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Application of Bacterial Endophytes to Control Bacterial Leaf Blight Disease and Promote Rice Growth

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***Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial leaf blight (BLB) disease in rice (*Oryza sativa* L.) and it is among the most destructive pathogen responsible for severe yield losses. Potential bacterial biocontrol agents (BCAs) with plant growth promotion (PGP) abilities can be applied to better manage the BLB disease and increase crop yield, compared to current conventional practices. Thus, this study aimed to isolate, screen, and identify potential BCAs with PGP abilities. Isolation of the BCAs was performed from internal plant tissues and rhizosphere soil of healthy and *Xoo*-infected rice. A total of 18 bacterial strains were successfully screened for *in vitro* antagonistic ability against *Xoo*, siderophore production and PGP potentials. Among the bacterial strains, 3 endophytes, *Bacillus* sp. strain USML8, *Bacillus* sp. strain USML9, and *Bacillus* sp. strain USMR1 which were isolated from diseased plants harbored the BCA traits and significantly reduced leaf blight severity of rice. Simultaneously, the endophytic BCAs also possessed plant growth promoting traits and were able to enhance rice growth. Application of the selected endophytes (BCAs-PGP) at the early growth stage of rice exhibited potential in suppressing BLB disease and promoting rice growth.**

Keywords **:** bacterial leaf blight (BLB), biocontrol agent, plant growth promotion (PGP), *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)

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Rice (*Oryza sativa* L.) is a type of grass cultivated as a form of staple food in most countries in Asia (Rajamoorthy et al., 2015). High-quality varieties of rice have been developed to meet consumers' demands and help reduce the reliance on imported fragrant rice. Among the varieties, MR 84 released in 1986 by the Malaysian Agricultural Research and Development Institute (MARDI), was one of the most popular rice varieties grown in Malaysia. Newer rice varieties with high yielding were later released and planted by farmers (e.g., MR220 CL1, MR220 CL2, MR263, MR253, and MR269) (Elixon et al., 2017). Unfortunately, the continuous planting of selected rice varieties in the same plots has caused widespread diseases called bacterial leaf blight (BLB) and bacterial leaf streak, especially during the wet seasons. The BLB disease was first encountered in 1884 by a farmer in Kyushu, Japan, and was later found in different parts of Asia, the United States of America, and Africa (Gnanamanickam, 2009). It caused approximately 70% yield loss to susceptible rice varieties under environmental conditions favorable for the disease infection (Saad, 1995; Saad and Habibuddin, 2010; Zou et al., 2010).

The BLB disease is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), a Gram-negative, rod-like shape, and motile bacterium with a single polar flagellum. The *Xoo* is a biotroph pathogen that keeps the host alive and feeds on

living plant tissues (Freeman and Beattie, 2008). Currently, no chemical control or bactericides is available to treat the disease and the farmers can only control it through cultural practices, e.g., using resistant varieties, planting space, keeping the farm clean, and using vector control. Suitable rice planting space can help reduce the disease severity index and the number of rice planted (Meah, 1987).

Previously, several findings were reported on the isolation and screening for potential biocontrol agents (BCAs) against BLB disease in rice (Azman et al., 2017; Ji et al., 2008; Ngalimat et al., 2021; Suárez-Moreno et al., 2019). Application of BCAs, which are able to produce metabolites against plant pathogens and perform spatial separation from the host plants via induced systemic resistance or systemic acquired resistance, may control the pathogens and suppress the disease (Kloepper et al., 1992). Locally isolated *Pseudomonas fluorescens* is an example of a potential BCAs for reducing BLB disease incidences when used to treat rice seeds (Shivalingalah and Umesha, 2013). *Acinetobacter* sp., *Bacillus* sp., *B. licheniformis*, *B. amyloliquefaciens*, *Burkholderia cepacia*, *Oceanobacillus oncorhynchi*, *Paenibacillus cineris*, *Pantoea* sp., *P. vagans*, *Pseudomonas putida*, and *Staphylococcus warneri*, were also known as antagonistic BCAs against the *Xoo* (Azman et al., 2017).

The BCAs would be more beneficial if the isolates are able to promote plant growth (Jiao et al., 2021; Ngalimat et al., 2021) and categorized as plant growth promoting bacteria (PGPB) (Qiao et al., 2017; Zhang et al., 2013). The PGPBs can directly promote plant growth by providing compounds (e.g., organic acids, siderophores) that increase mineral nutrient solubilization and availability (e.g., potassium, phosphate, and iron), performed nitrogen fixation process, and phytohormone production (Glick et al., 2007; Saharan and Nehra, 2011). Ngalimat et al. (2021) highlighted PGPB-mediated functional traits with the ability in biocontrol of rice pathogens (e.g., *Xoo*) and enhance rice growth. Other findings on BCAs-plant growth promotion (PGP) potentials were described by Huang et al. (2013) that selected rhizobacteria could function as biocontrol agents against bacterial wilt disease of tomato plants caused by *Ralstonia solanacearum*. Population densities of *R. solanacearum* in the rhizosphere soil and crown section of tomato plants were reduced due to the PGPB inoculation treatments. Additionally, Jiao et al. (2021) have proven that PGPB with biocontrol potentials is an environmentally friendly way of controlling plant disease, increasing crop yield, and bringing dual benefits to the host plants. The BCAs-PGP suppresses diseases by directly synthesizing pathogen-antagonizing compounds and triggering plant immune responses. Another indirect plant growth promotion potential of the PGPB was to lessen or prevent harmful effects of phytopathogenic organisms on the host plants due to the production of antagonistic substances or the induction of pathogen resistance by PGPB.

