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Characterisation of Plant Growth-Promoting Bacteria from Kacip Fatimah (*Labisia pumila*) under Natural Tropical Forest

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ABSTRACT

Medicinal plants play a major role in many cultures, not only as medicines, but also as trade commodities to fulfil the demands of distant markets. A study was conducted to characterise growth-promoting bacteria from two varieties of Kacip Fatimah *(Labisia pumila)* found at two different locations, Sungai Tekala, Semenyih, Selangor for *Labisia pumila* var. *lanceolata* and Bukit Slim Permanent Forest Reserve, Perak for *Labisia pumila* var. *alata.* Soil and plant samples were taken for the physico-chemical analyses and characterisation of the indigenous plant growth-promoting bacteria (PGPB). Both Labisia varieties were found to thrive in areas with quite similar soil chemical and physical properties in natural forest environments except for altitude, where *Labisia pumila* var. *alata* was found in higher elevation compared to *Labisia pumila* var. *lanceolata.* The soil in both places was found to be slightly acidic and low in nutrient content. Total bacterial population found on var. *alata* was higher than in var. *lanceolata* and the highest population was found in the root endosphere (8.68×10^7 cfu g^{-1} soil). Morphologically-isolated bacteria were circular in shape, with flat/raised elevation, entire margin, moist texture and smooth and glistening surface but varied in colour and size. Most of the bacterial strains showed several plant-growth promoting traits like plant-growth hormones (indole acetic acid (IAA)), N_2 fixation and P solubilisation activities and beneficial enzymes. Two of the bacterial isolates showing most of the beneficial properties were identified as *Exiguobacterium* sp. and *Stenotrophomonas* sp. These PGPB have the potential to enhance the growth of Kacip Fatimah.

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INTRODUCTION

Microorganisms play an important role in agriculture by supplying nutrients to plants and reducing the demand of chemical fertilisers (Cakmakci et al., 2006). Soil microbes affect soil fertility through their influence on organic matter turnover, mineral immobilisation and dissolution and soil aggregation (Davis & Abbott, 2006). Several bacteria have ability to fix atmospheric N_2 and are known as diazotrophs (Ladha & Reddy, 2000; Osivand et al., 2009). Poorly soluble P in soil can be solubilised by microorganisms and converted to soluble forms by the process of acidification, chelation and exchange reactions (Chung et al., 2005). Soil microorganisms such as the phosphate-solubilising bacteria (PSB) and arbuscular mycorrhizal fungi have the ability to mobilise P in soil and reduce inputs of chemical fertilisers (Arpana & Bagyaraj, 2007). Species of *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium* and *Erwinia* are well-known as plant growth-promoting bacteria (PGPB).

Higher populations of microorganisms occur in the rhizosphere than non-rhizosphere soil and they are metabolically more dynamic than those from other sources (Vazquez et al., 2000). Microorganisms are found everywhere in soils with various diversities in their functionalities. The population of these microorganisms is affected by soil physical and chemical properties, organic matter and P content, including cultural practices (Kim et al., 1998).

Several plant growth-promoting bacteria (PGPB) have been known to enhance growth of numerous plant species including some medicinal plants. Mixed bacterial inoculations were reported to have a higher success in improving plant growth as they provide more balanced nutrition. They significantly improved the adsorption of nitrogen, phosphorus and other mineral nutrients (Bashan & de-Bashan, 2005).

Kacip Fatimah *(Labisia pumila)* is one of the most popular traditional medicinal herbs found in the natural forests of Malaysia, Indonesia, Thailand, Indochina, Philippines and New Guinea (Wiart & Wong, 2002; Ong, 2004). Kacip Fatimah belongs to the family Myrsinaceae, and is a small wild sub-herbaceous plant with creeping stems growing naturally on the forest floor of 20-1500 m altitude. Malaysia is listed as the twelfth most bio-diverse nation in the world (Foster, 2009) with a diverse flora and fauna that are important for the phyto-pharmaceutical, phytocosmetic and nutraceutical industries. Herbal industry is one of the important industries in the development of health food. Currently, there is growing interest in medicinal plants as sources of new pharmaceutical products in Malaysia. Malaysia stands to gain from strong growth of natural herbal products. The growth rate of the herbal industry in Malaysia is estimated at 15% per annum and the industry is projected to have an

estimated market of RM15 billion and RM29 billion, respectively, for the years 2015 and 2020 (ECERDC, 2011).

Kacip Fatimah is a popular herb among local women (Ezumi et al., 2007); it is frequently consumed by women of reproductive age. It has been widely used by many generations for various ailments, especially those associated with childbirth and the feminine reproductive system. It has essential astringent, antibacterial and antifungal properties due to the presence of rich natural phytoestrogens. Other bioactive phytochemical contents are flavonoid (rutin, quercetin, kaempferol, myricetin), phenols (gallic acid, pyrogallol, caffeic acid), saponin, iron, benzoquinoid derivatives, alkenylresorcinols, triperpenoid and other antioxidative compounds consisting of β-carotene, vitamin C and anthocyanin (Foster, 2009; Norhaiza et al., 2009; Karimi et al., 2011). There is an increased demand for Kacip Fatimah but the plant source in natural forests are becoming scarce due to extensive harvesting. There are a few efforts to propagate the plant using seeds, leaves or root cuttings, but the growth rate in unnatural habitats is very slow and not profitable. In general, this plant thrives best in shady areas and non-waterlogged humus-rich soil.

