMB16	13.997 g
LMB 2	17.330 e	LMB 7	8.0567 h
LMB16	15.750 e	LMB 2	7.1230 h
Control	0.0000 f	Control	0.0000 i

Description: Numbers in the same column and followed by the same lowercase letter were not significantly different based on the LSD significance level of 5%

Table 3: Characterization of PGPBE as biocontrol agents

Isolate	Gram reaction	HCN production	Siderophore production	Protease production	Chitinase enzyme
LMA2	-	+	-	+	+
LMA3	-	+	-	+	+
LmA5	+	+	-	+	+
LmA6	+	+	-	+	+
LmB1	-	+	+	+	+
LmB2	+	+	-	+	+
LmB4	+	+	+	+	+
LmB6	-	+	-	+	+
LmB7	+	+	-	+	-
LmB8	-	+	-	+	+
LmB12	+	+	+	+	-
LmB16	+	+	+	+	-
LmB19	+	+	+	+	+
LmB20	-	+	-	+	+
LmB21	+	+	+	+	+
LmB22	+	+	-	+	+
LmB27	+	+	-	+	+
LmB33	+	+	-	+	+
LmB35	-	+	-	+	+
LmD11	-	+	+	+	-
LmD13	+	+	+	+	+
LmD14	+	+	-	+	+
LmD15	+	+	-	+	-
LmD16	-	+	-	+	+

Description: - : Absent, + : Present

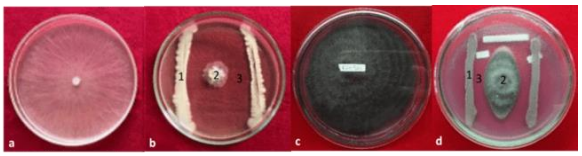

Fig. 1: Inhibitory power of PGPBE isolates against pathogenic fungi. **a.** *R. solani* (control), **b**. PGPBE LMD 13 and *R. solani*: b1. Endophytes isolate LMD13, b2. *R. solani*, b3. Clear zone. **c**. *C. lunata* on PDA media, **d**. PGPBE LMA5 and *C. lunata* isolates. d1. Endophytes islate LMA5, d2. *C. lunata*, d3. Clear zone

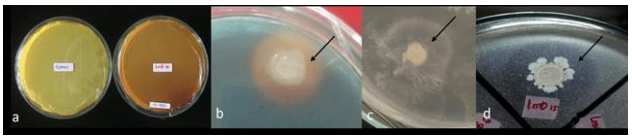

Fig. 2: Characterization of PGPBE as biocontrol agents. **a**. Production of HCN, **b**. Production of siderophores, **c**. chitinase enzymes and **d.** protease. Arrows show clear zones

with the best PGPBE from each organ of the rice plant forming a group with close relatives, namely *Bacillus*, *Lysinibacillus*, and *Pseudomonas fluorescens* bacteria as an outgroup. *Bacillus* bacteria closely related to isolates LMB21 and LMD13, while *B. cereus* closely relates to isolate LMB21, and *B. thuringiensis* closely relates to isolate LMD13. Meanwhile, the BLASTn results showed that isolates LMB4 and LMA5 were similar to *L. fusiformis*, but in the dendrogram, the two isolates formed their group, which was different from L. fusiformis. These results indicate that the two isolates are likely different species of *Lysinibacillus fusiformis*.