Numerous studies associated with the isolation and screening of BCAs isolated from healthy plants have been reported. However, the isolation and screening of BCAs-PGP from BLB-infected rice may be a better alternative for the isolation procedures. According to Smith et al. (2015), microbial strains that co-evolved with diseased plants for protracted periods and promote plant growth are likely to provide more than one benefit to the host plants. Phytomicrobiome members may synthesize and excrete a range of inter-organismal signal compounds that defend their host plant against pathogens and abiotic stresses. An earlier report by Huang et al. (2013) cited that the rhizosphere of a diseased host plant (tomato) could be a potential source for screening novel BCAs-PGP against bacterial wilt disease caused by *Ralstonia solanacearum*. Yuan et al. (2017) and Purahong et al. (2018) also shared similar thoughts that natural populations of rhizobacteria and endophytes in diseased plants were the most frequent sources of BCAs against specific pathogens. The relationship between diversity and biocontrol activity of endophytic bacteria obtained from healthy and diseased *Agave sisalana* was proven by De Souza et al. (2021). The results showed that diseased plants tend to offer a higher population of bacterial endophytes than healthy ones. However, the genetic diversity and the proportion or activity of potential BCAs against the pathogens of *A. sisalana* were not influenced by the health status of the host plant. The improved performances of the isolates from the diseased-plant rhizosphere may be due to their adaptations to the harsh environment (imposed by the pathogen) and were more efficient at colonization in the rhizosphere. Considering the above aspects of BCAs-PGP potentials, thus, this study was conducted with the following objectives: (1) to isolate, screen, and identify BCAs-PGP from local BLB disease-infected rice; (2) to determine the effects of selected BCAs-PGP on BLB disease suppression control and its effect on growth promotion of infected rice.

Materials and Methods

Isolation of bacteria from *in planta* tissues and rhizosphere soil of healthy and BLB disease-infected rice. A total of 3 healthy and 3 BLB disease-infected rice (*Oryza sativa* L.) variety (MR220 CL2) together with the plant rhizosphere soil were collected at MARDI station, Kam-

pung Sungai Sireh Batu Sebelas, 45500 Tanjung Karang, Selangor, Malaysia. All samples were collected randomly from the same rice field plot. The samples were kept in an icebox during transportation to the laboratory. Each plant sample was separated into leaves (1 g) and roots (1 g), followed by surface sterilization with 95% ethanol (10 s), soaked in 1% sodium hypochlorite (NaClO) (2 min; leaf and root) before rinsing with six changes of sterilized distilled water. Surface-sterilized plant samples were placed into universal bottles containing 9 ml of nutrient broth (NB) and crushed using a sterilized glass rod for separating the *in planta* endophytes from the plant tissues. Regarding bacterial isolation from rhizosphere soils, the samples (1 g) were placed into universal bottles containing 9 ml of NB and shaken for 1 min. A total of 1 ml solution was then transferred to another test tube containing 9 ml of NB for serial dilution procedure. The homogenized sample (0.1 ml) was then spread onto nutrient agar (NA) plates using a sterilized glass hockey stick and was incubated for 24 h at 28°C (Bashan et al., 1993). Bacterial colonies grown on NA plates were subcultured several times onto the same medium to obtain pure isolates and were preserved in 50% sterile glycerol stocks. A total of 3 potential endophytic bacterial BCAs-PGP were successfully isolated and deposited at the Centre for Chemical Biology, Microbial Diversity Library (CCB-MBL) of Universiti Sains Malaysia with accession numbers of CCB-MBL 5008 USML8, CCB-MBL 5009 USML9, and CCB-MBL 5010 USMR1.

***In vitro* pre-screening of antagonism assay against *Xoo*.** The stock culture of *Xoo* was obtained from Plant Protection Department, Faculty of Agriculture, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. The *Xoo* strain was cultured on NA plates for 72 h at 28°C. The bacterial cells were collected from NA plates and suspended in sterilized distilled water (1 ml), then determined cell density ($OD_{600\ nm}$) using a spectrophotometer. One hundred µl of *Xoo* culture (10^8 cfu/ml) was spread on NA plates for *in vitro* antagonism pre-screening of potential BCAs isolates. Ten µl suspension (10^8 cfu/ml) of each bacterial isolate was applied on a sterile paper disc (0.5 cm diameter) and placed on NA plates spread with *Xoo* culture in triplicates and incubated at room temperature for 24 h (El-shakh et al., 2015; Hastuti et al., 2012; Nagendran et al., 2013). The diameter of clear halo zones, which indicated *in vitro* antagonisms of the isolates against *Xoo,* was measured and recorded (Supplementary Data 1).

