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Screening of Endophytic Bacteria as Biocontrol Agents Against Bacteria Leaf Blight (*Xanthomonas oryzae*)

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ABSTRACT

Bacterial leaf blight (BLB) is one of major threats in rice production as it can cause 100% yield loss. Concern on the environment and human health has led an attempt to replace existing methods of chemical control and avoid extensive use of bactericides by using endophytic bacteria. The present study was conducted to screen and characterize bacteria isolated from different sources that has potential as antagonistic bacteria against *Xanthomonas oryzae* pv. *oryzae (Xoo)*, the causal agent of bacterial leaf blight of paddy. Two hundred and thirty-three endophytic bacteria were successfully isolated from roots and leaves from paddy field. Only 17 endophytic bacterial isolates showed positive antagonistic activity indicated by inhibition zone around bacterial colony against *Xoo* on nutrient agar plate with 2 endophytic isolates (BCA 3 and BCA 12) showed highest inhibitory effect with 35±0.00 mm in diameter. Molecular identification by 16S rRNA amplification successfully identified the antagonistic endophytic bacteria as *Pseudomonas fluorescens* and *Geobacillus thermoparaffinivorans*. Findings in this study revealed the biocontrol abilities of isolated endophytes as an excellent option to be used by agriculture sectors to have sustainable environment.

1. Introduction

Rice is the most important food crop of the developing world and the staple food of more than half of the world's population. More than 90% of rice is produces and consumed in Asia. In Malaysia, rice is cultivated on 8 major granary area with average rice consumption by Malaysian citizen is about 82.3 kilograms per year (Raweekul and Wuttitummaporn 2016). However, the average growth rate of rice yield in Malaysia was decreased due to the infection of rice plant from the microbial disease's incidence. Bacteria leaf blight (BLB) caused by *Xanthomonas oryzae* has recently become a common disease among rice crop in Malaysia. Moist conditions at the beginning of the season is the major factor enable the bacteria to grow from infected stubble to an adjacent seedling (Hogg *et al.* 2010). Farmers often heavily rely on using chemical bactericides to control these plant diseases as well as enhance the plant health. Unfortuntely, the continuing used of synthetic bactericides in agriculture have led to public concern on human and environmental health. Widely and extensive use of chemical bactericides has become a main environmental threat when its drivers to species extinction, leads to the reduction of global biodiversity and caused significant changes in ecosystem dynamics (Aktar *et al.* 2009).

Recently certain potential biocontrol agents such as *Bacillus amyloliquefaciens, Bacillus methylotrophicus* and *Bacillus subtilis* were isolated from rice plants showing the significant antimicrobial activities against *Rhizoctonia solani* and *Burkholderia glumae*, the two major rice pathogens that cause sheath blight and bacterial panicle blight diseases (Shrestha *et al.* 2016). For these and other reasons, nowadays endophytic bacteria are widely investigated by researcher to develop a sustainable environmentally friendly, microbial-based biocontrol agent as an alternative to agrochemicals.

Potential endophytic microorganisms are mainly consisting of bacteria and fungi that colonize inner

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part of plant tissues without causing any damage to their host plant (Schulz and Boyle 2006). The colonization of these endophytic microorganisms can be isolated from surface sterilized monocotyledonous and dicotyledonous plants (Hallmann *et al.* 1997). The main gateway for endophytes to penetrate the plant's tissues is from the root zone, but the earlier study also proved that endophytes also can invade through the aerial tissues of the host plant (chi *et al.* 2005). The pathways of endophytes also include the natural vents and wounds found at the plant tissues (Hallmann *et al.* 1997). Besides that, the microscopic opening such as stomata is a critical first step of the microbial entrance into host tissues (Schulze and Robatzel 2006).

Therefore the aim of the present study was to isolate and identify endophytic bacteria from commonly used rice cultivars in Malaysia, in order to evaluate their effect as biocontrol agents against rice bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae (Xoo)*.

2. Materials and Methods

2.1. Pathogen Inoculum Preparation

The *Xoo* strain (MXO 1410) was obtained from Laboratory of Plant Pathology, Rice and Industrial Crops Research Centre, MARDI Seberang Perai, Malaysia. The strain was sub-cultured in Potato Sucrose Agar (PSA) plate and in PSA agar slant for long term preservation in 20% glycerol and keep in -80°C. For inoculums preparation, the *Xanthomonas (Xoo)* culture was inoculated on PS broth media and incubated at 30°C for 48 hours. Then the *Xoo* culture was diluted with sterile distilled water. Cell suspensions containing 10^8 colony forming units (cfu) of *Xanthomonas oryzae* pv. *oryzae* using a spectrophotometer at 600 nm wavelength with 0.6 optical density volume was used (Azman *et al.* 2017).