Table 4: Percentage similarity of 16S rRNA gene sequence of PGPBE isolates from rice plant with its counterpart species in the GenBank data center

Isolate	Origin of isolate	Equivalent species	Similarity (%)	Accession No
LmA6	Root	*B. cereus* MD152	96.87	MT642947.1*
LmB2	Stem	*B. thuringiensis* ATCC 10792	98.20	CP020754.1*
LmB1	Stem	*O. intermedium* strain OI1	97.52	KT985368.1*
LmB35	Stem	*S. maltophilia* strain A1w2	97.92	AY512625.1*
LMA5	Root	*L. fusiformis* strain IRHB1-68	98.76	OP364549.1
LMD13	Leaf	*B. thuringiensis* strain Bt 35	99.86	KY921958.1
LMB4	Stem	*L. fusiformis* strain S10510	99.14	KF956556.1
LMB21	Stem	*Bacillus cereus* strain LXJ77	99.42	MN746190

*Rahma *et al.* (2022)

Discussion

PGPBE lives in plants and has more stable environments than microorganisms in the rhizosphere and soil. They are less susceptible to changes in environmental variables (Morales-Cedeño *et al.* 2021). Tsipinana *et al.* (2023) reported that endophytes and plants have developed a mutually advantageous partnership through a gradual process of co-evolution. Dubey *et al.* (2020) reported that plants can supply nutrients to endophytic microorganisms, which in turn assist plants in defending against pathogen invasion. PGPBE provides ecological benefits to the host plant, including increased stress tolerance, PGP, and resistance to pathogens (Hardoim *et al.* 2015). The PGPBE possesses anti-pathogenic properties and is capable of synthesizing antibiotic molecules. This bacteria can regulate plant pathogens through antibiosis, competition, lysis, promoting resistance, and creating growth chemicals. Bacteria can produce secondary metabolites that might hinder the growth or cause harm to pathogens.

The mechanisms of PGPBE in controlling the growth of fungal pathogens depend on their capabilities. There are several mechanisms of action, such as producing antibiotics, siderophores and lytic enzymes; competing for substrate and habitat; and activating the plant's systemic resistance (Meliah *et al.* 2021). Among bacteria, many members of the genus *Bacillus* and *Pseudomonas* have biocontrol ability. They have been reported to suppress fungal pathogen growth *via* secretion of antibiotics and siderophores and inducing the plants systemic resistance (Khabbaz *et al.* 2015; Shafi *et al.* 2017).

The cell walls of most filamentous fungi consist of chitin, *β*-glucan, mannan, and protein. The structural robustness of the cell wall directly influences its overall shape and stability. The cell wall plays a variety of important tasks throughout its contact with the environment (Meliah *et al.* 2021). Chitin, in particular, plays an important role in providing structural rigidity to fungal cell walls, enabling them to withstand physical and chemical attacks (Ajuna *et al.* 2013). Therefore, disrupting these cell wall constituents is an important strategy to regulate fungal survival. The hydrolytic enzymes from *Bacillus* species such as chitinases, *β*-1, 3-glucanases, proteases, lipases, amylases and cellulases in the biological control of

