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Isolation, screening and characterization of bacteria associated with cocoa tree roots for different plant growth promotion (PGP) activities

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ABSTRACT

Aims: Plant growth promoting rhizobacteria (PGPR) is a group of bacteria that colonise plant roots and enhance plant growth by a diverse range of mechanisms. This study aims to determine the capabilities of PGPR isolated from cocoa tree roots and their efficiency in enhancing plant growth under greenhouse conditions.
Methodology and results: Eight samples of healthy cocoa tree roots were collected from different locations in Malaysia. Isolated bacteria were screened based on nitrogen fixation, phosphate and potassium solubilization, and catalase activity. The efficiency of purified PGPR was evaluated from pot experiments of cocoa seedlings under greenhouse conditions. Out of 122 isolates, 18 isolates showed several traits of nitrogen fixation, phosphorus and potassium solubilization and were further screened for other plant growth promoting (PGP) traits like catalase and production of indole acetic acid (IAA). Out of all the PGP trait tests, seven isolates showed the most prominent results for *in vitro* tests and were further tested *in vivo* for growth promotion of cocoa seedlings under greenhouse conditions. In the presence of bacterial isolates with 2.5 g of inorganic fertilizer, *Leclercia adecarboxylata* resulted in increases in plant height, leaf number, root length, stem fresh weight and total fresh and dry weight of cocoa seedlings by 15.68%, 17.14%, 9.48%, 5.67%, 11.84% and 25.12%, respectively.
Conclusion, significance and impact of study: Based on the result, *L. adecarboxylata* incorporated with selected carrier material improve cocoa seedling growth and biomass. This formulation also reduces the production cost of inorganic fertilizer and increase the application and development of biofertilizer.

Keywords: Isolation, screening, characterization, cocoa tree root, plant growth promotion

INTRODUCTION

The cocoa industry in Malaysia has undergone a challenging phase recently. The industry started to decline in the 1990s due to the outbreak of disease, the replacement of cocoa estates by oil palm estates and the relatively high cost of fertilizer (Malaysian Cocoa Board, 2010). Besides the high cost of inorganic fertilizer, its prolonged use causes water eutrophication, soil acidification, groundwater contamination and atmospheric contamination. In addition, the usage of inorganic fertilizers affects microbial biodiversity by reducing the viability of beneficial microbes in the soil (Vassilev *et al.*, 2015). Therefore, there was an urgent need to find alternative strategies that could ensure competitive crop yields, reduce the use of inorganic fertilizer and maintain a long-term ecological balance in the agro-ecosystem. The alternative use of abundant plant growth-promoting rhizobacteria for the enhancement of biofertilizer was the perfect choice for use and application in cocoa cultivated areas.

Plant growth-promoting rhizobacteria (PGPR), which can be found in the rhizosphere of the plant root, are important in soil ecological environments in terms of plant-microbe interactions by assisting certain nutrient absorption, solubilizing mineral phosphates, increasing seed germination rate, yield, leaf area, chlorophyll content, plant nutrient uptake, protein content, shoot and root weight and delaying senescence (Bhattacharyya and Jha, 2012).

PGPR is a heterogeneous genera that comprises of *Pseudomonas*, *Burkholderia*, *Enterobacter*, *Rhizobium*, *Serratia*, *Arthrobacter*, *Azospirillum*, *Bacillus*, *Klebsiella*, *Flavobacterium*, *Erwinia*, *Alcaligenes* and *Acinetobacter*

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(Ahemad and Kibret, 2014). PGPR also enzymatically synthesise and modulate compounds, which assists the absorption of certain nutrients, solubilization of mineral phosphates (Bahadur *et al*., 2017), nitrogen biological fixations (Melo *et al*., 2016) and synthesis of plant hormones such as gibberellin, cytokinin, ethylene and indole-3-acetic acid (Spaepen *et al*., 2007). Besides that, PGPR is capable of preventing the harmful effects of phytopathogens using antibiotics (Martínez-Viveros *et al*., 2010) or siderophore production (Gupta *et al*., 2015). A possible explanation for the PGPR reaction mechanism was the ability of those bacteria to produce a vital enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the ethylene level in the host plant root, therefore increasing the root length and growth production (Hayat *et al*., 2010), the ability to produce several hormones such as auxin, gibberellin and cytokinin (Idris *et al*., 2007), the symbiotic nitrogen fixation (Pedraza, 2008), the solubilization of phosphorus and potassium, and the mineralization of nutrients (Ahemad and Khan, 2012).

