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Screening of fluorescent bacteria for growth promotion and biocontrol potential against *Pyricularia oryzae* on aerobic rice (MARDI Aerob 1)

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ABSTRACT

Aims: This study aimed to screen the plant growth-promoting fluorescent bacteria (FLB) which isolated from the healthy rice rhizophere and to evaluate its biocontrol and growth promotion properties against *Pyricularia oryzae* on aerobic rice seedling of MARDI Aerob 1.
Methodology and results: King's B agar with glycerol was used as the selective medium to isolate FLB from the healthy rice rhizosphere soil. All FLB obtained were *in vitro* screened for antagonistic activities against *P. oryzae* using dual culture, volatile substances and hydrogen cyanide productions. The potential FLB isolates were further evaluated on rice seedling early growth promotion before identified using 16S rRNA gene sequencing. A total of 24 FLB were isolated from the healthy rice rhizosphere soil in Setiu, Terengganu, Malaysia. Isolates: FLB4, FLB5, FLB7 and FLB10 scored the total of percentage inhibition radial growth (PIRG) values ranged 99.5-105.0%. Further seedling growth promotion screening revealed that FLB4, FLB7 and FLB10 were significantly improved seedling growth with vigor index of 378.32%, 461.53% and 335.60% over control (133.31%). 16S rRNA sequencing identified that FLB7 as *Bacillus subtilis* and the FLB4 and FLB10 as *Pseudomonas putida*.
Conclusion, significance and impact of study: The selected FLB isolates (FLB4, FLB7 and FLB10) are potential to be developed as biological control agents against *P. oryzae* with growth promoting property on aerobic rice seedling.

Keywords: Fluorescent bacteria, rice blast disease, *Pyricularia oryzae*, aerobic rice, biological control

INTRODUCTION

Rice is highly produced and consumed in Asia (Prasad, 2011). The outbreaks of rice blast disease caused by *Pyricularia oryzae* Cavara (synonym *Pyricularia grisea* Sacc., the anamorph of *Magnaporthe grisea*) had become a major threat to the world rice production. Rice blast infects all the plant parts at any growth stage, although it is more frequent happened at the seedling and flowering stages. The lesions are usually diamond shaped, 1.0–1.5 cm long with a gray or whitish center and brown or reddish brown at the margin (Scardaci *et al*., 1997).

Aerobic rice production system is a new way of rice cultivation, where the rice is cultivated in well-drained, non-puddled and non-saturated soils (Zainudin *et al*., 2014). Despite the advantages in cost saving, rice field that are not permanently flooded tend to have more weeds and diseases problem. The severity of rice blast disease was reported higher when the rice plant was cultivated under draught-stressed conditions (Scardaci *et al*., 1997). Chemical control of rice blast was suggested by International Rice Research Institute (IRRI), when the disease incidence is more than 30%. However, intensive fungicide applications have caused resistance (Titone *et al*., 2015). Therefore, the development in biological control through the application of plant growth-promoting fluorescent bacteria is suggested as an alternative in rice blast disease management.

Fluorescent bacteria (FLB) application has been reported as the most effective biocontrol agent in controlling soil and foliar diseases in plant (Sahayaraj, 2014) including *P. oryzae* (Reddy *et al*., 2007). However, no study was conducted to evaluate the bio-efficacy of FLB in *P. oryzae* management and growth promotion of aerobic rice variety MARDI Aerob 1. Hence, this study aimed to isolate and screen the indigenous fluorescent bacteria for growth promotion and biocontrol potential against *P. oryzae* on aerobic rice (MARDI Aerob 1). This potential approach offers an alternative in rice blast disease management with reducing the dependence to chemical pesticide in sustaining the aerobic rice production.

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MATERIALS AND METHODS

Isolation of fluorescent bacteria (FLB)

Rhizosphere soil samples from the healthy rice plants were randomly collected from the rice growing field in Setiu, Terengganu, Malaysia. Twenty rice plants were randomly collected from the ten localities and the rhizosphere soil was gently removed for FLB isolation. The isolation of fluorescent bacteria was conducted as described by Zhou *et al*. (2012). Five grams of rhizosphere soil sample was added into a conical flask containing 100 mL of sterilised distilled water and shake at 150 rpm using an orbital shaker for 30 min. Serial dilution of the soil suspension was conducted and 0.1 mL of the aliquot was spread onto King's B agar. King's B medium with glycerol (1% v/v) was used as a selective medium to isolate the fluorescent bacterial. After 24 h of incubation at 28 ± 2 °C, the colonies grown on the King's B agar were observed under Fluorescence Analysis Cabinet (model CM-10A, Spectronics, USA) at 365 nm wavelength to confirm for the fluorescent characteristic. The fluorescence forming bacterial colonies were selected and designated as FLB1 to FLB24 (Table 1).