Previous studies showed that PGPB can stimulate plant growth directly or indirectly in various ways through manipulation of their surrounding environment to support growth or through secretion of growth-promoting substances that enhance plant growth. Limited studies have been conducted on the beneficial effects of PGPB on the growth of Kacip Fatimah. The use of these beneficial bacteria may enhance sustainable production and reduce dependency on chemical fertilisers. The literature records many beneficial effects of PGPB in various crops but few studies have been conducted on Kacip Fatimah. Hence, the present study was carried out to isolate PGPB from the roots and shoots of Kacip Fatimah (*Labisia pumila*) obtained from natural forests and to characterise their plant-growth promotion traits.

MATERIALS AND METHODS

Plant and Soil Sampling

Kacip Fatimah plants were sampled from two different locations i.e. Sungai Tekala, Semenyih, Selangor for *Labisia pumila* var. *lanceolata* and Bukit Slim Permanent Forest Reserve, Perak for *Labisia pumila* var. *alata*. A total of 20 plants from each location were sampled together with the adhered soil for bacterial isolation. Soil samples at a depth of 15 cm were taken using an auger for nutrient analysis.

Locations and Climatic Conditions

The location and climatic conditions of the natural forest areas where Kacip Fatimah (*Labisia pumila*) were found were not significantly different. *Labisia pumila* var. *lanceolata* was found at elevation 66-116 m above sea level while *Labisia pumila* var. *alata* was found at the higher elevation of 266-356 m (Table 1). The light intensity and relative humidity of the area for var. *alata* was slightly higher than that for the location for var. *lanceolata* but the soil and

leaf temperatures were higher in area of var. *lanceolata* than that of var. *alata*.

Determination of Soil Chemical Properties

The pH of soil at ratio of 1:25 (soil:water) was determined using Beckman Digital pH meter. Nitrogen concentration in soil was determined using Kjeldahl method (Bremner, 1996). The available P was determined by the method of Bray and Kurtz (1945), while exchangeable K and cation exchange capacity (CEC) were determined using the modified leaching method of Chapman (1965).

Determination of Moisture in Soil

Ten grams of fresh soil were placed in a crucible and dried in an oven at 105 ^{o}C until a constant weight was obtained. The soil moisture content was expressed as follows:

$$\text{Soil moisture \%} = \frac{\text{Weight of water}}{\text{Dry weight of soil}} \times 100$$

Enumeration and Isolation of PGPB from Soil and Plant

Bacteria were isolated from the non-rhizosphere (bulk soil), rhizosphere and endosphere (root, stem and leaf) of Kacip Fatimah plants using serial dilution and plate count method (Johnson & Curl, 1972). Colony morphology of bacteria on a nutrient agar plate (NA) was observed and characterised according to shape, elevation, margin, texture, colour, surface and size within seven days of incubation.

Determination of Gram Reaction (Gram Staining and KOH Test)

The Gram reaction was determined using potassium hydroxide (KOH) test (Halebian et al., 1981) and the Gram-staining modified method of Hucker (1921). In a KOH test, Gram-negative bacteria turn viscous/ mucoid, while there is no change in Gram-positive bacteria.

Table 1
Chemical Properties of Soil Surrounding Kacip Fatimah Plant at 15cm Depth

Soil Characteristics	Location[a]	
	Sungai Tekala (*L.pumila* var.*lanceolata*)	Permanent Reserve Forest Bukit Slim *(L.pumila* var. *alata)*
pH	4.00 ± 0.03	4.42 ± 0.43
Total nitrogen (%)	1.03 ± 0.04	0.99 ± 0.7
Extractable phosphorus (mg kg^{-1})	5.23 ± 0.5	2.21 ± 0.18
Exchangeable potassium (mg kg^{-1})	20.68 ± 2.13	21.26 ± 1.49
Cation exchange capacity ($cmol_c\ kg^{-1}$)	18.09 ± 1.42	10.27 ± 0.48
Soil moisture (%)	37.51 ± 5.13	35.08 ± 1.36

[a]Average of five samples ± standard error of the mean

Screening for Phosphate Solubilising Activity

The ability of bacterial isolates to solubilise phosphate was determined using the National Botanical Research Institute's phosphate growth medium (NBRIP) modified method (Nautiyal, 1999). Four drops of 20 µl of bacteria from a nutrient broth was placed on a plate containing the NBRIP medium. The plate was incubated at a temperature of 28 ^{o}C±2 ^{o}C for 24 hours. Positive growth was observed through the visible halo zone surrounding the bacterial colony.

Screening for Potential Nitrogen Fixation Ability

The ability of the isolated bacteria to fix nitrogen was qualitatively determined using an N-free solid malate medium as used in the modified method of Dobereiner and Pedrosa (1987). Nitrogen fixing is indicated by a change in agar colour from green to blue.

