Diversity and Plant Growth Promoting Activities of Rice Epiphytic Bacteria

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Abstract

Plant-associated bacteria have been known for their direct and indirect abilities to promote plant growth. Because of their benefits on plants, they are generally recognized as plant growth promoting bacteria (PGPB). In the present study, epiphytic bacteria were isolated from surfaces of roots, stems and leaves of rice plants that were grown in organic farms. Of 113 bacterial isolates, 68 and 45 isolates were stained Gram-positive and Gram-negative, respectively. Based on their cell shapes, 106 isolates were bacilli while seven isolates were cocci. The use of the 16S rRNA gene sequence analysis indicated that they were members of phyla *Fimicutes* (54.87%), *Proteobacteria* (38.05%) and *Bacteroidetes* (7.08%). All isolates were assessed for their abilities in nitrogen fixation, phosphate solubilization, siderophore production, auxin production and 1-aminocyclopropane-1-caboxylic acid (ACC) deaminase activity. The result showed that 91 isolates were tested positives for at least one activity. The antagonistic activity against the pathogenic fungus *Pyricularia oryzae* was also tested. Twenty-three isolates inhibited the growth of the fungal mycelia. The percentage of growth inhibition of these isolates against *P. oryzae* ranged from 73.10±3.24% to 100.00±0.00%. The protease and chitinase activities were found in 19 and 2 isolates, respectively. However, crude preparation of bioactive compounds from bacterial cultures yielded negative results. Based on the results obtained in this study, the surfaces of rice plants were colonized by various bacteria with potentials in plant growth promotion.

Keywords: epiphytic bacteria, plant growth promoting bacteria, *Pyricularia oryzae*, rice
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1. Introduction

Diverse groups of bacteria were found associated with plants. They may colonize either plant rhizosphere as rhizobacteria, outer surfaces of plant organs as epiphytic bacteria or plant intercellular spaces as endophytic bacteria. A number of these bacteria were found to provide various direct benefits for plant growth as well as indirectly protect plants from phytopathogens. They are recognized as plant growth promoting bacteria (PGPB). Much research effort has been dedicated to study these bacteria for their application in agriculture in order to decrease our dependence on excessive use of chemicals that has led to the contamination of toxic compounds in environments [1].

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One of the direct mechanisms by PGPB is the increase of bioavailability of plant nutrients. Nitrogen is the most important nutrient for plant growth. However, it is mostly found as the diatomic gas that is not readily available for plants. It has to be converted into ammonia by either physical or biological nitrogen fixation. Several nitrogen- fixing bacteria in various genera including *Azosprillum*, *Herbasipirillum*, *Bacillus*, *Burkholderia* and *Pseudomonas* were found to provide fixed nitrogen to their plant hosts [2]. Phosphorus is considered the second- most important macronutrient for plants. However, a large amount of phosphorus is fixed or trapped in organic and inorganic compounds [3]. Phosphate- solubilizing bacteria are able to release phosphorus from its insoluble forms for plant absorption and utilization during growth and development. PGPB of genera *Bacillus*, *Pseudomonas* and *Burkholderia* are examples of phosphate- solubilizing bacteria [4]. Iron is one of the macronutrients required for plant growth. However, it is generally found as insoluble ferric (Fe^{3+}) hydroxides. This limits the uptake of iron by plants. Many PGPB were able to increase the availability of iron by secreting iron-chelating siderophores. Subsequently, the iron-bound form of siderophores is imported into the plant cells [5]. Members of the genus *Pseudomonas* were well known for their siderophore production [6]. Modulation of phytohormones is another direct mechanism found in PGPB. Auxin is an important hormone that regulates virtually all aspects of plant growth and development. The most common form of auxin in plants is Indole-3-acetic acid (IAA) that is also produced by many bacteria [7]. Examples of IAA- producing PGPB are *Azospirillum brasilense*, *Pseudomonas putida* and *Pantoea agglomerans* [8]. Ethylene is a phytohormone that is required for plant stress responses. The precursor of ethylene biosynthesis is 1-aminocyclopropane-1-carboxylic acid (ACC) that is converted into ethylene by the activity of the ACC synthase enzyme [9]. However, overproduction of ethylene induced by stress conditions could lead to growth reduction and senescence [10]. Previous studies showed that a number of PGPB were able to lower the ethylene level by producing ACC deaminase that converts ACC into α-ketobutyrate and ammonia. This resulted in the decrease of the ethylene levels and the increase of stress tolerance in plant hosts [11-13]. PGPB may indirectly confer disease resistance on the plant host through their antagonistic activities against phytopathogenic fungi. These activities may be derived from their ability to produce hydrolytic enzymes that disrupt cell wall components of the pathogens. Protease and chitinase degrade proteins and chitin that are components of the fungal cell wall [14]. Alternatively, many PGPB were shown to produce antifungal compounds that inhibited growth of the pathogens. Members of genera *Bacillus* and *Pseudomonas* were prominent PGPB in this regard because of their ability to synthesize various groups of antibiotics including ribosomal and non-ribosomal peptides and polyketides [5].