Siderophore production ability of isolates. The screening for siderophore production was performed on chrome azurol S (CAS) medium. To prepare the CAS agar medium, 100 ml of Minimal Media 9 salt solution was added to 750 ml of water, followed by the addition of 32.2 g PIPES (piperazine-1,4-bis(2-ethanesulfonic acid), completely dissolved in water by adjusting the pH to 6.8) and 15 g of bacteriological agar. After autoclaving, 30 ml of blue dye solution was mixed into the agar solution and poured onto plates aseptically. A loopful of bacterial isolates were streaked onto the CAS agar medium in triplicates and incubated for 24 h at 28°C. The formation of clear halo zones around the colonies due to the removal of iron from the dye indicated the production of siderophore (Hastuti et al., 2012; Hussein and Joo, 2014; Louden et al., 2011). The siderophore-producing isolates were later analyzed (in triplicates) for catecholate, carboxylate, and hydroxamate production in succinic broth (Sayyed et al., 2005). The broth was prepared as follows (in g/l): K_2HPO_4, 6.0; KH_2PO_4, 3.0; $MgSO_4.7H_20$, 0.2; $(NH_4)_2SO_4$, 1.0, and succinic acid, 4.0. The pH was adjusted to 7.0 with 2 N NaOH before autoclaving. The bacterial isolates (24 h culture; 1 ml) were inoculated into a conical flask containing 100 ml of succinic broth, incubated on an orbital shaker for 24 h at 28 ± 2°C, and harvested by centrifugation at 10,000 rpm for 10 min. A total of 0.2 ml of 10% (v/v) H_2SO_4 was added into a universal bottle containing 2.3 ml of sterile distilled water with 1 ml of culture supernatant for detecting catecholate siderophore (Rioux test) (Rioux et al., 1983). Then, 1 ml of 1% (w/v) ferric ammonium citrate ($C_6H_8FeNO_7$) (in 0.09 N H_2SO_4) was added, followed by the addition of 0.4 ml of 2 M ammonium fluoride (NH_4F), 0.4 ml of 1% (w/v) 1,10-phenanthroline monohydrochloride monohydrate (add 0.4 ml of water for blank) and 0.6 ml of 3 M hexamethylenetetramine. The mixture was heated to 60°C for 1 h and cooled down. A mixture with color changes from light yellow to orange or reddish-orange indicated a positive reaction. The isolates were also tested spectrophotometrically for carboxylate siderophore (Shenker et al., 1992). One ml of culture supernatant was mixed with 1 ml of 750 µM $CuSO_4$ and 2 ml of 0.2 M acetic acid ($C_2H_4O_2$). A positive reaction is presented by color changes in the mixture from blue to green. At the same time, hydroxamate siderophore production was tested using tetrazolium, Csaky, and Atkin tests. For the tetrazolium test, 1 ml of culture supernatant was added with 1 or 2 drops of NaOH and a pinch of tetrazolium salt (Snow, 1954). A positive reaction was shown by forming a deep red color solution. Another observation is called the Csaky test, which detected hydroxamic acid-based hydroxamate siderophores (Csaky, 1948). The test involved hydrolyzation of culture supernatant (1 ml) with H_2SO_4 (1 ml) in a boiling water bath for 6 h. Next,

3 ml of sodium acetate ($C_2H_3NaO_2$), 1 ml sulfanilic acid ($C_6H_7NO_3S$), and 0.5 ml iodine solution were added. Excess iodine was destroyed with 1 ml of sodium arsenite solution ($AsNaO_2$). One ml of alpha naphthylamine was added, and the total volume of the solution was made up to 10 ml with distilled water. The color was allowed to develop for 30 min, where the positive reaction showed color changes from colorless to red. For Atkin's assay, a total of 0.5 ml culture supernatant was mixed with 2.5 ml of reagent (0.1771 g iron triperchlorate ($Fe(ClO_4)_3$) dissolved in 100 ml of distilled water with 1.43 ml perchloric acid ($HClO_4$). The mixture was incubated at room temperature for 5 min to allow for color development. The negative control exhibited colorless to slightly yellow, while the positive reaction was orange in color (Atkin et al., 1970).

PGP properties of endophytes. Selected endophytes were tested for their potential in potassium and phosphate solubilization and indole acetic acid (IAA) production. For the potassium solubilization assay, 10 µl of endophytes suspension was dropped onto the Aleksandrov agar plate (in g/l: glucose, 5; $MgSO_4 \cdot 7H_20$, 0.5; $FeCl_3$, 0.005; $CaCO_3$, 0.1; $Ca(H_2PO_4)_2$, 2; potassium aluminum silicate [Muscovite mica], 2, and bacteriological agar, 15) and incubated for 24 h at 30°C (Hu et al., 2006). A clear halo zone formed around the colonies indicated a positive reaction and the ability of the isolates to solubilize potassium. Pikovskaya agar medium was used to determine the ability of isolates to solubilize phosphate. Composition of the medium were as follows (in g/l): glucose, 10; $Ca_3(PO_4)_2$, 5; $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; $MgSO_4 \cdot 7H_20$, 0.1; KCl, 0.2; yeast extract, 0.5; $MnSO_4 \cdot H_2O$, 0.002; $FeSO_4 \cdot 7H_2O$, 0.002, and bacteriological agar, 15. The medium was inoculated with 10 µl of endophytes suspension and incubated for 24 h at 28°C. The ability to solubilize phosphate was derived from the formation of a clear halo zone around the colony. The quantitative estimation of phosphate solubilization in a broth was carried out in Erlenmeyer flasks (250 ml) containing 100 ml of National Botanical Research Institute's Phosphate (NBRIP) growth medium and inoculated with 1 ml of culture (10^8 cfu/ml) (Nautiyal, 1999). The NBRIP broth was prepared as follows (in g/l): glucose, 10; $Ca_3(PO_4)_2$, 5; $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7H_20$, 0.25; KCl, 0.2, and $MgCl \cdot H_2O$, 5. The flasks were incubated for 48 h at 30°C on an incubator shaker at 180 rpm. Five ml of the broth culture from each flask were sampled and filtered through Whatman No. 1 filter paper and centrifuged at 10,000 rpm for 20 min. One ml of the supernatant was taken, followed by addition of 2.5 ml of Barton's reagent, made up to 50 ml with sterile distilled water and incubated for color (yellow) development. The intensity of the yellow color was detected by a spectrophotometer at 430 nm, and the amount of P-solubilized was extrapolated from the standard curve.