Screening for Hydrolytic Enzyme Reaction

The ability of the isolates to produce enzymes *viz*. cellulase and pectinase was determined using a carboxy methyl cellulose (CMC) and citrus pectin media, respectively. Two grams of substrate (CMC/citrus pectin) were added into 1 l of nutrient agar (NA). Bacterial cultures were dropped on a media plate containing CMC and citrus pectin substrates. The plates were incubated for 24 hours at a temperature of 28 ^{o}C±2^{o}C before staining with 0.1 % Congo red for 30 minutes. The plates were then washed with 1 m of sodium chloride (NaCl). A positive reaction was indicated by a visible halo zone around the bacterial colonies.

Screening for Starch Hydrolysis

Starch hydrolysis of bacterial isolates was carried out using the method of Seeley and Dain (1960). The bacterial isolates were incubated in a 250-ml Erlenmeyer flask containing 100 ml of a nutrient broth (NB) for 24 hours before inoculating a starch agar plate consisting of 5 g L^{-1} of starch, 14g L^{-1} of nutrient agar (NA) and a teaspoon of agar powder. After incubation for three to five days at a temperature of 28^{o}C ± 2^{o}C, the plate was flooded with a dilute iodine solution (Gram's iodine solution). The iodine was allowed to remain in the medium for 30 seconds and then decanted. Hydrolysis activity was indicated by a clear zone around the growth.

Screening for Catalase Production

Catalase production of bacterial isolates was determined according to the modified method of Duke and Jarvis (1972). The bacterial isolates were incubated in 250-ml Erlenmeyer flasks containing a 100-ml nutrient broth (NB) for 24 hours before streaking on nutrient agar (NA) plates. The plates were incubated for 24-48 hours at a temperature of 28 ^{o}C±2^{o}C. Three or four drops of 3% hydrogen peroxide were added over the entire surface of the bacterial culture. Catalase production was determined by the presence of bubbling or foaming on the culture.

Determination of Indole-Acetic Acid (IAA) Production

IAA production by bacterial isolates was determined using the colorimetric method, modified from Gordon and Weber (1951). The bacterial isolates were incubated in 250-ml Erlenmeyer flasks containing a 100-ml nutrient broth (NB) with addition of 0.2% of L-tryptophan. The bacterial cultures were grown on a shaker for 24 hours at a temperature of 28 ^{o}C±2 ^{o}C. Two mm of bacterial cultures were placed in an Eppendorf tube and centrifuged for 7 minutes at 7000 rpm. One ml of the supernatant from each isolate was mixed with 2 ml of Salkowsky's reagent. The colour absorbance was determined using a spectrophotometer at 535 ηm after 20-25 minutes.

Strain Identification Using Polymerase Chain Reaction (PCR)

Bacterial identification was determined using 16S rRNA gene partial sequence analysis. DNA for bacterial isolates was extracted using the Genomic DNA Mini Kit (blood cultured cell) Bacteria Protocol (GENEAID). The cell harvesting/pre-lysis for the isolates was conducted following the Gram-negative bacteria procedure. Cultured bacterial cells was transferred to a 1.5-ml microcentrifuge tube and centrifuged for 1 minute at 10 000 rpm. Then, 200µl of GT Buffer was added and the cell pellet was suspended using a vortex. For the lysis step, 200µl of GB Buffer was added to the sample and vortexed for 5 seconds. The sample was incubated at 70 ºC until the sample lysate was clear. For DNA binding, 200µl of absolute ethanol was added to the sample lysate and immediately vortexed for 10 seconds. All the mixture was transferred to the GD column and placed in a 2-ml collection tube and centrifuged at 12 000 rpm for 2 minutes. The 2-ml collection tube containing the flow-through was discarded and the GD column was placed in a new 2-ml collection tube. For DNA washing, 400µl of WI buffer was added to the GD column and centrifuged at 12 000 rpm for 30 seconds. The flow-through was discarded and the GD column was placed back in the 2-ml collection tube before 600 µl of wash buffer (ethanol added) was added to the GD column. The sample was centrifuged at 12 000 rpm for 30 seconds. The flow-through was discarded and the GD column was replaced in the 2-ml collection tube before being centrifuged again at 12 000 rpm for 3 minutes to dry the column matrix. For DNA elution, the dried GD column was transferred to a clean 1.5-ml microcentrifuge tube before 100 µl of pre-heated elution buffer was added to the centre of the column matrix. The mixture was allowed to stand for 3 minutes until the elution buffer was absorbed by the matrix before being centrifuged at 12 000 rpm for 30 seconds to elude the purified DNA. For sample mix preparations, Dream TAq Green PCR master mix-fermentas was used. The PCR amplification was carried out using the MJ Mini Personal Thermal Cycler, Bio-Rad. The universal primer-F used was 27f, AGAGTTTGATCMTGGCTCAG while the primer-R was 1492r,

GGTTACCTTGTTACGACTT. The DNA was visualised using gel electrophoresis under UV light in a UV transluminater. The PCR products were purified using a GeneJet PCR Purification Kit and the purified DNA was stored at -20 ºC before being sent to 1stBase DNA Sequencing Division for sequencing. The 16s rDNA gene sequence was compared with a nucleotide database of the National Centre for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) at http://blast.ncbi.nih.gov/Blast.cgi.