The aim of the present study was to investigate diversity and plant growth promoting activities of epiphytic bacteria of rice plants grown in organic farms that did not permit the use of chemical fertilizers and pesticides. We isolated epiphytic bacteria from the surfaces of roots, stems and leaves of rice plants collected from Bangkok, Chonburi, Saraburi and Suphanburi provinces, Thailand. Characterization of culturable isolates was determined based on their morphological characteristics and 16S rRNA gene sequences. Both direct and indirect plant growth promoting activities were examined. The result provided basic information of potential epiphytic PGPB for further *in planta* analysis and their application as biofertilizers and biocontrol agents.

2. Materials and Methods

2.1 Rice samples and isolation of epiphytic bacteria

Whole rice plants grown in organic farms were collected from Bangkok (13°52'56. 8" N 100°53'05.1"E), Chonburi (13°12'37.9"N 101°26'20.8"E), Saraburi (14°21'14.7"N 100°54'20.1"E)

and Suphanburi (14°32'35. 2" N 100°03'25. 6" E) provinces, Thailand. Samples from Bangkok location was of Hom-Nil variety while those from other locations were of Rice-Berry variety. Roots, stems and leaves were rinsed under running water and cut into small pieces.

2.2 Isolation of epiphytic bacteria

Samples were separately put in glass bottles containing 100 ml of sterilized distilled water. The bottles were shaken on a rotary shaker at 180 rpm for 30 min. Bacterial cell suspension was serially diluted and plated on nutrient agar (NA; HiMedia, India) plates and tryptone soya agar (TSA; HiMedia, India) plates. All plates were incubated at 30°C. Bacterial colonies were picked once they appeared on the surface of the media. This process was continued for seven days. Bacterial colonies were purified by repeatedly streaking on NA plates.

2.3 Morphological characterization

Pure isolates were examined for morphological characteristics including Gram-staining and cell shapes. The procedure was as follows: bacterial cells were smeared on a glass slide and fixed by passing through flame for a few times, crystal violet was used as the primary stain and removed after 1 min of staining. This was followed by addition of Gram's iodine on the smear for 1 min. The primary stain was removed by 95% ethanol within 20 s. The washing step was stopped by rinsing the slide with water. Safranin was used for staining the smear for 1 min and subsequently removed by rinsing with water. Bacterial cells were observed under a light microscope.

2.4 Identification of bacterial isolates using 16S rRNA gene sequences

Genomic DNA of all isolates was prepared using a commercial kit (Presto^{TM} Mini gDNA Bacteria Kit, Geneaid, Taiwan). Nearly complete 16S rRNA gene fragments were amplified using universal primers 41F (5'-GCTCAGATTGAACGCTGGCG-3') and 1492R (5'- TACGGYTACCTTGTTA CGACTT-3') [15-16]. Amplified products were cleaned using a commercial kit (Gel/PCR Purification Kit, Favorgen Biotech Corp, Taiwan) and sequenced using 41F and 1492R primers. Pairwise alignment of the 16S rRNA gene sequences was performed on the EzBioCloud database [17].