In vitro antagonistic screening against *P. oryzae*

The pure culture of the *P. oryzae* isolate was obtained from the Laboratory of Agri-Food Pests and Disease Management, Universiti Malaysia Terengganu (UMT) and was previously molecular identified as *Magnaporthe oryzae* (Teleomorph) strain Ar4 with accession number: KJ850439.

All the FLB isolates were *in vitro* screened for biocontrol properties against *P. oryzae* using dual culture testing as described by Maurya *et al*. (2014). The mycelium plug of *P. oryzae* (5 mm) obtained from the edge of 7 days old culture was placed perpendicular to the bacterial streaked with 2 cm apart on PDA. The Petri dish was incubated at 28 ± 2 °C for 14 days. The percent inhibition of radial growth (PIRG) was calculated using the formula:

$$\text{Percent of inhibition (PIRG)} = \frac{\text{C-T}}{\text{C}} \times 100$$

Where, C = mycelia radial growth of fungal in control; T = mycelia radial growth of fungal in treatment plate.

The production of volatile substances by the FLB was conducted as described by Mokhtar and Dehimat (2012). A disc of 5 mm *P. oryzae* plug was placed at the center of the potato dextrose agar (PDA) and the respective FLB isolated was streaked four times on Petri dish containing nutrient agar (NA). The Petri dish containing *P. oryzae* was placed at juxtaposed position with lids removed with FLB-streaked NA. Parafilm was used to seal the Petri dishes together and incubated at 28 ± 2 °C for 14 days. The PIRG was calculated as above formula.

Table 1: The *in vitro* antagonistic screening of FLB against *P. oryzae*.

Fluorescent pseudomonads bacteria (FLB)	Antagonistic screening of FLB against *P. oryzae*		
	Inhibition of radial growth (%)		
	Dual culture	Volatile substances	HCN production
FLB1	58.1^{ghi}	29.0^{abc}	-
FLB2	60.0^{efgh}	34.0^{ab}	-
FLB3	62.5^{defg}	24.5^{abcd}	-
FLB4	67.0^{bc}	34.0^{ab}	-
FLB5	70.5^{a}	30.5^{abc}	-
FLB6	63.0^{cdef}	29.0^{abc}	-
FLB7	69.5^{a}	35.5^{a}	-
FLB8	62.0^{efg}	34.5^{ab}	-
FLB9	68.8^{a}	22.5^{bcde}	-
FLB10	68.0^{ab}	31.5^{abc}	-
FLB11	61.5^{efg}	10.5^{e}	-
FLB12	58.5^{fgh}	13.0^{de}	-
FLB13	67.5^{b}	16.5^{de}	-
FLB14	56.0^{hij}	15.5^{de}	-
FLB15	53.5^{j}	19.5^{cde}	-
FLB16	56.5^{hij}	19.5^{cde}	-
FLB17	54.0^{ij}	20.0^{cde}	-
FLB18	62.0^{efg}	10.5^{e}	-
FLB19	67.5^{b}	23.0^{bcde}	-
FLB20	41.3^{k}	31.0^{abcd}	-
FLB21	69.0^{a}	11.0^{e}	-
FLB22	63.8^{bcde}	16.0^{de}	-
FLB23	57.0^{hij}	16.0^{de}	-
FLB24	66.5^{bcd}	16.0^{de}	-

Mean within the same column followed by the same letters are not significantly different at $p<0.05$.

The suppression effect of FLB against *P. oryzae* through hydrogen cyanide (HCN) production was conducted by streaking the FLB isolate on King's B agar that supplemented with glycine (1% v/v). Filter paper disc was then soaked in picric acid solution (0.5% picric acid and 2% Na_2CO_3 in 100 mL of distilled water) and placed in the inner lid of the Petri dish that containing the FLB. The Petri dish without FLB served as control. The plate was then sealed tightly and incubated at 28 ± 2 °C for three days. The formation of colour on the filter paper indicates positive reactions of HCN production, which are from yellow to light brown (weak, +), brown (moderate, ++), or reddish brown (strong, +++) (Suresh *et al*., 2010; Charulatha *et al*., 2013; Reetha *et al*., 2014).