Determination of Growth pH for Bacterial Strains

The bacterial strains were tested to determine the suitable pH condition for growth. The strains were cultured in 100-ml Erlenmeyer flasks containing a sterilised nutrient broth adjusted to pH 3, pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9 for 24 hours at 28±2 °C on an orbital shaker. A ten-fold serial dilution was made and total bacterial population was determined using the drop-plate technique and was calculated as follows:

$$\text{cfu} = \frac{\text{(Mean plate count) (Dilution factor)}}{0.1 \text{ ml}}$$

Determination of Optimum Growth of Bacterial Strains

The bacterial strains were tested to determine optimum cell growth. The strains were cultured in 250-ml Erlenmeyer flasks containing a 100-ml sterilised nutrient broth with adjusted pH 3, pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9 accordingly on the orbital shaker. Two ml of the nutrient broth was taken after 12 hours, 24 hours and 36 hours of culturing and put into Eppendorf tubes. The Eppendorf tubes were centrifuged at 7000 rpm for 7 minutes. The supernatant was taken out, leaving only the bacterial cells. The cells were then washed with 0.85% of phosphate buffered saline (PBS). The final solution was read using a spectrophotometer at 600 nm for an optical density reading.

RESULTS

Chemical Properties

Different species of *Labisia pumila* were sampled from two different locations. Table 2 shows that the two species could grow in slightly acidic soil at pH 4.00±0.03 (var. *lanceolata*) and 4.42±0.43 (var. *alata*). For the soil nutrient content, total soil nitrogen in surrounding soil was not significantly different for both species, which was 1.03±0.04 % for the area with var. *lanceolata* sampled from Sungai Tekala and 0.99±0.7 % for var. *alata* sampled from the Permanent Reserve Forest Bukit Slim. Extractable phosphorus of var. *lanceolata* soil was higher (5.23±0.5 mg kg^{-1}) than in var. *alata* soil but exchangeable potassium was higher in var. *alata*. (21.26 mg kg^{-1}). Cation exchange capacity in var. *lanceolata* soil was also greater at 18.09±1.42 $cmol_c$ kg^{-1} compared to 10.27±0.48 $cmol_c$ kg^{-1} for var. *alata*.

Table 2
Location and Climatic Conditions of Kacip Fatimah (Labisia pumila) in a Natural Forest

Species	Location and climatic conditions						
	N (^{0})	E (^{0})	Elevation (m)	Light intensity (µmol $m^{-2}S^{-1}$)	Temperature (^{o}C)	Leaf temperature (^{o}C)	Relative humidity (%)
L.pumila var. *lanceolata* (Sungai Tekala)	3.0598-3.0605	101.8718-101.8722	66-116	10.2 ± 1.56	29.58 ± 0.25	27.44 ± 0.09	80.6 ± 0.75
L.pumila var. *alata* (Permanent Reserve Forest Bukit Slim)	3.9658-3.9671	101.4223-101.4226	266-356	11.4 ± 1.72	27.66 ± 0.11	25.62 ± 0.18	82.62 ± 0.49

[a]Average of five samples ± standard error of the mean

Total Bacterial Population

Total bacterial population of var. *alata* was higher than var. *lanceolata* in most plant parts except in phyllosphere (Table 3). The bacterial populations were highest in the root endosphere (8.68×10^7) for both species and the lowest was in the non-rhizosphere of var. *lanceolata* and in phyllosphere of var. *alata*.

Plant Growth-Promoting Traits of Bacterial Isolates

There were 20 bacterial strains isolated from the soil and Kacip Fatimah plants obtained from Sungai Tekala, Selangor for var. *lanceolata* (Table 4). Six of them were isolated from the stem, five from the root, seven from the rhizosphere and two from the soil. Results of the Gram reaction showed that 16 isolates were Gram-positive and four were Gram-negative. All bacteria were able to grow in the N-free solid malate medium except for one of the isolates, which was KLR 06. Out of 20 isolates, only five strains were able to solubilise calcium phosphate and none of the strains were able to synthesise either cellulase or pectinase. However, 15 of the strains isolated were able to synthesise catalase enzyme while half of the strains were able to hydrolyse starch (Figure 1).

There were 17 bacterial strains isolated from which six were isolated from the rhizosphere, six from the roots, four from the soil and one from leaf of Kacip Fatimah (*L. pumila* var. *alata*). Results of the Gram reaction showed that nine isolates were Gram-positive and eight were Gram-negative. All the bacteria were able to grow in the N-free solid malate medium except two of the isolates (KAH03 and KAR18). Nine of the isolates were able to solubilise calcium phosphate while the rest showed a negative result. For hydrolytic enzyme reaction, six of the isolates were positive for

Table 3
Total Bacterial Populations from Non-Rhizosphere, Rhizosphere and Endosphere of Kacip Fatimah

Soil/plant parts	Total bacterial population ($cfug^{-1}$ soil $plant^{-1}$ dw)[a]	
	Sungai Tekala (*L. pumila* var. *lanceolata*)	Permanent Reserve Forest Bukit Slim (*L. pumila* var. *alata*)
Non-rhizosphere	1.04×10^{4d}	4.28×10^{5c}
Rhizosphere	2.53×10^{6b}	3.28×10^{7a}
Root endosphere	3.14×10^{6b}	8.68×10^{7a}
Stem endosphere	2.94×10^{4d}	1.86×10^{5c}
Phyllosphere	1.13×10^{6b}	1.57×10^{5c}

[a]Average of 19 samples
Means within the same column followed by the same letters are not significantly different at $P>0.05$.