The production of phytohormone, IAA, was determined colorimetrically (Gordon and Weber, 1951; Patten and Glick, 2002). One ml of the pre-culture of endophytes in NB broth was transferred into a new 100 ml of NB and added with 5 ml of L-tryptophan, which acted as the precursor for IAA production. The bacterial culture (1.5 ml) was transferred into a sterile Eppendorf tube and centrifuged at 7,000 rpm for 7 min. The supernatant (1 ml) was transferred into a cuvette, followed by addition of 1 ml of Salkowski's reagent (2 ml of 0.5 M $FeCl_3$ mixed with 98 ml of 35% perchloric acid). The mixture was incubated for 30 min for the pink color to develop, indicating IAA production (Asghar et al., 2002; Patten and Glick, 2002). The absorbance of each sample was detected by using a spectrophotometer at 535 nm. The values were compared to the IAA standard curve to obtain the concentrations.

BLB disease control and growth promotion of inoculated rice. The rice seeds (*Oryza sativa* L.) variety MR220 CL2 obtained from the MARDI, Kepala Batas, Pulau Pinang, Malaysia, were sterilized with 70% ethanol for 1 min and rinsed with three changes of sterile distilled water. Surface-sterilized seeds were placed into glass bottles containing 1% agar and incubated for 72 h at 28 ± 2°C in the dark for germination. Fully germinated rice seedlings (7 days old) were planted into pots containing 1 kg sterilized sand and fertilized with Yoshida nutrient (Ahmad et al., 2016; Yoshida et al., 1976) (Supplementary Table 1) for 45 days after transplanting (DAT) under standard plant house conditions with natural light and natural fluctuations of temperature and humidity. The seedlings were treated with the following treatments: (1) –BCA, –*Xoo*, (2) +*Azospirillum brasilensis* Sp7 (ATCC 29145; a well-known plant growth enhancer), –*Xoo*, (3) –BCA, +*Xoo*, (4) +Sp7, +*Xoo*, (5) +USML8, +*Xoo*, (6) +USML9, +*Xoo* and (7) +USMR1, +*Xoo*. Infection of rice seedlings with *Xoo* (10^9 cfu/ml) was conducted at 30 DAT via leaf clipping method. Respective BCAs-PGP (USML8, USML9, and USMR1) were inoculated to the seedlings (5 ml/seedlings at 10^9 cfu/ml) at 1 DAT, 29 DAT, 1 day after infection (DAI), 3 DAI, 5 DAI, 7 DAI, and 13 DAI (Ji et al., 2008). Additionally, *A. brasilense* Sp7 was used as a positive control treatment for plant growth enhancement. Observations and data were collected at 15 DAI, which includes lesion length (cm), diseased leaf area (%DLA) (Yasmin et al., 2017), disease scale (International Rice Research Institute, 2002), disease suppression (%) (Ji et

al., 2008), shoot length (cm), shoots and roots dry weight (g), and root volume (cm^3) of the host plants (Supplementary Data 1, Supplementary Table 2). The leaf greenness of the youngest fully expanded leaves for each plant was also recorded using a portable leaf-chlorophyll meter (MINOLTA SPAD-502, Minolta Camera Co., Osaka, Japan) (Neufeld et al., 2006). The leaf chlorophyll content was determined based on the standard curve of leaf greenness values and total leaf chlorophyll content (mg chlorophyll/mg fresh leaf weight) (Amir et al., 2001). The experiment was laid out in a Completely Randomized Design with 6 replications and harvested at 45 DAT. The data were statistically analyzed by IBM SPSS Statistics version 26.0 (http://www.ibm.com/products/spss-statistics). Differences between inoculation treatments were investigated with Welch's analysis of variance (ANOVA) followed by Tukey's honest significant difference test. Differences were considered significant at the $P < 0.05$ level. Similar experiments were repeated 3 times in the glasshouse, and all repetitions were independent.

Molecular identification and phylogenetic analysis of endophytes. The genomic DNAs of selected potential BCAs-PGP endophytes (USML8, USML9, and USMR1) were extracted using GenCheck DNA Extraction Reagent (FASMAC Co. Ltd., Midorigaoka, Atsugi, Japan). The 16S rRNA gene of the three selected strains was amplified by polymerase chain reaction (PCR) using the universal primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR products were sequenced, and the partial 16S rRNA sequences (approximately 1,350 bp) were used to identify the strains using the Basic Local Alignment Search Tool (Blastn) analysis available in the National Centre for Biotechnology Information (NCBI) server. The sequences of USML8, USML9, and USMR1 were submitted to the DDBJ/EMBL/GenBank databases under the accession numbers MZ820861, MZ820862, and MZ820863, respectively. The sequences and the reference strain were aligned using MUSCLE (https://www.megasoftware.net) (Edgar, 2004). The phylogenetic trees were reconstructed via neighbor-joining (Saitou and Nei, 1987), implemented in

Table 1. Pre-screening of bacterial BCAs isolated from tissues of root, leaf and rhizosphere soils of healthy and BLB disease-infected disease rice via *in vitro* antagonism assay against *Xoo* and siderophore production

No.	Isolate	Plant condition	*In vitro* antagonism test[a]	Siderophores production[b]
1	*Azospirillum brasilensis* Sp7 (Control)		++	++
2	USMR3	Healthy rice	+	+
3	USMS5		++	+
4	USML3		+	++
5	USML4		++	+
6	USML5e		+	+
7	USML10	Diseased rice	+	++
8	USMR10		++	–
9	USMR10f		++	++
10	USMR9		++	+
11	USMS10		++	+
12	USMS10c		+	+
13	USMS2		+	++
14	USMR10b		++	+
15	USMS10a		++	+
16	USML8a		+	+
17	USML8		+++	++
18	USML9		+++	+
19	USMR1		+++	+

Data were averages of three replicates.
Samples: R, root tissues; L, leaf tissues; S, rhizosphere soil.
BCA, biocontrol agent; BLB, bacterial leaf blight; *Xoo*, *Xanthomonas oryzae* pv *oryzae*.
[a]Halozone diameter (mm) for *in vitro* antagonism assay against *Xoo* (Supplementary Data 1): +++, $\Delta\gamma \geq 20$ mm; ++, $\Delta\gamma \geq$ 10-19 mm; +, $\Delta\gamma \geq$ 5-9 mm; –, $\Delta\gamma < 5$ mm.
[b]Halozone diameter (mm) for siderophores production: ++, 16-25 mm; +, 5-15 mm; –, no siderophores production.