2.5 Plant growth promoting activities

For nitrogen fixation, bacterial cells were washed in glucose-nitrogen-free broth [18] and centrifuged at 5,000 rpm for 15 min with a Minispin microcentrifuge (Eppendorf, Germany). The rotor diameter was 12 cm. The supernatant was discarded, and the same liquid medium was used for cell suspension. Two µl of the suspension were placed on glucose-nitrogen-free agar. Plates were incubated at 30°C for 4 days. The ability to grow on the same solid medium supplemented with 3 mM $(NH_4)_2SO_4$ was used as the indication of a positive result. To determine the phosphate solubilizing activity, bacterial colonies were spotted on Pikovskaya's (PVK) medium and National Botanical Research Institute's phosphate growth medium [19]. Bacteria were grown at 30°C for 4 days and checked for phosphate solubilization on both media. Chrome azurol S (CAS) agar medium [20] was used to examine the production of siderophores. Bacterial colonies were streaked on the medium and grown at 30°C for 48 h.

To determine IAA production, bacterial cells were inoculated in 50 ml of nutrient broth (NB; Himedia, India) containing 1% (w/v) L-tryptophan and grown at 30°C for 48 h on a rotary shaker at 160 rpm. Bacterial cells were separated from the culture by centrifugation at 2,500 rpm

for 5 min with a Minispin microcentrifuge (Eppendorf, Germany). The rotor diameter was 12 cm. One-hundred µl of the supernatant were tested for the presence of IAA by mixing with an equal volume of Salkowski's reagent [21]. For ACC deaminase activity, bacterial cells were washed in 1 ml of Dworkin and Foster (DF) salt minimal broth [22] once by resuspension and centrifugation. Harvested bacterial cells were then resuspended in 1 ml of fresh DF salt minimal broth and 2 µl of the cell suspension were inoculated on DF salt minimal agar supplemented with 2mM ACC as the sole nitrogen source. Bacteria were incubated at 30ºC for 4 days. Negative and positive control groups were bacteria grown on DF salt minimal agar and DF salt minimal agar containing 2 mM $(NH_4)_2SO_4$, respectively.

2.6 Indirect plant growth promoting activities

The antagonistic activity against *P. oryzae* was determined using the dual culture test. Epiphytic bacteria were streaked on potato dextrose agar (PDA; SRL, India). A mycelium plug of *P. oryzae* was placed 2 cm away from the bacteria. Plates were incubated at 30ºC for 14 days. Control plates were prepared by inoculating *P. oryzae* without epiphytic bacteria. The inhibition zone was the indicator of a positive result. To determine the percentage of growth inhibition, the experiment was repeated with all antagonistic isolates. Three replicates were performed for each isolate. Control plates were prepared by placing the mycelium plug on the medium only. The percentage of growth inhibition in each replicate was calculated as shown in below equation.

$$\text{Percentage of growth inhibition} = [(R_c-R_e)/R_c] \times 100$$

R_c is the radius of the fungal colony in control plates. R_e is the radius of the fungal colony in experimental plates. For statistical analysis, one-way ANOVA was performed and followed by the Tukey test. $P<0.05$ was considered statistically significant.

To test for their protease and chitinase activities, epiphytic bacteria were inoculated on skim-milk agar medium and NA medium that was supplemented with 1% colloidal chitin, respectively [23]. All plates were incubated at 30ºC for 48 h.

For extraction of extracellular antifungal compounds, antagonistic isolates were cultured in 50 ml NB at 30ºC for 7 days on a rotary shaker with 160 rpm. Subsequently, cultures were subjected to liquid-liquid extraction using an equal volume of ethyl acetate as the organic solvent. Each culture was extracted three times and the exhausted supernatant was collected. The solvent was evaporated using a rotary evaporator at 40˚C under 240 bars. Remaining crude extracts were washed off the bottle using methanol and left in a desiccator until methanol completely evaporated. The extracts were weighed and resuspended in methanol at 150 mg/µl and 300 mg/µl concentrations. The suspension was placed on sterilized paper discs No. 1 (GE Healthcare, USA). The discs were left to dry and tested for growth inhibition on *P. oryzae* in place of epiphytic bacteria as described in the dual culture test.