Table 4
Plant Growth Promoting Trait of Bacterial Isolates in Kacip Fatimah (L. pumila var. lanceolata)

Bacterial strain	Gram reaction	N_2-fixation ability (N- free solid malate medium)	Phosphate solubilising activity	Hydrolytic enzyme reaction		Starch hydrolysis	Catalase production
				CMC	Citrus pectin		
KLS01	+	+	-	-	-	+	+
KLS02	+	+	-	-	-	+	+
KLS03	-	+	+	-	-	-	+
KLR05	+	+	-	-	-	-	-
KLR06	+	-	-	-	-	+	+
KLR07	+	+	-	-	-	+	-
KLS09	+	+	-	-	-	+	+
KLR10	-	+	+	-	-	-	+
KLR11	+	+	-	-	-	+	-
KLH12	-	+	+	-	-	-	+
KLH13	-	+	+	-	-	-	+
KLS14	+	+	-	-	-	-	-
KLH19	+	+	-	-	-	-	-
KLT22	+	+	-	-	-	-	+
KLT23	+	+	-	-	-	+	+
KLH24	+	+	-	-	-	+	+
KLH25	+	+	+	-	-	-	+
KLH27	+	+	-	-	-	+	+
KLH29	+	+	-	-	-	-	+
KLS35	+	+	-	-	-	+	+

both the carboxymethyl cellulase (CMC) and citrus pectin test but one isolate (KAR18) showed positive for carboxymethyl cellulase (CMC) and negative for citrus pectin. Nine isolates produced catalase and 11 isolates could hydrolyze starch (Table 5 and Figure 1).

Indole-Acetic Acid (IAA) Production

All the strains isolated from var. *lanceoalata* were able to produce IAA. The highest production was 5.77 µg/ml from KL S14, while the lowest was 1.45 µg/ml from KL T24 strains isolated from *L. pumila* var. *lanceolata* (Figure 2). However, the bacterial strains isolated from the *L. pumila* var. *alata* were also able to produce IAA, except for one strain (KAT14). The highest production was 5.84 µg/ml from KAH19, while the lowest was 1.14 µg/ml from KAR12 isolated from *L. pumila* var. *alata* (Figure 3).

Bacterial Identification Using Polymerase Chain Reaction (PCR)

Two of the isolates (KAR12 and KAR23) that possessed the most beneficial traits were taken for identification, while similar characteristics possessed by the isolates were not chosen for isolation, such as KAT 13. Both are from *L. pumila* var. *alata* sampled from the Permanent Forest Reserve Bukit Slim, Perak. BLAST output of isolate KAR12 after the 16s rRNA gene partial

Table 5
Plant Growth Promoting Trait of Bacterial Isolates in Kacip Fatimah (L. pumila var. alata)

Bacterial strain	Gram reaction	N_2-fixation ability (N- free solid malate medium)	Phosphate solubilising activity	Hydrolitic enzyme reaction		Starch hydrolisis	Catalase production
				CMC	Citrus pectin		
KAL01	+	+	+	-	-	+	-
KAH02	+	+	-	-	-	+	+
KAH03	+	-	+	+	+	+	-
KAH05	-	+	+	-	-	-	-
KAH06	+	+	-	-	-	+	+
KAR08	-	+	+	-	-	-	-
KAR09	-	+	-	-	-	-	+
KAR12	+	+	+	+	+	+	+
KAT13	+	+	+	+	+	+	+
KAT14	+	+	-	-	-	+	-
KAT15	-	+	-	+	+	-	+
KAT16	+	+	+	+	+	-	-
KAR17	-	+	-	-	-	+	+
KAR18	+	-	-	+	-	+	-
KAH19	-	+	+	-	-	+	+
KAR20	-	+	-	-	-	-	+
KAR23	-	+	+	+	+	+	-

sequence PCR procedure showed that the strain belonged to *Exiguobacterium* sp. NIU-K2 (Table 6), while isolate KAR23 belonged to *Stenotrophomonas* sp. Y06 (Table 6) with 100% and 99% similarity, respectively.

Growth of Bacterial Strains at Different pH

Growth of both bacterial strains was significantly affected by culture pH. The strains were able to grow at a pH range of 5-9 and could not grow at pH 3 (Figure 4). After 24 hours, growth of *Exiguobacterium* sp.

Table 6
BLAST Output of Isolates KAR12 and KAR23 (Sequences Producing Significant Alignment)

Isolates	Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KAR12	HQ385945.1	*Exiguobacterium* sp. NIU-K216S ribosomal RNA gene, partial sequence	939	1848	100%	0.0	100%
	HQ385944.1	*Exiguobacterium* sp. NIU-K416S ribosomal RNA gene, partial sequence	939	1848	100%	0.0	100%
	HM047519.1	*Exiguobacterium acetylicum* strain KNUC604 16S ribosomal RNA gene, partial sequence	939	1848	100%	0.0	100%
	JF227852.1	Uncultured bacterium clone ncd2602b09c1 16S ribosomal RNA gene, partial sequence	939	1848	100%	0.0	100%
	JF71564.1	Uncultured bacterium clone ncd1967d06c1 16S ribosomal RNA gene, partial sequence	939	1848	100%	0.0	100%
KAR23	JF220295.1	Uncultured bacterium clone ncd2628f03c1 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%
	JF163965.1	Uncultured bacterium clone ncd1904d07c1 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%
	HM161884.1	*Stenotrophomonas* sp. Y06(2010) 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%
	HM161883.1	*Stenotrophomonas* sp. Y05(2010) 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%
	HM161882.1	*Stenotrophomonas* sp. Y04(2010) 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%
	HM161866.1	Uncultured bacterium clone hdb_SIPB653 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%