MEGA X (https://www.megasoftware.net) (Kumar et al., 2018) with a bootstrap of 1,000 replications.

Results

Pre-screening of potential bacterial biocontrol agents against *Xoo*. A total of 18 bacterial strains potential as BCAs-PGP were successfully isolated and positively responded to both *in vitro* antagonism tests against *Xoo* and siderophore production (Table 1). Thirteen potential isolates were obtained from internal root tissues (5 isolates), internal leaf tissues (4 isolates), and rhizosphere soils (4 isolates) of BLB diseased plants. The remaining five isolates were obtained from healthy rice plants of internal root tissues (1 isolate), internal leaf tissues (3 isolates), and rhizosphere soils (1 isolate). Out of 13 bacterial isolates of BLB diseased host plants, isolates USML8, USML9, and USMR1, which were the endophytes, recorded the largest halo zone diameter of *in vitro* antagonism test against *Xoo* ($\Delta\gamma \geq$ 20 mm) (Table 1). Additionally, for siderophores production, USML3, USML10, USMR10f, USMS2, and USML8 also recorded the largest halo zone diameter (in a range of 16-25 mm) (Table 1). Therefore, the potential endophytic BCAs (USML8, USML9, and USMR1) were selected for a more in-depth analysis of *Xoo* biocontrol and PGP potentials.

Secondary screening of biocontrol (BCAs) and PGP potentials of endophytes. Based on the positive and promising results of the pre-screening test of the endophytes (Table 1), thus more in-depth testing was conducted on the potential isolates as BCAs-PGP (Table 2). The results proved that the endophytes (USML8, USML9, and USMR1) could produce catecholate siderophore, similar to that of *Azospirillum brasilense* Sp7. Additionally, isolate USML9 and USMR1 were able to produce hydroxamate siderophores which were not produced by *A. brasilense* Sp7 (Table 2, Supplementary Fig. 1). Table 2 showed that isolate USMR1 was able to solubilize potassium (K) and exhibited the highest P solubilizing ability (at 22.35 ± 2.38 µg/ml). Additionally, all isolates also showed capability in producing IAA, which was similar to the control treatment (+*A. brasilensis* Sp 7) (Table 2).

BLB disease severity control and growth promotion of rice at 45 DAT. Inoculation of endophytic BCAs-PGP had reduced the length of leaf lesions in *Xoo*-infected rice plants compared to the control (–BA, +*Xoo*) (Table 3). Inoculation of USML9 recorded the lowest leaf lesion length of only 1.5 ± 1.3 cm. Inoculation of USML9 and USMR1 also showed a significant reduction in %DLA of 4.02 ± 3.9% and 7.60 ± 6.1%, respectively. Furthermore, a lower disease scale (scale 5) was recorded for BLB-infected rice plants inoculated with both isolates. In addition, the disease suppression (%) was much higher, up to 85.3 ± 12.5% for rice plants inoculated with USML9, and the suppression rate was significantly higher than that of *A. brasilense* Sp7 with *Xoo* sample (62.9 ± 29.9) (Table 3, Supplementary Fig. 2). The ability of the isolates to function as BCAs was supported by their potential as PGP agents (Table 4). Inoculation of USML9 and USMR1 on rice plants successfully showed significant effects on the shoot length and leaf chlorophyll content of the host plants compared to the controls (–BA, –*Xoo* and –BA, +*Xoo*). Isolate USMR1 high-

Table 2. Secondary screening of siderophores (catecholate, carboxylate, and hydroxamate) and PGP properties of selected BCAs-PGP endophytes

Isolate	Siderophores production					PGP properties		
	Catecholate	Carboxylate	Hydroxamate			Potassium solubilization ability	Phosphate solubilization ability[a] (µg/ml)	Indole-3-acetic acid production[a] (µg/ml)
	Rioux test	Spectrophotometric test	Tetrazolium test	Csaky-test	Atkin test			
Azospirillum brasilense Sp7 (control)	+	–	–	–	–	+	30.86 ± 1.73 c	1.81 ± 0.05 a
USML8	+	–	–	–	–	–	ND	2.37 ± 0.60 a
USML9	+	–	+	+	+	–	5.99 ± 0.13 a	3.79 ± 2.24 a
USMR1	+	–	+	+	+	+	22.35 ± 2.38 b	1.82 ± 0.22 a

(+) A positive reaction forms a clear zone around the colony for siderophore production and potassium solubilization ability. (-) A negative reaction for siderophore production and potassium solubilization ability. Data were averages of three replicates.
PGP, plant growth promotion; BCA, biocontrol agent; ND, not determined; SD, standard deviation.
[a]Mean followed by the different letters in each column for phosphate solubilization and indole-3-acetic acid productions indicated significant differences among treatments and were analyzed separately by Tukey honest significant difference at $P < 0.05$. Values are mean ± SD of triplicate.