2.2. Rice Plant Sampling Materials

Three-month-old of rice plants samples was randomly collected from three different rice fields in Pendang (Kedah), Teratak Pulai (Kelantan), and Seberang Prai (Pulau Pinang). The whole rice plant will be carefully uprooted and place in a sterile plastic bag, label and tied and further put in a box containing ice (Raweekul and Wuttitummaporn 2016).

2.3. Endophytic Bacterial Isolation

The roots and leaves were washed with slow running tap water and then cut into small pieces. The surface of the leaves and roots performed surface sterilized by using 10% (v/v) of Sodium Hypochlorite and a few drops of Tween-20 was added. The samples were rinsed with the sterilized distilled water five times and then a mortar and a pestle were used to ground the tissues and obtain the bacterial suspension. Serial dilutions were done from the suspension and the concentration were prepared up to 10^{-3}, then the sample was streaked on nutrient agar (NA) and incubated at 30°C for 7 days. Water that was used for the final rinse was kept to plate on NA plates to act as a control (Raweekul and Wuttitummaporn 2016). Isolated endophytic bacteria were plated on Nutrient agar medium after performed serial dilution method as mentioned by (Yasmin *et al.* 2017). The purified isolated bacteria strain was stored in NA slants at 4°C and in -20°C for further used.

2.4. Antagonistic Bacteria Screened

The inhibition of *Xoo* were screened *in vitro* by well diffusion method as described by (Abdulkadir and Waliyu 2012). The fresh culture of *Xoo* was spread on TSA media. The isolated bacteria strain that was grown on Nutrient broth for screened of antagonistic activity were spotted about 60 µl in the well on TSA plates that already spread with *Xoo* strain. The plates were kept in the incubator at 30°C for 24 h and the clear zone of inhibition was recorded by measuring the diameter (Yasmin *et al.* 2017).

2.5. Antagonistic Bacteria Identification by Using Molecular Method

The antagonistic endophytes grown in Nutrient Broth and incubated at 37°C for 2 days. The DNA of each endophytes was extracted using RBC HiYield™ Genomic DNA Mini Kit (Bacteria) as per manufacturer instruction. The DNA was amplified by Mastercycler® Nexus Gradient machine for polymerase chain reaction amplification. The gene fragments of antagonistic endophytes were sequenced using universal primers pair 518F (5'-CCAGCAGCCGCGGTAATACG3') and 800R (5′TACCAGGGTATCTAATCC-3′) that was synthesised by MYTACG Bioscience Enterprise, Malaysia. The 50 µl of PCR reaction mixture contained 5X Green GoTaq

(5 µl), dNTPs (1 µl), Forward primer (1 µl), Reverse primer (1 µl , genomic DNA (2 µl), Taq Polymerase (0.5 µl) and nucleus free water (39.5 µl) modified from Azman *et al.* (2017) method. Amplifications of gene was performed for 30 cycles in Eppendorf gradient thermal cyclers programmed (Kathleen 2006). The reaction will proceed as follows: 1 cycle for 3 minutes at 94°C (Denature), 40 cycles for 1 minutes at 94°C (Denature), followed by 50°C for 1 minutes (Anneal) and 1 minutes at 72°C (Elongation).

The PCR product was proceeded for Gel Electrophoresis, 1% agarose gel was used by preparing 1g agarose powder with 100 ml 1X TBE buffer then was stained with 2.5 µl DNA Gel Stain. Gel was visualized by using Bio Rad™ gel documentation system under UV light to observe the DNA band, 1 kb and 100 bp of ladder acted as marker. The sequencing process was submitted to MYTACG Bioscience Enterprise for outside services. The pairwise alignment analysis of partial 16S rRNA gene sequences was performed on the MEGA X database (Kim *et al.* 2012). The 16S rRNA gene sequences of known bacterial species were subjected to Basic Local Alighment Search Tool (BLAST) for identification and all obtained sequence were submitted to NCBI GenBank database (Azman *et al.* 2017).