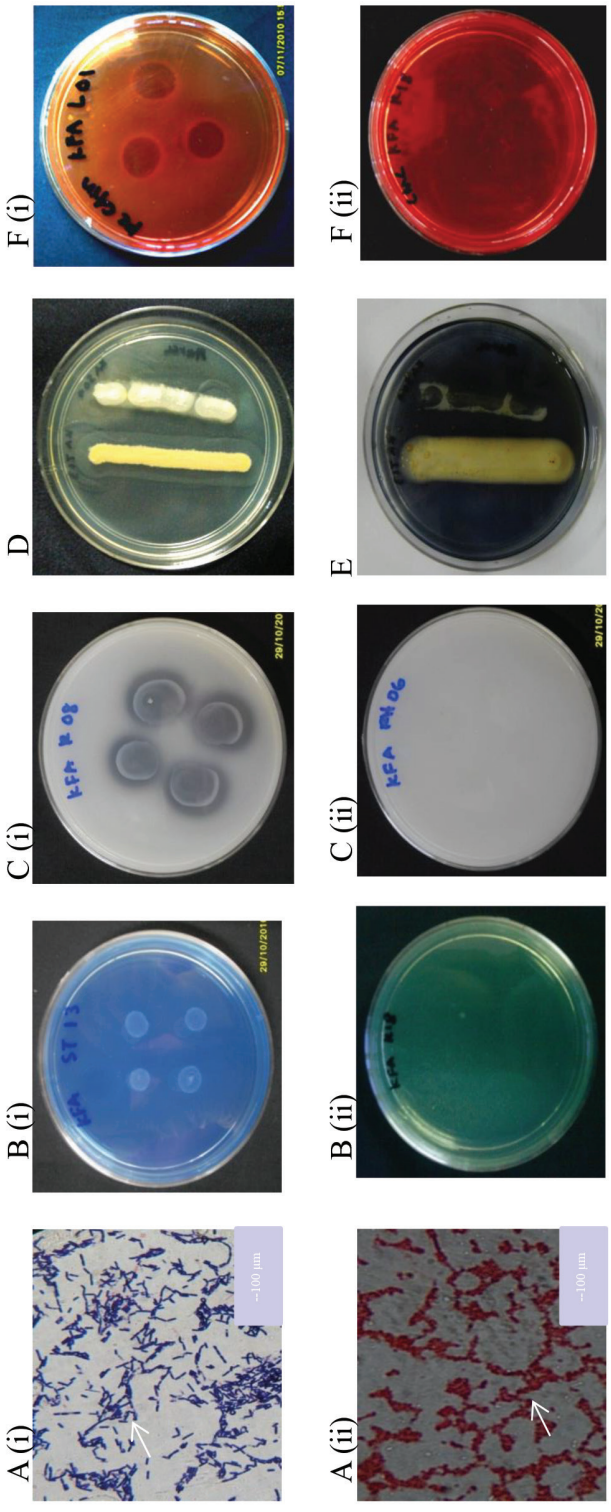

Figure 1. Plant-growth promoting trait of bacterial isolates in agar plates. A (i) Positive result, and A (ii) Negative result of Gram staining. B (i) Positive result and B (ii) Negative result of N_2-fixation ability on N-free solid malate medium. C (i) Positive result and C (ii) Negative result of phosphate solubilisation on NBRIP media plate. D, Negative result (left) and positive result (right) of catalase production on nutrient agar (NA) plate. E, positive result (left) and negative result (right) of starch hydrolysis on starch agar media plate. F (i) positive result and F (ii) negative result of hydrolytic enzyme on citrus pectin media (CMC) plate.

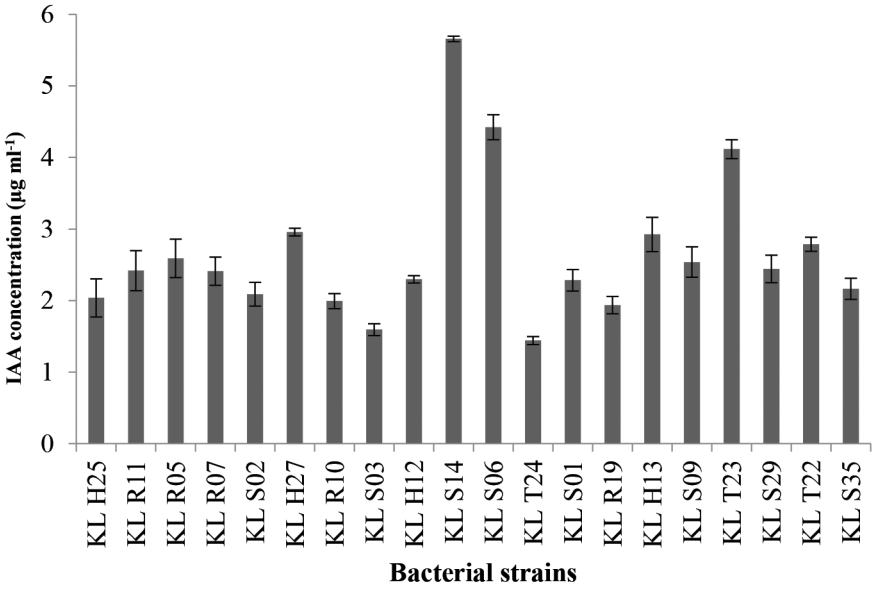

Figure 2. Indole-acetic acid (IAA) production by bacterial strains isolated from *L. pumila* var. *lanceolata.* Bars indicate standard error, n=3.