During the last two decades, the use of biofertilizer in agriculture has increased and several studies clearly demonstrated the positive effects of PGPR on the growth and yield productivity of different crops. Inoculation with *Serratia plymuthica* showed significant increase in plant height (up to 15%), dry weight (62%) and fruit yield (32%) in cucumber compared to un-inoculated plants (Egamberdieva *et al*., 2011). Majeed *et al.* (2015) conducted experiments on wheat, which showed significant increase by 25-45% in shoot and 29-52% in root length compared to un-inoculated plants. Moreover, inoculation of *Azotobacter* with biofertilizer reduced the use of inorganic fertilizer with 25-30 kg N/ha (Narula *et al*., 2005). Unfortunately, only a few studies have been conducted on PGPR in the cocoa environment. With increasing awareness about the long-term effects of inorganic fertilizer and the need to reduce the cost of cocoa production, it is important to identify a specific microbial strain with diverse capabilities which can be used in growth promotion to achieve the desired cocoa growth and production.

Therefore, this study is designed to isolate and screen PGPR associated with healthy cocoa tree roots in different locations in Malaysia. These bacteria were isolated and screened through *in vitro* for plant growth promoting potential. Bacterial isolates showing the maximum PGP traits were further tested *in vivo* pot studies under greenhouse conditions and the effects on the cocoa seedling growth were investigated. The potential bacteria were then identified by 16S rRNA sequence analysis.

MATERIALS AND METHODS

Sampling locations

All the samples were collected at eight different locations in Malaysia. Five samples were collected from a cocoa smallholder's farmer plot [located in Bukit Kerayong, Selangor (3.19082, 101.40338); Serian, Sarawak (0.91074, 110.53961); Beseri, Perlis (6.56215, 100.20868); Tanah Merah, Kelantan (5.832383, 102.161123) and Kg. Lapis Sg Ayam, Batu Pahat, Johor (1.782019, 102.937640)] and three from demonstrational plots at the Cocoa Research and Development Centres (CRDC) around Malaysia [CRDC Jengka, Pahang (3.61679, 102.50913), CRDC Tawau (4.26185, 118.01332) and CRDC Bagan Datuk (3.89426, 100.86634)] which showed better performance in growth and yield production. The samples were collected from matured cocoa trees (10 to 15 years after planting), regardless of the clones, healthy cocoa trees (free from vascular-streak dieback or black pod incidents) and those which yielded more than 0.5 tonne/ha. The locations were chosen based on the locality of states and represented each region of Malaysia to ensure the availability of bacteria in each location. Each location was selected with the aim of isolating and screening of PGPR and endophytic bacteria with several capabilities to enhance the growth of cocoa trees.

Root samples

Healthy cocoa tree roots were collected from the rhizosphere and endosphere of cocoa tree roots growing at different locations in Malaysia as mentioned above. Intact root systems (approximately 10-15 cm) were dug out and the root samples were cut, carefully placed in plastic bags and stored at 4 °C. Eight root samples were collected for the isolation of rhizospheric and endophytic bacteria. Samples of whitish cocoa root tips were collected (45-60 cm) from tree base using a surface-sterilized hoe.

Isolation and screening of the potential bacteria based on laboratory and pot experiment

This experiment was carried out at the Microbiology and Physiology Laboratory of Cocoa Research and Development Centre, Jengka, Pahang. Briefly, 1 g of fresh and whitish cocoa root tips was washed twice and transferred into McCartney bottles containing 15 mL of sterile distilled water. The whitish cocoa root tips were then taken out and the surface was sterilized by soaking in 95% ethanol for 10 sec, 1% sodium hypochlorite for 2 min and washing 6 times with sterile distilled water. The roots were cut into small pieces using a sterilized blade. These disserted root pieces were transferred into another McCartney bottle containing 15 mL of sterile distilled water. These roots were homogenized for 30 sec by using vortex mixer. These solutions were used to isolate the endophytic bacteria. A series of dilutions up to 10^{-9} were prepared for both roots. For each dilution, 100 µL was spread on nutrient agar (Merck, Germany) and incubated for 24 to 72 h at 28 °C. Predominant and morphologically distinct colonies were purified by repeated culturing and maintained on nutrient agar (Merck, Germany) slants. All pure isolates were screened for their ability to fix nitrogen, solubilize phosphorus and potassium, and produce plant

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hormones (indole-3-acetic acid, IAA) through plate assays and colorimetric method, respectively. All isolates were maintained at 4 °C with equal volumes of nutrient broth and 30% glycerol.