Table 3. Disease severity control of rice inoculated with BCAs-PGP endophytes at 45 DAT under plant house condition

Treatment	Leaf lesion length (cm)	Diseased leaf area (%)	Disease scale	Disease suppression (%)
–BA, –*Xoo*	ND	ND	ND	ND
+*Azospirillum brasilensis* Sp7, –*Xoo*	ND	ND	ND	ND
–BA, +*Xoo*	10.4 ± 2.4 d[a]	52.81 ± 11.8 c	7	ND
+*A. brasilensis* Sp7, +*Xoo*	3.9 ± 3.1 bc	11.48 ± 11.7 ab	5	62.9 ± 29.9 ab
+USML8, +*Xoo*	4.9 ± 2.0 c	17.70 ± 10.1 b	5	52.1 ± 19.4 a
+USML9, +*Xoo*	1.5 ± 1.3 a	4.02 ± 3.9 a	5	85.3 ± 12.5 c
+USMR1, +*Xoo*	2.7 ± 1.7 ab	7.60 ± 6.1 a	5	73.6 ± 16.7 bc

Values were mean ± SD of six replicates and the experiment was repeated three times with all repetitions independent. The differences between inoculation treatments were investigated with Welch's analysis of variance (ANOVA).
Disease scale of bacterial leaf streak disease (International Rice Research Institute, 2002): 0, no lesions observed; 1, small brown specks of pinpoint size or larger brown specks without a sporulating center; 3, lesion type is the same as in scale 2, but a significant number of lesions are on the upper leaves; 5, typical blast lesions infecting 4-10% of the leaf area; 7, typical blast lesions infection 26-50% of the leaf area; 9, more than 75% leaf area affected.
BCA, biocontrol agents; PGP, plant growth promotion; DAT, days after transplanting; *Xoo*, *Xanthomonas oryzae* pv *oryzae*; ND, not determined; SD, standard deviation.
[a]Mean followed by the different letters in a line indicates significant differences among treatments and were analyzed separately by Tukey honest significant difference at $P < 0.05$.

Table 4. PGP of rice inoculated with BCAs-PGP endophytes at 45 DAT under plant house condition

Treatment	Shoot length (cm)	Leaf chlorophyll content[a]	Shoot dry weight (g)	Root dry weight (g)	Root volume (cm^3)
–BA, –*Xoo*	22.1 ± 3.3 b[b]	0.30 ± 0.03 a	0.03 ± 0.01 a	0.02 ± 0.01 b	0.61 ± 0.33 b
+*A. brasilensis* Sp7, –*Xoo*	37.3 ± 12.2 d	0.36 ± 0.04 b	0.14 ± 0.12 c	0.06 ± 0.09 d	1.07 ± 0.69 d
–BA, +*Xoo*	19.8 ± 3.3 a	ND	0.03 ± 0.02 a	0.01 ± 0.01 a	0.28 ± 0.26 a
+*A. brasilensis* Sp7, +*Xoo*	39.5 ± 13.5 e	0.34 ± 0.08 ab	0.17 ± 0.14 d	0.08 ± 0.06 e	1.10 ± 0.71 e
+USML8, +*Xoo*	32.2 ± 10.3 c	0.34 ± 0.07 ab	0.09 ± 0.07 b	0.05 ± 0.03 c	0.63 ± 0.39 b
+USML9, +*Xoo*	42.0 ± 11.3 g	0.37 ± 0.04 b	0.18 ± 0.13 e	0.08 ± 0.07 f	0.93 ± 0.58 c
+USMR1, +*Xoo*	40.1 ± 13.9 f	0.37 ± 0.05 b	0.21 ± 0.24 f	0.11 ± 0.11 g	1.30 ± 1.36 f

Values were mean ± SD of six replicates and the experiment was repeated three times with all repetitions independent. The Differences between inoculation treatments were investigated with Welch's analysis of variance (ANOVA).
PGP, plant growth promotion; BCA, biocontrol agents; DAT, days after transplanting; *Xoo*, *Xanthomonas oryzae* pv *oryzae*; ND, not determined; SD, standard deviation.
[a]Chlorophyll content: mg chlorophyll/mg leaf fresh weight.
[b]Mean followed by the different letters in a line indicates significant differences among treatments and were analyzed separately by Tukey honest significant difference at $P < 0.05$.

lighted significant effects on the shoot dry weight, root dry weight, and root volume of the host plants compared to the controls (Table 4, Supplementary Fig. 2). Moreover, shoot length, shoot dry weight, and root dry weight of USML9 and USMR1 were improved compared to those of the +*A. brasilensis* Sp7, +*Xoo*. These findings demonstrated the ability of endophytic BGAs-PGPs to promote the growth of the host plants, even though the plants were experiencing significant biological stress effects due to *Xoo* infection.

Table 5. Sequence analysis of 16S rRNA of BCAs-PGP endophytes isolated from BLB-infected rice

Isolates	Identification	Closest relative	Query coverage (%)	E-value	Maximum identity (%)
USML8	*Bacillus* sp.	*Bacillus tropicus* N24^T	100	0.0	99.93
USML9	*Bacillus* sp.	*Bacillus altitudinis* 41KF2b^T	100	0.0	100.00
USMR1	*Bacillus* sp.	*Bacillus paramycoides* NH24A2^T	100	0.0	100.00

BCA, biocontrol agents; PGP, plant growth promotion; BLB, bacterial leaf blight.