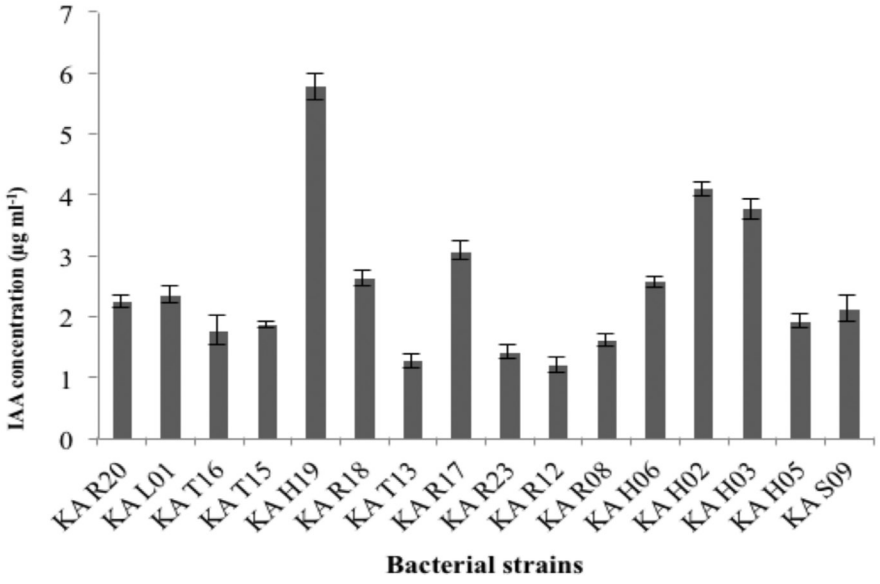

Figure 3. Indole-acetic acid (IAA) production by bacterial strains isolated from *L. pumila* var. *alata.* Bars indicate standard error, n=3.

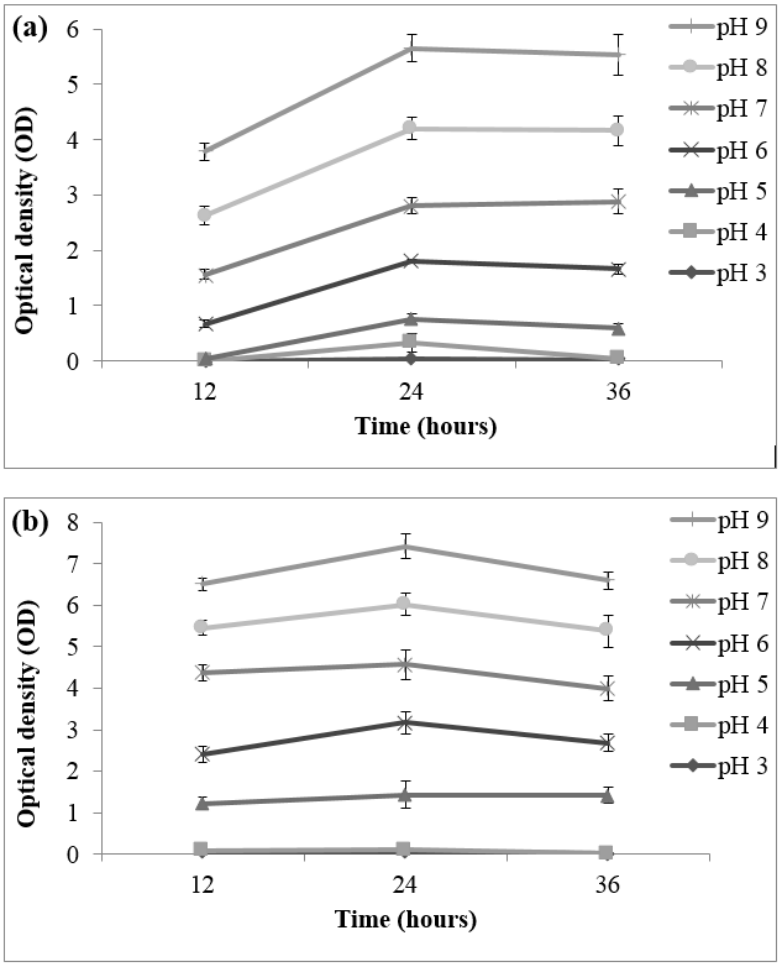

Figure 4. Cell growth of *Exiguobacterium* sp. (a) and *Stenotrophomonas* sp. (b) at different pH of culture solution. Bars indicate standard error, n=3.

was optimum at pH 8 (Figure 4a). However, the growth of *Stenotrophomonas* sp. was optimum at pH 6 and the highest growth was observed after 24 hours culturing (Figure 4b).