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Rice seed germination and vigor testing

Aerobic rice (variety MARDI Aerob 1) seeds were obtained from Malaysian Agriculture and Research Development Institute (MARDI). The rice seeds were surface sterilised using the method as described by Oyebanji *et al*. (2009). The selected FLB isolates (FLB4, FLB5, FLB7 and FLB10) were grown in nutrient broth (NB) for 24 h before inoculated to the surface sterilised rice seeds. Sterilised rice seeds without FLB inoculation served as control. The rice seed germination rate was assessed after five days of incubation (28 ± 2 °C) and calculated using the following formula:

$$\text{Rice seed germination rate (\%)} = \frac{\text{Number of germinated seeds}}{\text{Number of the total seeds tested}} \times 100\%$$

The plumule and radicle lengths were also measured with a vernier caliper at 5th days after incubation. The vigor index and percentage of growth increment were calculated using the following formula as described by Gummert (2010) and Ng *et al*. (2012):

$$\text{Vigor index} = \text{Mean of (plumule + radicle lengths)} \times \text{Germination rate (\%)}$$

$$\text{Growth increment (\%)} = \frac{\text{(Vigor index in treatment - Vigor index in control)}}{\text{Vigor index in control}} \times 100\%$$

Identification of fluorescent bacteria

Gram staining of the FLB isolates was conducted as described by Saida *et al.* (1998). The respective FLB smear was heat fixed on a slide before staining with two drops of crystal violet solution for 1 min and thoroughly washed with distilled water. The specimens were observed under a light microscope and the colour (blue or red) of the bacterial cells was recorded.

The 16S rRNA gene regions were amplified with the universal primer (Frank *et al*., 2008) for all the selected FLB isolates (FLB4, FLB5, FLB7 and FLB10). The DNA of the FLB isolates were extracted following the manufacturer's protocol for genomic DNA isolation provided by Promega Wizard® Genomic DNA Purification. Polymerase chain reaction amplifications were conducted using the master mix with the preparation for one sample as follows: 5 µL of GoTaq polymerase buffer, 0.5 µL of dNTP, 1.5 µL of $MgCl_2$, 1 µL of forward primer 27F (5' AGAGTTTGATCCTGGCTCAG 3'), 1 µL of reverse primer 1492R (5' TACCTTGTTACGACTT 3'), 0.25 µL Taq polymerase, 13.75 µL nuclease-free water. Aliquot of 2 µL of DNA template of each bacterial isolates was added into the master mix to achieve the total volume of 25 µL. Aliquot 2 µL of nuclease free water was added into a master mix to serve as a control. The reactions were performed in a thermal cycler with an initial denaturation at 95 °C for 30 sec, followed by 30 cycles of 30 sec denaturation at 95 °C, 60 s of annealing at 45–68 °C, elongation for 1 min/kb at 68 °C and 68 °C for 5 min of final extension. The PCR products were resolved using electrophoresis on a 0.8% agarose gel prepared in 1× Tris-borate-EDTA (TBE) buffer.

For DNA sequencing, the reactions were performed by First BASE Laboratories. The data were analyzed using Basic Local Alignment Tools (BLAST). The sequences obtained were aligned using Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 and blasted in NCBI (National Centre for Biotechnology Information) database website using the nucleotide basic local alignment for identification.

Statistical analysis

The experimental units were arranged in complete randomized design with 5 replications per treatment. All data collected were subjected to analysis of variance and tested for significance using Duncan Multiple Range Test (DMRT) at $p \leq 0.05$ with the SPSS software.

RESULTS

Isolation and *in vitro* antagonistic screening of fluorescent bacteria against *P. oryzae*

A total of 24 FLB isolates were obtained and screened for antagonistic activity against *P. oryzae* based on dual culture testing and volatile substances production (Table 1). The clear inhibition zone formed within the *P. oryzae* colony with FLB streak demonstrated strong antagonistic effect on PDA (Figure 1). Generally, all isolates exhibited inhibition activity against *P. oryzae* with the PIRG values for dual culture testing ranged 41.3–70.5%. Among the 24 FLB isolates tested, isolates FLB5, FLB7, FLB9 and FLB21 showed significantly high PIRG against *P. oryzae* with 70.5, 69.5, 68.8 and 69.0%, respectively (Table 1 and Figure 1). The inhibition capability of FLB against *P. oryzae* based on the volatile substances production were ranged 10.5–35.5% (Table 1). The colony growth of *P. oryzae* was strongly inhibited by the produced volatile substances from respective antagonist FLB (Figure 2). The highest percentage of inhibition against *P. oryzae* was exhibited by FLB7 with 35.5%. However, none of the 24 FLB isolates tested producing HCN as a mechanism to suppress the growth of *P. oryzae* by changing the colour