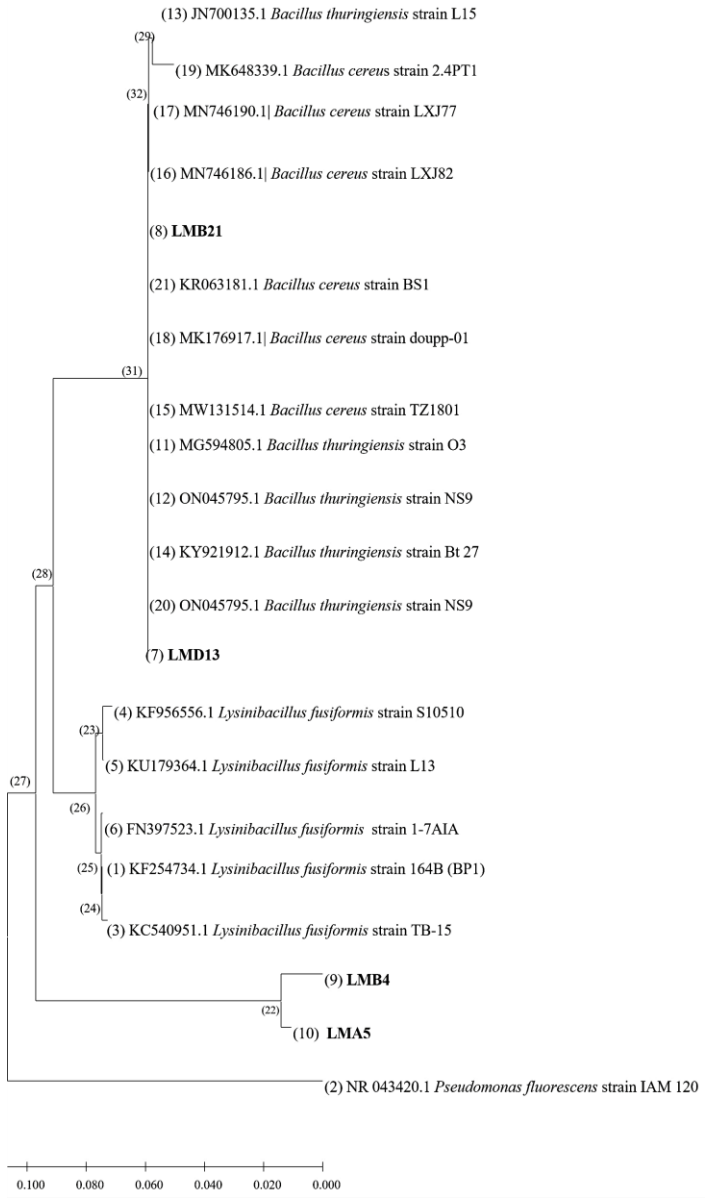

Fig. 3: Dendrogram of PGPBE isolates based on the 16S rRNA gene with the Neighbor-Joining Tree method using MEGA X

phytopathogens which could be a more sustainable alternative to chemical pesticides. Hydrolytic enzymes such as chitinase and proteinase produced by bacterial groups have long been reported to be able to cut the glycosidic and peptide bonds of filamentous fungi.

Bacteria that produce siderophores have been identified in several genera, including *Bacillus* (Kesaulya *et al.* 2018), *Pseudomonas* (Baune *et al.* 2017), *Rhodococcus* (Sah and Singh 2015), *Serratia* (Gerc *et al.* 2014), *Streptomyces* (Gáll *et al.* 2016) and others. Bacterial synthesis of siderophores is advantageous for plants and is regarded as a notable characteristic of plant growth-promoting bacteria (PGPB), which can impact plant growth (Timofeeva *et al.* 2022). Soil microorganisms create siderophores to provide plants with iron, which enhances their growth. Nevertheless, bacterial siderophores are accountable for inhibiting the growth of certain plant-pathogenic fungi and bacteria (Scavino and Pedraza 2013).

Kumar *et al.* (2020) stated that bacteria of the genus *Bacillus*, particularly Gram-positive PGPBE, are widespread in rice plant and play a dominant role in controlling pathogenic fungi. They produce enzymes comprising lipopeptides that function as antifungi agents, effectively destroying the cell walls of fungi. Rais *et al.* (2017) added that *Bacillus* can produce siderophores, proteases, and glucanases, inhibiting the development of *R. solani*. According to Won *et al.* (2019), *Bacillus* produced cell wall-degrading enzymes such as

chitinase and *β-1,3-glucanase*. Putri *et al.* (2022) stated that PGPBE from the leaves, stems and roots of healthy rice plant can produce HCN compounds, dissolve phosphate, and degrade chitin, showcasing their potential as biocontrol agents capable of suppressing the development of *Pyricularia* and *Xanthomonas oryzae*.

According to Johnson *et al.* (2019), the 16S rRNA gene sequence spans approximately 1550 bp, comprising both conserve and variable regions. The nucleotide base sequence encoding the 16S rRNA gene is a fundamental standard for bacteria identification, with specific sequences for different bacteria. Identification relies on the best-matching sequences of bacteria with all known 16S rRNA gene sequences in the GenBank data center.