2.6. Control of Bacterial Leaf Blight Disease under Greenhouse Conditions

The rice seeds from variety MR284 was performed seed bacterization using 2% sodium hypochlorite for 30 second, rinsed with sterile distilled water and lastly was dried overnight. The seeds were soaked in potential endophytes suspension for 2 hours before kept it dried under shade. The treated seeds were sown in pots along with the untreated seeds that acted as control. At day 22 after sowing, the positive control of plant was sprayed with chemical bactericide and negative control were sprayed with sterile distilled water (Krishnan and Muthurajab 2014). At 30 days after sowing, the leaves were clip inoculated with sterile scissors that was dipped in 10^8 cfu ml^{-1} of *Xoo*, then the treated plants were covered with a polythene bag. At 14 days after clip inoculation, the effectiveness of the treatments on bacterial leaf blight disease intensity was observed using 0^{-9} scale followed the Standard Evaluation System for rice (IRRI). The disease severity was calculated using formula:

$$PDI = \frac{Sum\ of\ all\ numerical\ ratings}{Total\ number\ of\ leaves\ graded} \times \frac{100}{Maximum\ grade}$$

In addition, the biocontrol effect of potential endophytes against *Xanthomonas oryzae* like disease severity and suppression efficacy were collected at different time intervals were collected from the following treatments.
Treatment 1: *Xoo*
Treatment 2: *Xoo* + Chemical bactericide
Treatment 3: *Xoo* + *Enterobacter* sp.
Treatment 4: *Xoo* + *Bacillus subtilis*
Treatment 5: *Xoo* + *Bacillus cereus*
Treatment 6: *Xoo* + *Gamma proteobacterium*
Treatment 7: *Xoo* + *Pseudomonas fluorescent*
Treatment 8: *Xoo* + *Geobacillus thermoparaffinivorans*

2.7. Statistical Analysis

All collected data were analysed by Statistical Package for Social Sciences (SPSS, version 23). The data were subjected to the analysis of variance (ANOVA). A completely randomized design was used for all the experiments, with 3 replications for each treatment. Differences between experimental outcomes were analysed using Tukey's HSD test and $p \leq 0.05$ was considered significantly different.

3. Results

3.1. Isolation of Potential Endophytes

Three different rice field in Kedah, Kelantan, and Pulau Pinang were selected in this study to evaluate the potential of isolated bacteria endophytes as biocontrol agents against *Xoo*. *In vitro* microbiological techniques was used to observe phenotypic functionality of these endophytes to supress the bacterial pathogen and genome analysis approach to identify the bacterial endophytes.

3.2. The Distribution of Endophytes

Based on the study, a total of 233 endophytes were successfully isolated from surface-sterilized leaves and roots of rice plant from three different rice field in Kedah, Kelantan and Pulau Pinang, Malaysia. The number of isolated endophytic bacteria were varied from different rice field and part of rice plant (Figure 1). Kedah rice field demonstrated the highest number of isolated endophytes isolates from root with 77.78% while leaves in Pulau Pinang showed the highest number of endophytes isolates from leaves samples with 34.78%.

3.3. *In vitro* Screening

All endophytes isolates were screened for their activity against *Xanthomonas oryzae* pv. *oryzae (Xoo)*,