3. Results and Discussion

A total number of 113 epiphytic bacterial isolates were obtained from the rice plants. Forty-seven isolates were obtained from root surfaces. Thirty-one and thirty-five isolates were from stem and leaf surfaces, respectively. Gram-staining of these isolates showed that 68 and 45 isolates were Gram-stained positive and Gram-stained negative, respectively. Based on their cell shapes, 106 isolates were bacilli while the remaining seven isolates were cocci.

For molecular characterization, partial 16S rRNA gene sequences of all isolates were amplified and sequenced. The sequences were used for the pairwise alignment analysis on the EzBiocloud database. Details of the result are provided in Table1. The result showed that 34 isolates displayed 100% sequence similarity with the sequences on the database. The sequence similarity of the remaining 79 isolates ranged from 98.50% to 99.99% when compared with the database. Sixty-two isolates (54.87%) of the isolates belonged to the phylum *Firmicutes* and represented the largest group of the population. They were classified into four different genera including *Bacillus* (51 isolates), *Staphylococcus* (6 isolates), *Fictibacillus* (4 isolates) and *Exiguobacterium* (1 isolate). The second-largest group comprised of 43 isolates (38.05%) that were determined as members in the phylum *Proteobacteria*. They were divided into seventeen genera. Seven genera were found with more than one isolates. These included *Pseudomonas* (12 isolates), *Acinetobacter* (6 isolates), *Burkholderia* (4 isolates), *Enterobacter* (4 isolates), *Klebsiella* (3 isolates), *Aeromonas* (2 isolates) and *Chromobacterium* (2 isolates). One isolate was recorded for each of the following genera: *Aquitalea*, *Brevundimonas*, *Citrobacter*, *Kinneretia*, *Pandoraea*, *Rahnella*, *Roseateles*, *Serratia*, *Vogesella* and *Xanthomonas*. The smallest group contained eight isolates (7.08%) and belonged to phylum *Bacteroidetes*. They were divided into three genera including *Chryseobacterium* (4 isolates), *Chitinophaga* (3 isolates) and *Sphingobacterium* (1 isolate). Based on the isolation sources, the bacterial communities were relatively different (Table 1). On root surfaces, members of *Firmicutes* constituted 53.19% of the population. This was followed by *Proteobacteria* (34.04%) and *Bacteroidetes* (12.77%). In contrast, the dominant group (54.84%) on stem surfaces consisted of members in phylum *Proteobacteria* and was followed by *Firmicutes* (38.719%) and *Bacteroidetes* (6.45%). On the other hand, only those of phyla *Firmicutes* (71.43%) and *Proteobacteria* (28.57%) were recovered from leaf surfaces. At the genus level, 18, 12 and 7 different genera were found on roots, stems and leaves, respectively. This result indicated that root-surface colonizing bacteria were likely the most diverse group.

The diversity of culturable bacteria observed in the present study was consistent with previous reports. Members of phyla *Firmicutes*, *Proteobacteria* and *Bacteroidetes* were commonly isolated from rhizosphere, organ surfaces and internal tissues of various plant species. For example, a previous study investigated the diversity of bacteria on grape plants that were grown in two different vineyards. Despite the locations, *Firmicutes* and *Proteobacteria* were two of the three largest groups that colonized berries, leaves and barks of the plants. Another group of isolated bacteria belonged to the phylum *Actinobacteria* [24]. Similarly, our previous study showed that endophytic bacteria isolated from rice plants were members of *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* [25]. The culture-dependent approach had a limitation for the study of bacterial diversity since not all bacteria could grow on synthetic media. It could only reveal small fractions of an actual bacterial population. To overcome this limitation, several studies employed next-generation sequencing technologies and the metagenomic approach to obtain more inclusive information regarding the bacterial diversity. A recent study used Illumina MiSeq sequencing for identifying bacterial operational taxonomic units (OTUs) that were endophytes of rice sprouts, roots and stems. Although members of the common phyla mentioned above still represented the dominant groups, OTUs of other phyla including *Fusobacteria*, *Acidobacteria*, *Deinococcus-Thermus* and *Verrucomicrobia* were also detected. Additionally, the study observed a high level of bacterial diversity at the genus level [26]. In the present study, the level of diversity observed in root-colonizing bacteria was relatively higher than that found in stem- and leaf-colonizing bacteria. Roots and other plant underground organs were well known for their richness in bacterial compositions. An important contributing factor was derived from the secretion of root exudates that provided various types of organic compounds which may act as either nutritional resources that support bacterial growth and signaling molecules for bacterial chemotaxis [2].