The analysis results of the nucleotide sequences of the antifungal-producing endophytic bacteria are shown in Table 4. The isolates showed high similarities with some species in the genera *Bacillus, O. intermedium, S. maltophilia, L. fusiformis. Lysinibacillus* sp. is a bacterium that belongs to the family Bacillaceae. It is Gram-positive and has a rod-shaped structure. These microbes were once classified as part of the Bacillus genus, but they have been reclassified under the genus Lysinibacillus due to recent taxonomic changes (El-Sayed *et al.* 2022). The genus Bacillus produces bioactive metabolites with a diverse range of structures and a broad variety of bioactivities, including antifungal, antibacterial, anticancer and antineoplastic activities (Liu *et al.* 2017). The *Bacillus* sp. Recognized for their ability to manufacture antifungal antibiotics includes *B. subtilis, B. brevis, B. licheniformis, B. circulans,* and *B. cereus* (Meliah *et al.* 2021). Clarridge (2004) reported that bacteria identification similarity value ≥ 94% shows the same species as its counterpart in GenBank. The 16S rRNA gene is a highly accurate marker, a key component in the ribosome pathway for protein biosynthesis. The molecular identification results showed that PGPBE found in rice plant was highly diverse and possessed the characteristics of biocontrol agents. This was in line with the observation of Rahma *et al.* (2022), showing that isolates LMA6, LMB2, LmB 1 and LmB 35 closely resembled *Bacillus cereus* MD152, *B. thuringiensis* ATCC 10792, *Ochrobactrum intermedium* strain OI1, and *S. maltophilia* strain A1w2. All bacteria had been extensively documented for their role as potent biocontrol agents against plant pathogens.

Additionally, Kumar *et al*. (2020) identified the broad-spectrum antagonistic effects of bacteria isolation from root and stem tissues against pathogenic bacteria and fungi. Approximately five PGPBE isolates from rice stems, including *B. subtilis, B. cereus*, and *B. pumilus*, had high antagonism against *R. solani*. Whipps (2001) identified crucial secondary metabolites such as ammonia, butyrolactone, 2,4-diacetylphloroglunicol, hydrogen cyanide (HCN), and extracellular enzymes to suppress pathogen growth. Tashi-Oshnoei *et al.* (2017) reported Gram-negative bacteria such as *Stenotrophomonas* sp. can produce siderophores, protease, and HCN, along with phosphate dissolution. Alijani *et al.* (2020) observed that Gram-negative PGPBE *S. maltophilia*, isolation from strawberry leaves, can suppress the growth of *C. nymphaeae* and simultaneously produce chitinase, pectinase, and siderophores.

This research established the significant contribution of endophytic bacteria in the rice plant as producers of antifungal substances. Endophytic bacteria can indirectly enhance plant growth by combating plant diseases. Their ability to excrete secondary metabolites, which contribute to the destruction of fungal cell walls, further reinforces their capacity to impede fungal proliferation. Research on the colonization of these endophytic bacteria in plants is anticipated to be conducted soon as an initial measure to harness their potential as biocontrol agents.

Conclusion

In conclusion, the study identified four of the best PGPBE isolates from various parts of rice plants with the ability to suppress the growth of two pathogenic fungi, namely isolates LMA5 and LMA6 hailed from rice roots, LMB35 from rice stems and LMD13 from rice leaves. These isolates had characteristics indicative of their potential as biocontrol agents, producing secondary metabolites with antifungal properties, including hydrogen cyanide, siderophores, protease enzymes, and chitinase enzymes. Molecular identification based on the 16S rRNA gene showed that LMA5 resembled *L. fusiformis* strain IRHB1-68, LMB21 was similar to *B. cereus* strain BS1, LMB4 mirrored *L. fusiformis* strain S10510 and LMD13 shared similarity with *B. thuringiensis* strain Bt 35. The PGPBE isolates had significant potential as biocontrol agents against pathogenic fungi in rice plants.

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Conflicts of Interest

All authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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