DISCUSSION

The bacterial populations of Kacip Fatimah differed between plant varieties and the location from which they were collected. The bacterial population in most of the plant parts except for phyllosphere was higher in var. *alata* than in var. *lanceolate*, whereas the population in phyllosphere for var. *lanceolata* was higher than for var. *alata* perhaps because the leaf surface of var. *lanceolata* was greater than that of var. *alata.* Variety *lanceolata* lives on a higher surrounding where the temperature is 29.58 ^{o}C±0.25 ^{o}C) and leaf temperature is 27.44

°C±0.09 °C as compared to 25.62 °C±0.18 °C of var. *alata*. Higher leaf temperature may increase leaf dehydration, reduce water availability and restrict phyllosphere bacterial populations as the bacterial populations within leaves are strongly correlated with water availability. Leaf water availability may have direct impact on bacterial growth as it can restrict bacterial growth (Freeman & Beattie, 2009). Most of the bacterial strains were Gram-positive and showed the ability to grow under fixed-nitrogen limiting conditions (N-free solid medium), indicating that there was strong possibility that the strains could fix nitrogen. Nitrogen fixation is a biological reaction where atmospheric N_2 is converted into NH_3; this is carried out by nitrogenase enzyme complex, which is activated under nitrogen-limiting conditions (Ladha & Reddy, 2000; Naher et al., 2011). The bacteria isolated from Kacip Fatimah could be said to be diazotrophs, which have the ability to fix nitrogen from the atmosphere and supply it to plants.

Phosphate-solubilising bacteria (PSB) produce organic acids to solubilise inorganic insoluble phosphorus. The starch hydrolysis test indicated the ability of bacterial strains to produce certain exoenzymes, including α-amylase, amylomaltase and α-glucosidase (Hii et al., 2012). The exoenzymes are able to degrade starch molecules into subunits that can be utilised as a source of carbon and energy for growth (Mutalib et al., 2012).

The catalase test was carried out to determine the ability of bacterial cultures to degrade hydrogen peroxide by producing the enzyme, catalase. Catalase-positive organisms use catalase to deactivate peroxide radicals, thus allowing them to survive unharmed within the host (Rao et al., 2003).

Bacterial strains can directly influence shoot development by secreting IAA as a plant-growth promoting hormone. The IAA is one of the most physiologically active auxins and several microorganisms including PGPB have the ability to produce IAA through the L-tryptophan metabolism. The IAA-producing bacteria use this phytohormone to interact with plants as part of the colonisation strategy, including phytostimulation and basal plant defense mechanisms (Spaepen et al., 2007). Several studies showed that IAA-producing bacteria promote plant growth and yield (Tsavkelova et al., 2007; Giongo et al., 2007). However, the growth of both bacterial strains was significantly affected by the pH culture. Generally ,strains were able to grow at the pH range of 5-9 and could not grow at the low pH level of pH 3. Similar findings were reported by Small et al. (1994).

The bacterial strain that possessed the most beneficial traits tested was *Exiguobacterium* sp., which has been characterised as a Gram-positive, facultative anaerobe that can grow profusely under aerobic conditions (Tatiana et al., 2014). Several researchers found that Exiguobacterium strains possess unique properties for application in biotechnology, bioremediation, industry and agriculture (Lee et al., 2009; Margesin & Feller, 2010; Kumar et al., 2010). This genus can also

survive in a wide range of temperatures from -12 to 55 °C and environmental conditions and have been isolated from different habitats (Vishnivetskaya & Kathariou, 2005; Vishnivetskaya et al., 2007). Hence, these two strains were able to survive well in a wide range of temperatures in the Permanent Reserve Forest Bukit Slim. Earlier studies also showed that *Exiguobacterium* sp. can influence growth and nutrient uptake of seedlings, improve yield and enhance the content of secondary metabolites of a salt-tolerant plant and wheat (Selvakumar et al., 2010; Bharti et al., 2013).

The other bacterial strain identified was *Stenotrophomonas* sp., which is a Gram-negative, aerobic bacterial strain that can be found in a wide range of environments particularly in close association with soil and plants (Kim et al., 2010; Ramos et al., 2011). *Stenotrophomonas* sp. has been isolated from various plants such as coffee (Nunes & de Melo, 2006), dune grass (Dalton et al., 2004) and medicinal herb (Lata et al., 2006). This strain can survive in a broad range of temperatures from 4-35 °C, has potential to be used for bioremediation, phytoremediation, biocontrol and also as a plant-growth promoter (Liu et al., 2007; Ryan et al., 2009).

CONCLUSION

Two varieties of Kacip Fatimah were found to thrive in areas with quite similar soil chemical and physical properties in natural forest environments except for the altitude where var. *alata* was found in higher elevation compared to var. *lanceolata*. The study also showed that there is a diverse growth of microorganisms in Kacip Fatimah plants. Most of the bacterial strains isolated from *Labisia pumila* showed plant growth-promoting traits and several possessed multiple traits. Two of the bacterial isolates showing most of the beneficial properties were identified as *Exiguobacterium* sp. and *Stenotrophomonas* sp. These PGPB have the potential to enhance growth of Kacip Fatimah for future plant propagation.

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