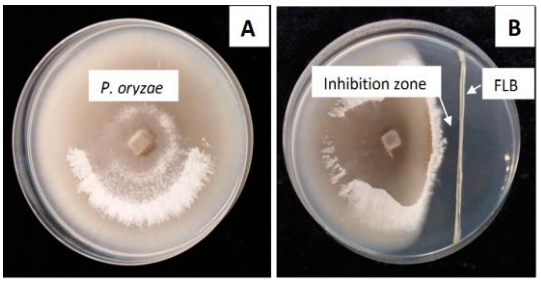

Figure 1: The clear inhibition zone formed within the *P. oryzae* colony with FLB streak demonstrated strong antagonistic effect on PDA (B) as compared to control (without FLB) (A).

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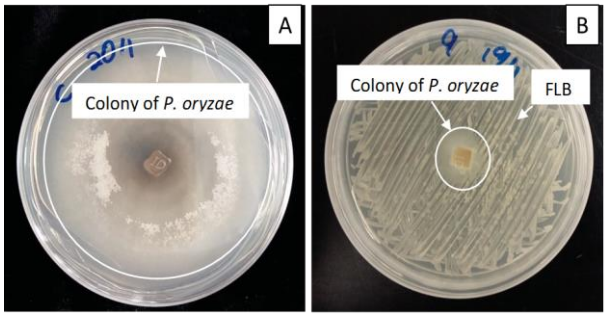

Figure 2: The inhibitory effect of volatiles substances produced by FLB on the colony diameter of *P. oryzae* (B) as comparison with control (without FLB) plate (A).

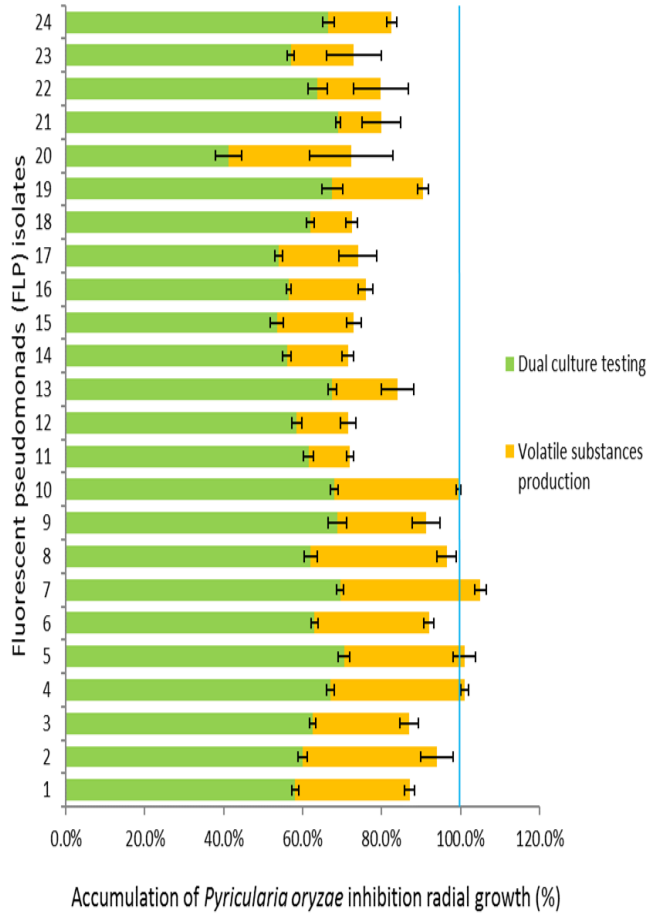

Figure 3: The accumulation inhibition effects of FLB against *P. oryzae* with the cutoff point at 100% (blue vertical line). Four FLPs (FLB4, FLB5, FLB7 and FLB10) were selected.

of filter paper from yellow to reddish brown after incubation.