All epiphytic bacteria were examined for their direct plant growth promoting activities including nitrogen fixation, phosphate solubilization, siderophore production, IAA production and

ACC deaminase activity. Ninety-one isolates displayed at least one positive activity (Table 2). Forty-nine isolates (43.36%) showed one activity. The second largest group of 33 isolates (29.20%) was tested positive for two activities. Only eight (7.08%) and one (0.88%) isolates displayed three and

Table 1. Characterization of 113 epiphytic bacteria from rice plants based on the analysis of the 16S rRNA gene sequences. Numbers in parentheses indicate the number of isolates that were affiliated with each genus.

Phylum	Genus		
	Roots	Stems	Leaves
Firmicutes	*Bacillus* (20), *Exiguobacterium* (1), *Fictibacillus* (3), *Staphylococcus* (1),	*Bacillus* (10), *Staphylococcus* (2),	*Bacillus* (21), *Fictibacillus* (1), *Staphylococcus* (3)
Proteobacteria	*Acinetobacter* (1), *Aeromonas* (2), *Aquitalea* (1), *Burkholderia* (2), *Enterobacter* (1), *Klebsiella* (1), *Padoraea* (1), *Pseudomonas* (3), *Rahnella* (1), *Serratia* (1), *Vogesella* (1), *Xanthomonas* (1)	*Acinetobacter* (4), *Burkholderia* (2), *Chromobacterium* (2), *Citrobacter* (1), *Enterobacter* (3), *Kinneretia* (1), *Klebsiella* (2), *Pseudomonas* (2)	*Acinetobacter* (1) *Brevundimonas* (1), *Pseudomonas* (7), *Roseateles* (1)
Bacteroidetes	*Chitinophaga* (3), *Chryseobacterium* (3)	*Chryseobacterium* (1), *Sphingobacterium* (1)	–

Table 2. Distribution of plant growth promoting activities among isolated epiphytic bacteria. N: nitrogen fixation, P: phosphate solubilization, S: Siderophore production, I: IAA production, A: ACC deaminase activity.

Activities	Number of isolates
N	17
P	3
S	21
I	4
A	4
N+P	2
N+S	6
N+A	5
N+I	1
P+A	2
S+A	8
I+A	4
I+S	5
N+P+A	1
N+S+I	1
N+S+A	3
P+S+A	1
S+I+A	2
P+S+I+A	1

four activities, respectively. Twenty-two isolates (19.47%) were tested negative for all activities. None of the isolates displayed all five activities.

Details of bacterial groups that displayed the positive activities are provided in Table 3. For nitrogen fixation, 36 positive isolates were able to grow on the glucose-nitrogen-free agar. Members of the phylum *Firmicutes* represented the dominant group (23 isolates) and were classified as *Bacillus*, *Exiguobacterium*, *Fictibacillus* and *Staphylococcus*. The second-largest group was *Proteobacteria* with 11 isolates that belonged to genera *Acinetobacter*, *Aeromonas*, *Brevundimonas*, *Klebsiella*, *Pandoraea* and *Pseudomonas*. Two isolates were affiliated with genera *Chitinophaga* and *Chryseobacterium* of phylum *Bacteroidetes*. For phosphate solubilization, the positive result was determined based on the presence of the clear zone around bacterial colonies on PVK and NBRIP media. Among 113 isolates, we found 9 isolates that displayed the phosphate solubilization activity. They were members of genera *Bacillus*, *Burkholderia*, *Pseudomonas* and *Roseateles*. Forty-nine isolates were able to produce siderophores as indicated by the presence of the orange halo around the colonies that were grown on CAS agar medium. Thirty-one isolates that belonged to the phylum *Firmicutes* were members of genera *Bacillus*, *Exiguobacterium*, *Fictibacillus* and *Staphylococcus*. Fifteen isolates were of the phylum *Proteobacteria*. They included isolates of genera *Acinetobacter*, *Aeromonas*, *Burkholderia*, *Kinneretia*, *Klebsiella*, *Pandoraea*, *Pseudomonas* and *Serratia*. The remaining three isolates were of the phylum *Bacteroidetes*. They were classified as members of genera *Chitinophaga*, *Chryseobacterium* and *Sphingobacterium*.