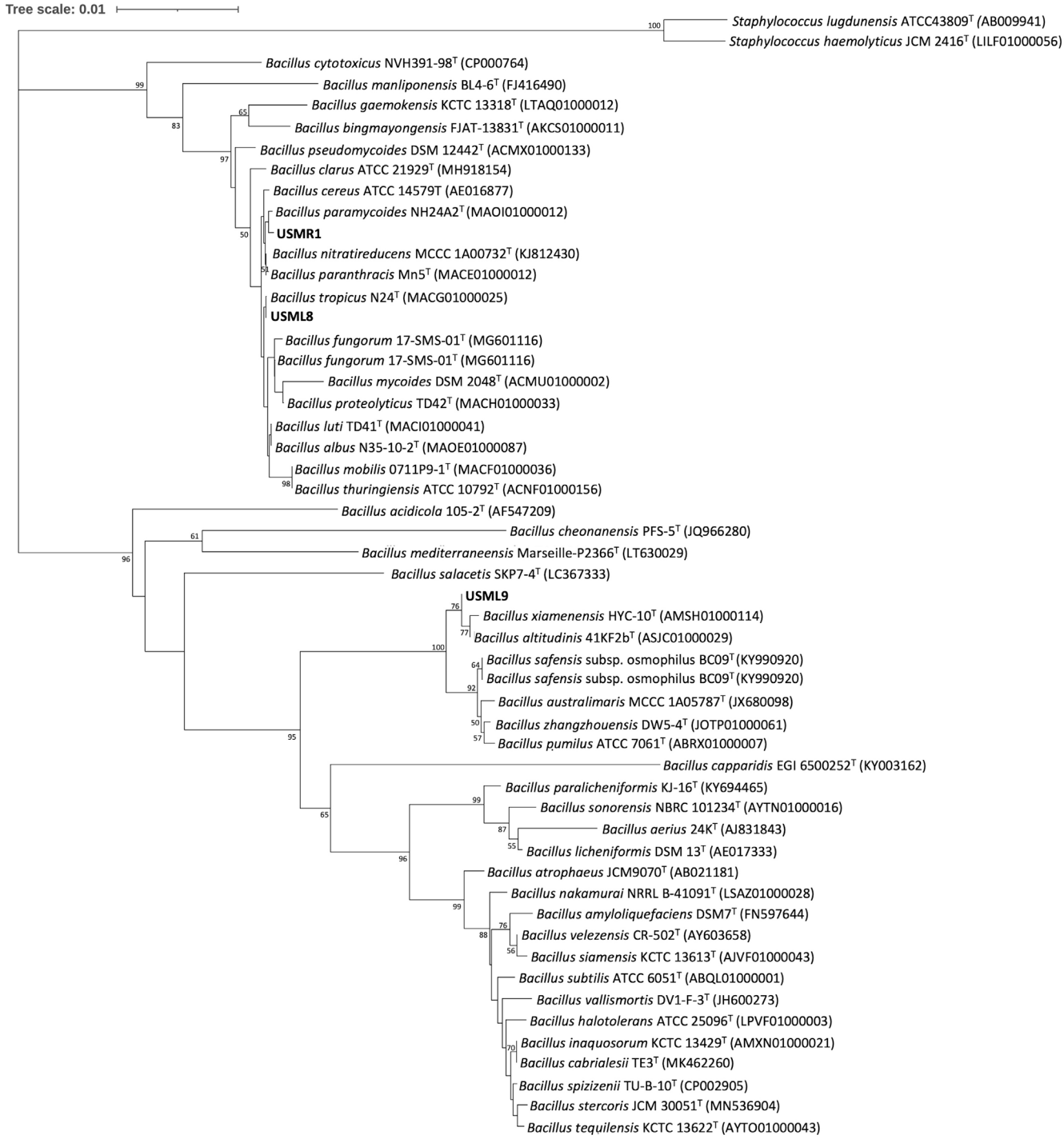

Fig. 1. Phylogenetic tree of three BCAs-PGP endophytes constructed via neighbor-joining, implemented in MEGA X with a bootstrap of 1,000 replication. BCA, biocontrol agents; PGP, plant growth promotion.

Molecular characterization of endophytes. The endophytes USML8, USML9, and USMR1 were identified through molecular identification analysis based on partial 16S rRNA gene sequences and the phylogenetic tree analysis. Strain USML8, USML9, and USMR1 were identified as the genus *Bacillus* (Table 5, Fig. 1). The closest related strains of strain USML8, USML9, and USMR1 were *Bacillus tropicus* N24^T (99.93% similarity), *Bacillus altitudi-*

nis 41KF2b^T (100% similarity), and *Bacillus paramycoides* NH24A2^T (100% similarity), respectively (Table 5).

Discussion

All 3 endophytic isolates were identified as *Bacillus* sp. strain USML8, *Bacillus* sp. strain USML9, and *Bacillus* sp. strain USMR1. The endophytes were proven as potential BCAs against *Xoo* and simultaneously PGP for *Xoo*-infected rice host plants. A report by Ngalimat et al. (2021) found that certain selected PGPB, e.g., *Bacillus, Pseudomonas, Enterobacter*, and *Streptomyces*, were potential as biocontrol agents against BLB disease-infected rice. Similarly, our findings also demonstrated the potential of the isolate USML9 as BCAs against BLB disease-infected rice, based on the bacterial effects on promoting disease suppression effects (85.3 ± 12.5%) of the treated host plants. The promising results were as expected due to the ability of the endophytes to produce siderophores (catecholate and hydroxamate) and function as plant growth promoters (potassium solubilizing bacteria, phosphate solubilizing bacteria, and IAA producers). Since all selected BCAs-PGP isolates were categorized as endophytes, the isolates can instantly adapt to the *in-planta* environment of the infected host plants, efficiently suppress the pathogen in controlling BLB disease infection, and promote plant growth.