In vitro screening of isolates for different plant growth-promoting activities

Nitrogen fixation test

For the nitrogen fixation test, a nitrogen-free solid malate (Nfb) medium was prepared (Döbereiner and Day, 1976). The composition of the medium was (g/L) dl-malic acid (5), K_2HPO_4 (0.5), $MgSO_4{\cdot}7H_2O$ (0.2), KOH (4), NaCl (0.1) and $CaCl_2$ (0.02). Other components included were (mL/L) trace element solution (2), alcoholic solution of 5% bromothymol blue (2), Fe-EDTA (4) and vitamin solution (1). The composition of the trace element solution was (mg/200 mL distilled water) $NaMoO_4$ (200), $MnSO_4{\cdot}H_2O$ (235), H_3BO_3 (280), $CuSO_4{\cdot}5H_2O$ (8) and $ZnSO_4{\cdot}7H_2O$ (24). For the vitamin solution (mg/100 mL distilled water), the components were biotin (10) and pyridoxine (20). After all the components had been mixed, pH was adjusted to 6.8 using NaOH before sterilization. One loopful of bacterial colony was streaked onto the media. The plate was incubated at 28 °C for 24 h. The colour changes were recorded. The change of media colour from pale green to blue indicated the nitrogen fixation process carried out by the bacteria. The colour change resulted from the increase of pH due to the formation of ammonia and nitrates.

Phosphate solubilization test

Bacteria with the ability to solubilize phosphorus were tested on Pikovskaya agar (Pikovskaya, 1948). The medium was prepared using several compositions (g/L): glucose (10), $Ca_3(PO_4)_2$ (5), $(NH_4)_2SO_4$ (0.5), NaCl (0.2), $MgSO_4{\cdot}7H_2O$ (0.1), KCl (0.2), yeast extract (0.5), $MnSO_4{\cdot}H_2O$ (0.02), $FeSO_4{\cdot}7H_2O$ (0.002) and agar (15). The components were mixed and sterilized. One loopful of bacterial colony was streaked onto the media and incubated for 24 to 72 h at 28 °C. The appearance of a clear halo zone indicated the bacteria's ability to solubilize phosphate.

Potassium solubilization test

The selected isolates were grown on modified Aleksandrov agar to determine their ability to solubilize potassium (Hu *et al.*, 2006). The composition of the media was as follows (g/L): glucose (5), $MgSO_4{\cdot}7H_2O$ (0.5), $FeCl_3{\cdot}6H_2O$ (0.01), $CaCO_3$ (0.1), potassium aluminium silicate (muscovite mica) (3), $Ca(H_2PO_4)_2$ (2) and agar (20). One loopful of bacterial colony was streaked onto the media, incubated at 33 °C and observed for five to ten days after incubation. The ability to solubilize muscovite mica as a source of potassium was indicated by the formation of a clear halo zone around the colony.

Detection of indole-3-acetic acid (IAA)

Culture growth conditions

Phytohormone production was determined using the colorimetric method (Gordon and Weber, 1951). The selected isolates were grown in nutrient broth and shaken for 24 h. A volume of 1 mL of bacterial culture was transferred to a new nutrient broth with the addition of 5 mL L-tryptophan as an indicator of indole-3-acetic acid (IAA). Nutrient broth without any bacterial isolate was used as the control. The bacterial control (1.5 mL) was centrifuged at 10,000 rpm for 10 min at 5 °C. One millilitre of supernatant was mixed with 2 mL of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) and absorbance of the result showed the development of a pink colour after 25 min at 535 nm in UV/visible spectrophotometer. The IAA production was calculated from the regression equation of the standard curve and the result was expressed as µg/mL over control.

Catalase activity

A drop of 48 h old bacterial colony was placed on a clean glass slide and 3% hydrogen peroxide was added before mixing with a sterile toothpick. Effervescence indicated catalase activity.