The results on bacterial activities involved in the increase of plant nutrients were relatively consistent with previous reports. Except for *Fictibacillus*, *Staphylococcus* and *Chitinophaga*, strains of the other nine genera were described for their nitrogen-fixing capability on the basis of either growth on nitrogen-free medium or the ability to reduce ethylene by nitrogenase or the presence of the *nifH* gene which codes for a nitrogenase subunit [27-32]. For phosphate-solubilizing activity, positive genera found in the present study were also previously reported as phosphate solubilizers [31-33]. The only exception was genus *Roseateles*. For siderophore production, other strains of most genera found in the present study were capable of producing siderophores [25, 34-41]. In contrast, up to the date of manuscript preparation, there were no reports of plant-associated bacteria in genera *Fictibacillus*, *Kinneretia* and *Chitinophaga* that displayed siderophore production.

The test for IAA production was performed by mixing culture supernatant with Salkowski's reagent. The pink color is the indicator of the presence of IAA. The positive result was obtained from the supernatant of 19 bacterial isolates. The predominant group consisted of 16 isolates of the phylum *Proteobacteria*. They were classified into genera *Acinetobacter*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pandoraea*, *Pseudomonas* and *Rahnella*. Three other isolates were all members of the phylum *Firmicutes* and belonged to genera *Bacillus* and *Staphylococcus*. Determination of ACC deaminase was based on the utilization of ACC as the sole nitrogen source in DF minimal agar. Thirty-one isolates were able to produce ACC deaminase. Among these, seventeen isolates belonged to the phylum *Firmicutes* and were classified as members of genera *Bacillus* and *Exiguobacterium*. Fourteen positive isolates of the phylum *Proteobacteria* consisted of members in genera *Acinetobacter*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Roseateles* and *Serratia*. Other bacterial strains in the corresponding genera previously demonstrated for IAA production [25, 33, 37, 42-46] and ACC deaminase activity [27, 47-51] were consistent with our observation. One exception was found with the genus *Roseateles* whose members, thus far, were not known for producing the ACC deaminase enzyme.

All 113 epiphytic bacterial isolates were screened for the antagonistic activity against *P. oryzae*, the causal agent of the leaf blast disease, on the basis of the inhibition zone formation in the dual culture test (Figure 1). Twenty-three isolates were found as the antagonists of the fungal pathogen. They were members of four different genera including *Bacillus* (16 isolates), *Burkholderia* (4 isolates), *Chitinophaga* (2 isolates) and *Pseudomonas* (1 isolate). The percentage

Table 3. Affiliation and the number of isolates of epiphytic bacteria that were tested positive.

Activities	Affiliation		
	Firmicutes	***Proteobacteria***	***Bacteroidetes***
Nitrogen fixation	4 genera, 23 isolates	6 genera, 11 isolates	2 genera, 2 isolates
Phosphate solubilization	10 genera, 3 isolates	2 genera, 6 isolates	-
Siderophore production	4 genera, 31 isolates	8 genera, 15 isolates	3 genera, 3 isolates
IAA production	2 genera, 3 isolates	7 genera, 16 isolates	-
ACC deaminase	2 genera, 17 isolates	6 genera, 14 isolates	-
Antagonism against *P. oryzae*	1 genera, 16 isolates	2 genera, 5 isolates	1 genus, 2 isolates