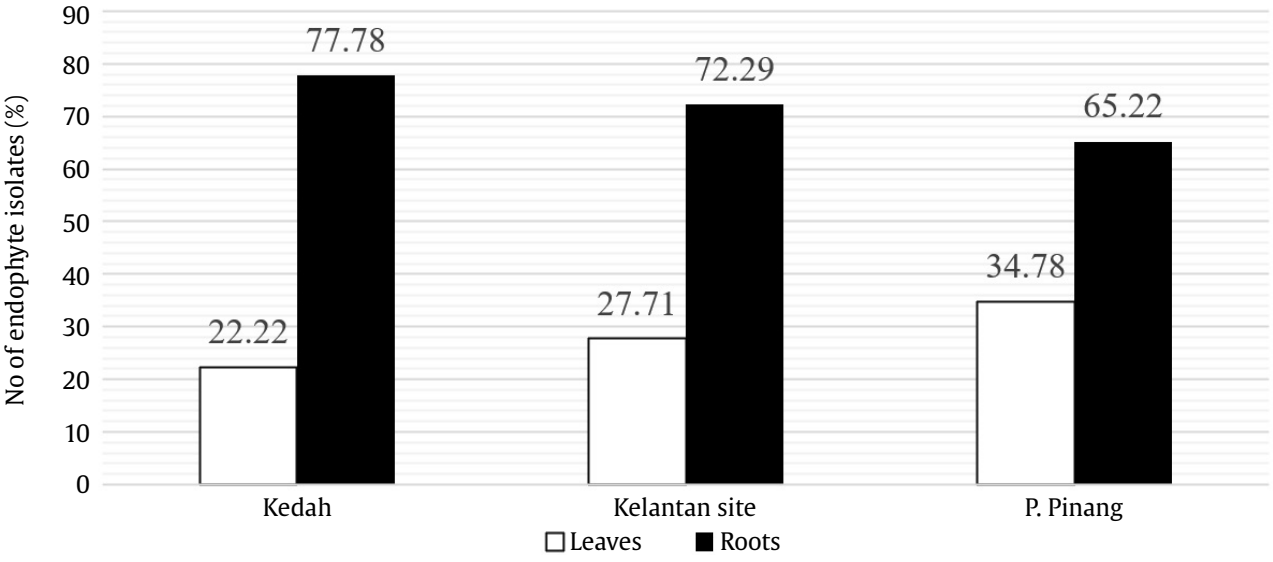

Figure 1. The percentage of endophytic bacteria isolated from the respected rice plant tissues

the causal agent of bacterial leaf blight disease. Out of 233 isolated endophytes, only 17 showed positive result in terms of inhibitory against *Xanthomonas oryzae* pv. *oryzae*. Based on the 16S rRNA sequences, all 17 endophytes were closely related to *Enterobacter* sp., *Geobacillus thermoparaffinivorans, Gamma proteobacterium, Pseudomonas fluorescent, Bacillus subtilis,* and *Bacillus cereus*.

All the 7 identified species of endophytes were found to have antagonism properties against test pathogens. There was a significant difference ($p<0.05$) among potential endophytes against *Xoo* according to Tukey's test (Table 1). Maximum diameter of inhibition was observed on *Pseudomonas fluorescent* and *Geobacillus thermoparaffinivorans* with 35±0.00 mm each (Table 1).

Table 1. Antibacterial activity of endophytes against bacterial leaf blight.

Endophytes	Diameter of inhibition zone (mm)
Enterobacter sp.	30.5±0.58[a]
Geobacillus thermoparaffinivorans	35.0±0.00[b]
Gamma proteobacterium	31.3±0.58[c]
Pseudomonas fluorescent	35.0±0.00[d]
Bacillus subtilis	33.3±0.58[e]
Bacillus cereus	32.3±0.58[f]

3.4. Effects of Endophytes in Suppression of Disease under Glasshouse Experiment

The glasshouse screening was performed to all the potential endophytes and its showed different effect on development of bacterial leaf blight disease in rice (Table 2). Generally, all potential endophytes demonstrated a substantial reduction of bacterial leaf blight disease compared to control. Endophyte *Geobacillus thermoparaffinivorans* and *Bacillus* sp. showed the highest disease control efficacy, 83.49% and 81.56% respectively. The lowest suppression efficiency was observed in *Enterobacter* sp. with 33.01%.

4. Discussion

Endophytic is a microorganism that live within the plant tissues without causing any harms to the host plant. Endophytic bacteria likely utilize plant

Table 2. Effects of potential endophytes in controlling bacterial leaf blight disease in glasshouse experiment

Treatment	Disease severity (%)	Suppression efficacy (%)
Xoo	76.30	-
Xoo + Chemical bactericide		
Xoo + Enterobacter sp.	57.78	24.27
Xoo + Bacillus subtilis	14.07	81.56
Xoo + Bacillus cereus	36.30	52.42
Xoo + Gamma proteobacterium	43.70	42.73
Xoo + Pseudomonas fluorescent	51.11	33.01
Xoo + Geobacillus thermoparaffinivorans	12.60	83.49

tissues through several entry sites (Hardoim *et al.* 2015). The opening or cracks at the root, stomata, hydathodes and wound at the shoots are determined as main entry point of endophytes to enter the host plant (Naveed *et al.* 2014). In the present study indicate that, root part showed a high colonization of endophytic bacteria compare to leaves part in three rice field. Similar finding also was reported by Kandel *et al.* (2017) which showed a high distribution of endophytes bacteria isolated from root parts. This factor influences by the soils condition itself dominated by many microorganisms and the roots endophytes are recruited from the soils, then move to the stems and leaves (Chi *et al.* 2005; Liu *et al.* 2017). Furthermore, the physical and chemical of soils, nutrient availability and moisture directly affected the endophytes bacterial communities at roots and also is a place for complex interaction between the plant and surrounding soil microorganisms because plant needs great variety of bacteria to facilitate the uptake of nutrients, water uptake and utilization of organic pools (Aletaha *et al.* 2018).