This study was conducted to observe the efficiency of BLB biocontrol endophytes isolated from rice infected with BLB disease. Initially, it was based on the general assumption that microorganisms isolated from healthy host plants may have more potential BCAs than those isolated from disease-infected host plants (Fravel, 2005). However, Huang et al. (2013) asserted that the rhizosphere zone of disease-infected plants is a much better source for isolating potential BCAs than healthy plants. Their findings indicated that the rhizosphere zone of diseased tomatoes is a better source for BCAs isolation samples in controlling *Ralstonia solanacearum* infection. Similarly, our findings successfully demonstrated potential *Xoo* biocontrol endophytes with PGP ability (*Bacillus* sp. USML8, *Bacillus* sp. USML9, and *Bacillus* sp. USMR1) isolated from BLB-diseased infected rice. Several findings have also shown that infectious phytopathogens can stimulate host plants to produce several different compounds, which ultimately affects the composition and activity of potential BCAs that inhabit the rhizosphere zone of the host plants (Compant et al., 2010; Huang et al., 2013; Rudrappa et al., 2008; Trivedi et al., 2012). Similar findings by Huang et al. (2013) showed that the rhizosphere zone of diseased-infected plants could be an excellent sampling site for isolating BCAs against bacterial wilt disease in tomatoes. The BCAs-PGP have a potential to control the pathogenic microbes due to better adaptation of the isolates against challenging environments imposed by the pathogenic microbes infecting the host plants. For instance, it was reported that phytomicrobiome members of the host plants synthesize and excrete a range of inter-organismal signal compounds to defend the host plant against pathogens' biotic stresses (Smith et al., 2015). The compounds include broad-spectrum antibiotics, lytic enzymes, organic acids, and other metabolites, such as proteinaceous exotoxins and antimicrobial peptides (bacteriocins). The endophytes can be potentially effective biocontrol agents in preventing *Xoo* from further invading the vascular system of rice (Hastuti et al., 2012; Yadeta and Thomma, 2013). Similarly, Widiantini et al. (2017) also isolated effective biocontrol endophytes from healthy rice plants against rice blast disease (*Pyricularia oryzae* Cav.). The isolates were found to inhibit the growth of *P. oryzae* by more than 50% and caused mycelia malformation. However, according to De Souza et al. (2021), higher population densities of endophytes observed for diseased plant tissues of sisal plants caused by *Aspergillus welwitschiae* were not always translated into superior biocontrol potentials in the endophytic community.

The genus *Bacillus* is widely used as antagonistic bacteria against *Xanthomonas oryzae* pv. *oryzae* (Azman et al., 2017). Additionally, Fira et al. (2018) also described that *Bacillus* spp. could outcompete pathogenic microbes colonizing the rhizosphere zones of the host plants *via* the production of specific metabolites, including surfactins, difficidin, and bacilysin (Sarwar et al., 2018; Wu et al., 2018). Coinciding with these early discoveries, our isolates were also identified as *Bacillus* spp. (USML8, USML9, and USMR1) with BCAs-PGP properties that benefit the host plants. In agreement with earlier findings by Rais et al. (2017) and Shafi et al. (2017), our findings have demonstrated the potential of the endophytic *Bacillus* spp. as a biocontrol agent against *Xoo.* Siderophore production is one of the sustainable strategies of indigenous microbes to eliminate any pathogenic microbes *via* acquisition of readily available iron in the rhizosphere zones of the host plants (Haas and Défago, 2005; Shanmugaiah et al., 2016). Li and colleagues also suggested that *in vitro* antagonism reactions (e.g., against *Xoo*) were due to siderophore and hydrogen cyanide production (Li et al., 2008). The response might be caused by synergistic interaction of siderophore with other metabolites (Yasmin et al., 2016) or bioactive compounds, which act as antibiotics and cell wall degrading enzymes in the decision-nutrient competition (El-Tarabily et al., 2000; Hastuti et al., 2012; Siddiqui and Shaukat, 2003). In this

study, catecholate- and hydroxamate-type siderophores productions were observed in three isolates. On the other hand, *A. brasilense* Sp7 could produce only catecholate-type siderophores. The less productivity of siderophores in *A. brasilense* Sp7 might involve its the lower disease suppression on BLB diseased rice compared to that of USML9 and USMR1.

PGPBs could directly promote plant growth by providing compounds that increase mineral nutrient solubilization (e.g., potassium and phosphate), nitrogen fixation, and phytohormones production (Glick et al., 2007; Saharan and Nehra, 2011). The ability of the isolates to produce IAA contributed to improving the rooting system of rice for the absorption of more nutrients and water (Overvoorde et al., 2010). Phosphate-solubilizing ability aided in enhancing the development and growth of the host plants and induced the production of specific metabolites (e.g., siderophores and phytohormones) involved in the control of the phytopathogens (Vassilev et al., 2006). Besides that, several investigations demonstrated that Bacillus spp. were beneficial to rice planting (Elekhtyar, 2016; Radhakrishnan et al., 2017; Shakeel et al., 2015; Win et al., 2018). In addition, PGPBs, such as *Azospirillum*, *Azotobacter*, and *Pseudomonas*, have also acted as BCAs and promoted seed germination rates, plant height, and root length (known as bioenhancer) (Ashrafuzzaman et al., 2009; Manivannan, 2011). Azman et al. (2017) also cited other PGPB, including *Pseudomonas putida* PSP450, *Acinetobacter* sp. NP18, and *Bacillus amyloliquefaciens* V3 as potential biocontrol agents against *Xoo.* The isolates produced hydrolytic enzymes (protease, cellulase, lipase), siderophore, IAA, fixed nitrogen, and solubilized phosphate. In conclusion, endophytic BCAs-PGP designated *Bacillus* sp. strain USML8, *Bacillus* sp. strain USML9, and *Bacillus* sp. strain USMR1 were isolated from BLB diseased plants. Application of the endophytes at the early growth stage of the rice seedlings exhibited the prevention of BLB disease transmission, the reduction of disease severity on infected plants, and the increase in plant growth. In addition, compared to *A. brasilensis* Sp 7, higher disease suppression and plant growth promotion were observed by USML9 and USMR1. These findings proved that locally isolated BCA-PGP endophytes were effective in controlling BLB disease in rice plants in Malaysia.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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