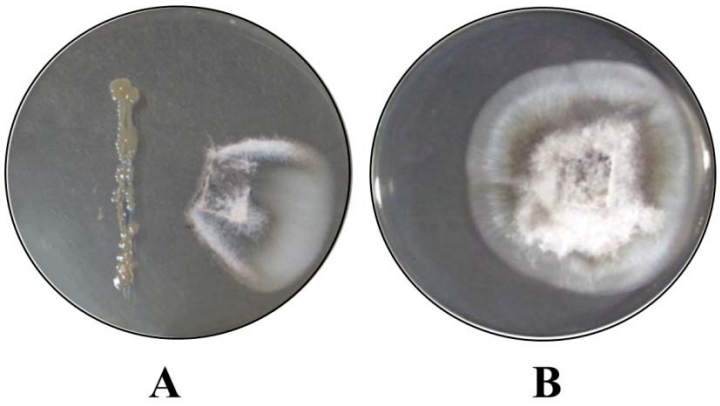

Figure 1. Formation of the inhibition zone between *Bacillus* sp. 1110 and *P. oryzae* (A) indicated the antagonistic activity of the isolate as opposed to the control plate (B) that was inoculated with the fungal pathogen only.

of growth inhibition was determined in each of the antagonistic isolate (Figure 2). The highest level (100% growth inhibition) was observed in *Bacillus* sp.1021, *Bacillus* sp. 1110, *Bacillus* sp. 2211, *Bacillus* sp. 2306, *Bacillus* sp. 3210, *Bacillus* sp. 3308, *Bacillus* sp. 3310 and *Pseudomonas* sp. 2302. The remaining isolates displayed the percentage of growth inhibition that ranged from 73.10% to 98.12%. Enzymatic protease and chitinase activities were tested on all 23 antagonistic isolates. The formation of the clear zone around bacterial colonies on skim-milk agar and NA supplemented with 1% colloidal chitin indicated the positive results of protease and chitinase, respectively. The result showed that 17 isolates produced proteases only. There were no isolates that only showed chitinase activity. Two isolates including *Burkholderia* spp. 1208 and 2208 displayed both protease and chitinase activities. To investigate the production of antifungal compounds, crude ethyl-acetate extracts of the antagonistic isolates were prepared. Although these isolates displayed relatively high levels of *P. oryzae* growth inhibition in the dual culture test, the crude extracts were unable to inhibit the mycelial growth of the fungal pathogen.