Previous researcher find out that endophytic bacteria have several potential applications in drug discovery and pharmaceutical (Strobel 2006; Guo *et al.* 2008). Extensive field research has addressed endophytic bacteria might enhance biocontrol efficacy and consistency in performance since there are an interaction between plant and microbes and these interactions contribute to the plant health for better growth and development, soil fertility as well as yield (Souza *et al.* 2015).

Biocontrol is one of the most effective alternate strategies that can be used to treat the bacteria caused plant diseases (Pal and Gardener 2006). Biological control of plant diseases can be defined as the process in which the pathogenic bacteria that caused plant disease will be inhibit or impacting their activities by one organism (Dicklow 2013). Endophytic bacteria have shown effective control of disease such as *Bacillus* sp., *Acinetobacter* sp., *Bacillus licheniformis,* and *Pseudomonas putida* (Palaniyandi Velusamy *et al.* 2013), Burkholderia cepacia, *Bacillus amyloliquefaciens, Staphylococcus warneri, Panteo vagans, Pantoea* sp., *Oceanobacillus oncorhynchi,* and *Paenibacillus cineris* (Azman *et al.* 2017).

Two hundred and thirty-three endophytic bacteria were isolated from leaves and roots of rice plant but only 17 endophytes showed positive antagonistic activity against *Xanthomonas oryzae*. The potential endophytes when identified by molecular 16s rRNA amplication were *Enterobacter* sp., *Geobacillus thermoparaffinivorans, Gamma proteobacterium, Pseudomonas fluorescent, Bacillus subtilis,* and *Bacillus cereus*. Similarly, Walintang *et al.* (2017) and Liu *et al.* (2017) were also reported the presence of *Bacillus* sp., *Pseudomonas* sp., and *Gamma proteobacterium* endophytes isolates from rice plants. The results of antagonistic activity showed *Geobacillus thermoparaffinivorans* and *Pseudomonas fluorescent* seem to effectively inhibit the growth of *Xanthomonas oryzae* with 35 mm in diameter of inhibition zone. Similarly, according to Durairaj *et al.* (2017), *Pseudomonas* and *Bacillus* strain are found to have broad spectrum of antagonistic activity against bacterial phytopathogens. Besides that, the ability of *Pseudomonas fluorescent* to suppress pathogens is mainly connected to their secondary metabolites secretion such as hydrogen cyanide phenazines phloroglucinols, pyrrolnitrin, and pyoluteorin (Palleroni 2005; Hass and Defago 2005; Muller *et al.* 2018).

The potential endophytes then was proceed for glasshouse evaluation. Glasshouse evaluation included assessment of effects due to the local environment, seasonal variation and crop management practice such as pesticide use, which cannot be assessed in the laboratory experiment (A. Nicholas *et al.* 2007). Bacillus subtilis was not showing highest inhibitory effect when tested in laboratory, but some significant effects were detected in the glasshouse evaluation when *Geobacillus thermoparaffinivorans* and *Bacillus subtilis* showed highest of disease control efficacy by 83.49% and 81.56%. As far is our concern, this is the first study reported the efficiency of *Geobacillus* sp. against *Xoo*. According to Hussein *et al.* (2015), *Geobacillus* sp. are grouped together as *Bacillus* sp. but then being reclassification according to the thermophile's characteristic to a separate genus, *Geobacillus* sp. Hussein *et al.* (2015) reported the use of *Geobacillus* sp. in controlling and protecting plants against *Fusarium* wilt, the wide spread plant pathogen. Therefore, the result in present work suggested *Geobacillus thermoparaffinivorans* together with *Bacillus* sp. to be developed and commercialize as promising biocontrol agent in agriculture sector in future.

5. Conclusion

Present study showed the potential of *Geobacillus thermoparaffinivorans* and *Bacillus subtilis* endophytes for

bacteria leaf blight disease reduction. Further research is needed for the identification of secondary metabolites production of both isolates and its effectiveness as biocontrol agent against other important pathogens of rice as well as its efficacy in the pot trial.

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