Selection of the most potential FLB

From the *in vitro* antagonistic screening against *P. oryzae*, four FLB isolates: FLB4, FLB5, FLB7 and FLB10 which scored the total PIRG values ranged 99.5–105% (Figure 3) were selected for further exploitation in early aerobic rice seeding growth promotion properties.

Rice seed germination and vigor test

The bio-efficacy of rice seedling early growth promotion using the four selected FLB isolates was conducted using seed germination and vigor testing (Table 2). Rice seeds (MARDI Aerob 1) inoculated with the respective FLB isolates (FLB4, FLB5, FLB7 and FLB10), improved the germination rate to 86.00, 82.00, 89.00 and 92.00%, from 76.25% (control-without FLB inoculation). The highest increment of rice seed germination rate was 20.66% by FLB10 (Table 3). In addition, rice seeds inoculated with FLB4 and FLB7 significantly increased plumule length with 0.87 cm and 0.96 cm over control (0.33 cm). The high percentage increments of plumule length were recorded by FLB4 (163.64%) and FLB7 (190.91%) over control (Table 3). Similarly, the radical lengths of rice seedlings were also increased significantly after inoculation with FLB4 (3.53 cm), FLB7 (4.22 cm) and FLB10 (3.12 cm) compared to control (1.26 cm). The maximum increment in radical length was exhibited by FLB7 with 234.92% over

Table 2: Growth promotion of aerobic rice seed MARDI Aerob 1, inoculated with the selected FLB after five days of incubation.

Treatments	Plumule length (cm)	Radicle length (cm)	Percentage of germination (%)	Vigor index
Control	0.33^{b}	1.26^{b}	76.25^{b}	133.31^{d}
FLB4	0.87^{a}	3.53^{a}	86.00^{ab}	378.32^{b}
FLB5	0.32^{b}	1.50^{b}	82.00^{ab}	149.40^{d}
FLB7	0.96^{a}	4.22^{a}	89.00^{ab}	461.53^{a}
FLB10	0.52^{b}	3.12^{a}	92.00^{a}	335.60^{c}

Mean within the same column followed by the same letters are not significantly different at $p \leq 0.05$.

Table 3: Increment percentage early rice seedling growth performances after inoculated with the respective FLP fluorescent bacteria (FLB).

Increment of early rice seeding growth performances (%)				
Treatments	Plumule length	Radicle length	Germination rate	Vigor index
FLB4	163.64	180.16	12.79	183.79
FLB5	-	19.05	7.54	12.07
FLB7	190.91	234.92	16.72	246.21
FLB10	57.58	147.62	20.66	151.74

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control (Table 3). Interestingly, the root hair formation of rice seedlings after inoculated with the selected FLB, was greatly improved (Figure 4).

All rice seeds inoculated with FLB showed significantly increased in vigor index compared to control at $p \leq 0.05$, except FLB5. A highly significant of vigor index was recorded for rice seedling inoculated with FLB7 (461.53), with 246.21% of increment over control, followed by FLB4 (378.32) with 183.79% of increment, FLB10 (335.60) with 151.74% of increment. In contrast, the vigor index of rice seedling inoculated with FLB5 had no significant different with the control (Table 3). Therefore, isolates FLB4, FLB7 and FLB10 were suggested as the most prominent FLB in early aerobic rice (MARDI Aerob 1) seedling growth promotion and also strong antagonist against *P. oryzae*.

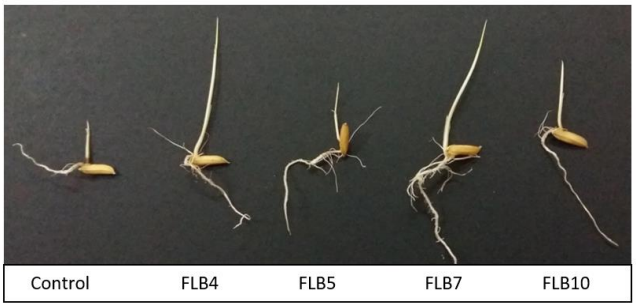

Figure 4: Early growth promotion effects of aerobic rive seedling (MARDI Aerob 1) after inoculated with the respective FLB exhibited improvement of plumule, radicle and root hairs formation after 5th days of incubation.