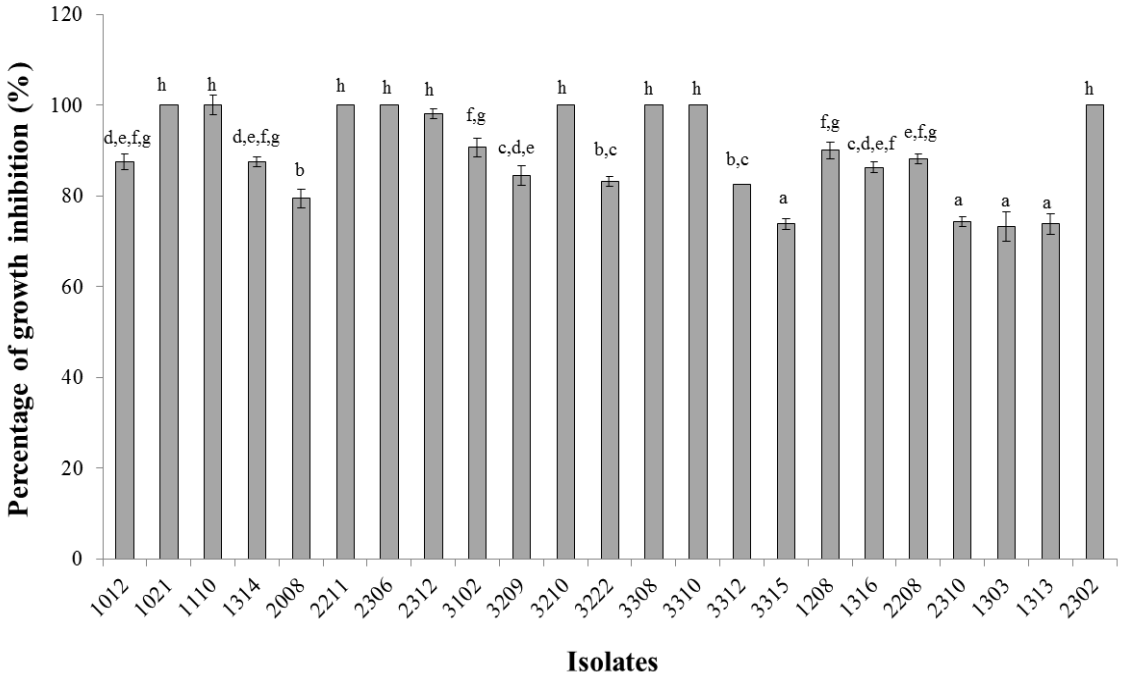

Figure 2. The percentage of growth inhibition of 19 antagonistic isolates against *P. oryzae*. Values represent mean ± standard deviation (n = 3). Different letters indicate statistically significant differences between the percentage of growth inhibition of the isolates (P<0.05).

The antagonistic isolates obtained in our study belonged to four different genera including *Bacillus*, *Pseudomonas*, *Burkholderia* and *Chitnophaga*. This was somewhat in agreement with previous reports. Strains of genera *Bacillus* and *Pseudomonas* were shown for their capability to inhibit the mycelial growth of *P. oryzae* [52-53]. In contrast, *Burkholderia glumae* 411 gr-6 was the only strain of the genus *Burkholderia* that was characterized and reported for its inhibitory effect on the fungus [54]. However, because of its pathogenicity in causing the panicle blight disease [55], its direct application as the biocontrol agent may be limited. Members of the genus *Chitinophaga* were scarcely associated with inhibition of phytopathogen growth. An isolate of the genus was able to reduce the severity of the damping off disease in potato that was caused by *Rhizoctonia solani* [56]. Inhibition and suppression of pathogen growth and colonization by PGPB occur through various mechanisms. One of them is the production of cell-wall degrading enzymes. Protease and chitinase were important for the disruption of proteins and chitin of the fungal cell wall, respectively [5]. In our study, protease was likely more involved in the inhibition of *P. oryzae* growth than chitinase. This was because 17 out of 23 isolates were protease-positive. Additionally, two isolates showing chitinase activity also produced protease. Another major mechanism of disease suppression by PGPB is the production of antibiotic compounds. Despite the strong antagonisms of the 23 isolates against *P. oryzae* in the dual culture test, the crude extracts of all isolates obtained in this study failed to suppress the fungal growth. This result suggested the influence of culture media on antifungal compound production. The dual culture test was carried out on PDA while bacterial culture used for the extraction of antibiotic compounds was grown in NB. The influence of culture

media and growth conditions on antibiotic production was previously demonstrated in other studies. For example, optimization of culture media and growth conditions of *Streptomyces rimosus* MY02 increased the production rate of an antifungal compound against *Fusarium oxysporum* [57].

4. Conclusions

Epiphytic bacteria were isolated from roots, stems and leaves of rice plants. Characterization of these isolates based on the 16S rRNA gene sequences showed that they belonged to phyla *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. The bacterial community on root surfaces was the most diverse group. Bacterial diversity from each location also followed the same trend. Ninety-one isolates displayed at least one of the tested direct plant growth promoting activities. The percentages of positive isolates from the four isolation sources were different. Isolates from Saraburi province showed the highest percentage (91.43%) followed by those from Bangkok (77.78%), Cholburi province (75.68%) and Supanburi province (75.00%). Twenty-three isolates showed the antagonistic activity against *P. oryzae*. The percentage of *P. oryzae* growth inhibition ranged from 73.10% to 100%. Protease and chitinase activities were detected in some of the antagonistic isolates. In contrast, crude extracts of all isolates were tested negative for the inhibition of *P. oryzae* growth. Several epiphytic bacteria isolated in the present study were potential candidates as PGPB for further *in planta* analyses.

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