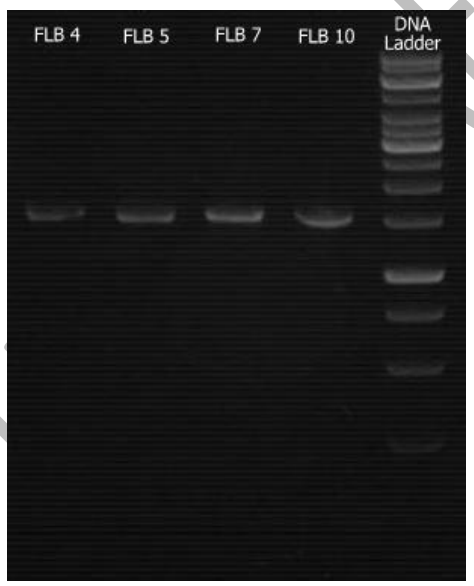

Figure 5: Agarose gel electrophoresis of beneficial rhizobacterial DNA. Lanes (from left to right) denote sampled DNA of FLB4, FLB5, FLB 7, FLB10 and DNA ladder (1kb). All bands presented at approximately 1500 bp.

Identification of the selected fluorescent bacteria

The Gram staining of bacterial cells indicated that all the selected FLB isolates were Gram-negative bacteria except FLB7 as Gram-positive bacteria. All FLB isolates were confirmed by molecular identification using 16S rRNA sequences and the PCR products were presented at approximately 1500 kB in 0.8% agarose gel (Figure 5). The isolates obtained were belonging to the two genera of *Pseudomonas* and *Bacillus* after comparing the obtained 16S rRNA gene sequences using BLAST tool to the GenBank database (Figure 6) and highly similar to *Pseudomonas putida* (FLB4, FLB5, FLB 10) and *Bacillus subtilis* (FLB7) with 99% sequence similarity, respectively.

DISCUSSION

Fluorescent *Pseudomonas* is widely used as plant growth-promoter and abundantly distributed in the rhizosphere soil. Most of the *Pseudomonas* are capable to produce yellow-green pigment siderophores that fluorescent under UV light at 365 nm (Lamichhane and Varvaro, 2013). The prominent inhibition effects of *P. fluorescens* against various plant pathogens such as *Botrytis fabae* (Alemu and Alemu, 2013), *Fusarium moniliforme*, *Rizoctonia solani*, and *Alternaria alternata* (Maurya *et al*., 2014) were reported. Similarly, Suryadi *et al*. (2013) reported that the culture filtrates of a consortium in which containing *P. aeruginosa* and *Bacillus* spp. had significantly reduced the growth of *P. oryzae* at 66–83%. Thus, the FLB isolates obtained from the healthy rice rhizosphere soil have the biocontrol potential against *P. oryzae.*

Volatile compounds produced by plant growth promoting rhizobacteria (PGPR) have been reported as one of the mechanisms in biocontrol (Sarangi *et al*., 2010). Volatile substances production using indirect confrontation method has been widely used to evaluate the inhibitory effect of isolates against the pathogen (Bendahmane *et al*., 2012). *Pseudomonas* spp. show high versatility in metabolic capacity in synthesis of antibiotics, siderophores or HCN (Charest *et al*., 2005). These metabolites work effectively to suppress deleterious microorganism and indirectly improve plant health. Application of HCN-produced PGPR helps to suppress plant pathogen through induce systemic resistance in plant (Meena, 2014). However, in the current study, none of the FLB isolates obtained were capable to produce HCN. Other volatile substances produced by *Pseudomonas* spp. could be employed in the suppression of *P. oryzae* in this study. For instance, volatile substances such as benzathiozole, cyclohexanol, n-decanal, dimethyl trisulfide, 2-ethyl-1-hexanol and nonanal were also reported associate to the inhibition of *Sclerotinia scleretiorum* (Weisskopf, 2013). Those volatile substances could contribute to the inhibition of *P. oryzae* when incubated with the selected FLB isolates. Further purification and identification of the volatile compounds produced by the FLB is warranted.

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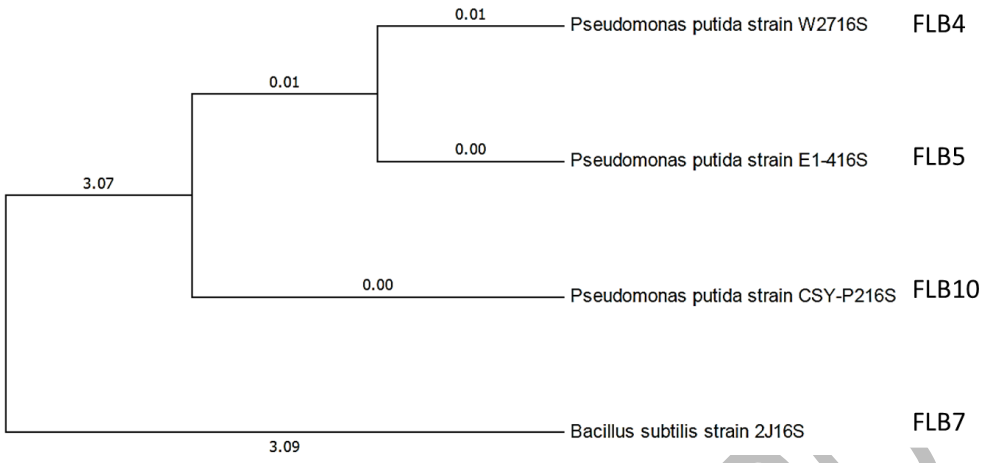

Figure 6: The evolutionary relationships taxa of the potential FLB isolates with the Neighbor-Joining tree developed using MEGA7 software.

This study explored the diverse inhibition potential of the selected FLB was relation to other volatile compounds produced exclude HCN. The capability of fluorescent pseudomonads isolated from 13 states of India by Kumar *et al.* (2015) were comparatively low in HCN production to other metabolites produced. The quantitative measurement of HCN using liquid media based on a modified colorimetric methemoglobin method (von Rohr *et al*., 2009), initially described by Baumeister and Schievelbein (1971) was necessary to determine the low HCN production capability. This method stimulates the production of HCN, thus enabling the determination of the maximum potential of rhizobacteria for HCN production (Rijavec and Lapanje, 2016).

In this present study, the inoculation of rice seedlings with FLB exhibited diverse in their ability to promote rice seedling growth. The production of indole acetic acid (IAA) by FLB could play an important role to improve seed germination (Hayat *et al*., 2010). Besides, various phytohormones such as gibberellic acid, cytokinins and ethylene produced by PGPR were also reported to increase the primary nutrients supply to the host plant (Bhattacharyya and Jha, 2012). The outcome of this study was in agreement with Kumar *et al*. (2015), where PGPR-inoculated pigeon pea seeds exhibited higher growth than the control was related to the production of phytohormones.

The selected FLB isolates (FLB4, FLB5 and FLB10) were molecular identified as *Pseudomonas putida* and isolate FLB7 was *Bacillus subtilis*. The *Pseudomonas* and *Bacillus* spp. were abundant in the rhizosphere soil of cereal plant, for instant on biochemical characterization basis, 44% of *Bacillus* spp. and 24% of *Pseudomonas* spp. were identified in wheat rhizosphere as reported by Joshi and Bhatt (2011). In *Pseudomonads fluorescent* groups, the fluorescent pigments produced are pyocyanin or pyoverdine during growth in low-iron condition that involve in inflammatory response and tissue damage during pathogenesis (Hossain, 2014). While in *Bacillus* spp., the fluorescent compound produced is chlorxanthomycin, both are visible under long-wavelength of UV light (Magyarosy *et al*., 2002). Both of these genera are the most widely studied due to their high efficacy being use as biological agents. Most of the bacteria from the genus of *Bacillus* and *Pseudomonas* have effectively suppressed the development of phytopathogenic fungi (Weisskopf, 2013). In addition, *Pseudomonas* spp. were also exhibit the plant growth-promoting effect that improve and cure the pathogen infected plants (Selvakumar *et al*., 2015). Besides, rice straw compost fortified with *Pseudomonas* sp., *Bacillus* sp. and other beneficial microbes was reported significantly increased plant growth and productivity of aerobic rice (Ng *et al*., 2012).

CONCLUSION

The three selected FLB isolates (FLB4, FLB7 and FLB10) are potential to be developed as alternative control approach against *P. oryzae* on aerobic rice variety MARDI Aerob 1 with prominent early seedling growth promotion properties. Further exploration on the mechanism of the selected FLB in suppression of *P. oryzae* is suggested especially under field conditions. In addition, the bio-efficacy of the microbial consortium of these FLB isolates is needed to be explored as biological control agent and growth promoter to control rice blast disease and also to promote the seedling growth of aerobic rice MARDI Aerob 1.

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