Pot experiments

Pot experiments were carried out in the greenhouse of the Cocoa Research and Development Centre, Jengka, Pahang. Pots were filled with 3 kg of sterile soil. The soil used was a Bungor series, which is fine, kaolinitic, isohyperthermic, red-yellow Tipik Lutualemkuts. The soil used in the experiment had a pH of 5.2. It was sieved (2 mm) and placed into clean plastic pots and arranged in an experimental design layout. Cocoa seeds of BR 25 clone were surface sterilized for 15 min using 70% ethanol. The seeds were put into a double layer of sterilized wet gunny sacks for germination. After three days, two germinated seeds were transferred into pots and watered at field capacity. After a month of sowing, the seedlings were thinned out and only one healthy and uniform seedling was retained in the pots.

The growth-promoting effect of selected PGPR was studied on 2-month-old cocoa seedlings. A total of 81 pots were sown: three seedlings for each replicate (nine treatments with three replicates) were arranged in a randomised complete block design (RCBD). The treatment details are shown in Table 1.

Bacterial cultures and fertilizer were applied after 2 months of planting (after the cotyledon had fallen) and bacterial cultures were applied to the seedlings near the root zone of the cocoa seedlings. The plants were harvested 6 months after planting. The root length and fresh and dry weight of the plant components were measured. Plants were oven-dried at 70 °C for 96 h.

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Table 1: List of treatments for efficacy of PGPR isolates in this study.

No.	Treatment	Details
1	T1	1 mL of BR7 + 2.5 g of inorganic fertilizer (N 12%, P_2O_5 12%, K_2O 17%, MgO 2% + TE)
2	T2	1 mL of FE6 + 2.5 g of inorganic fertilizer (N 12%, P_2O_5 12%, K_2O 17%, MgO 2% + TE)
3	T3	1 mL of GR3 + 2.5 g of inorganic fertilizer (N 12%, P_2O_5 12%, K_2O 17%, MgO 2% + TE)
4	T4	1 mL of GR6 + 2.5 g of inorganic fertilizer (N 12%, P_2O_5 12%, K_2O 17%, MgO 2% + TE)
5	T5	1 mL of GR1 + 2.5 g of inorganic fertilizer (N 12%, P_2O_5 12%, K_2O 17%, MgO 2% + TE)
6	T6	1 mL of HE8 + 2.5 g of inorganic fertilizer (N 12%, P_2O_5 12%, K_2O 17%, MgO 2% + TE)
7	T7	1 mL of HR3 + 2.5 g of inorganic fertilizer (N 12%, P_2O_5 12%, K_2O 17%, MgO 2% + TE)
8	T8	Control (uninoculated bacteria with no fertilizer)
9	T9	Control (2.5 g of inorganic fertilizer (N 12%, P_2O_5 12%, K_2O 17%, MgO 2% + TE)

Bacteria identification through genotypic characterization

The bacterial genomics DNA extraction was done by using the protocol suggested by Sambrook and Russel (2001) with some modifications. The bacteria were cultured overnight for 18 to 24 h at 30 °C. The culture was then centrifuged and the pellets obtained were suspended in 0.5 mL of 10% (w/v) SDS and 0.5 mL of lysis solution [8 M urea (Merck, Germany), 0.3 M NaCl (Merck, Germany) and 10 mM Tris-HCL (Merck, Germany)]. The suspension was incubated at 37 °C for 20 min and extracted by adding 2 volumes of phenol. The mixture was then centrifuged at 9,750× *g* for 10 min. An equal volume of chloroform and isoamyl alcohol was then added and centrifuged at 9,750× *g* for 5 min. The aqueous phase containing genomic DNA was precipitated by adding 2.5 volumes of 100% ethanol and 1/10 volumes of 3 M sodium acetate (pH 5.2) buffer. The mixture was then incubated at –20 °C for 1 h and then centrifuged at 9,750× *g* for 5 min. The pellets consisting of DNA was washed with 70% (v/v) ethanol. TE buffer [10 mM Tris-HCl (pH7.5) and 1 mM EDTA (pH 8.0)] were used to dissolve the dried DNA pellet and stored at –20 °C.

Polymerase Chain Reaction (PCR) was conducted according to the manufacturer's instructions (Lucigen, USA) for amplification of 16S rRNA sequence of the bacteria. The PCR was carried out by using thermal cycler (peqSTAR, Germany) using master mix of 14 µL deionized water, 2.5 µL of 10× reaction buffer containing 15 mM $MgCl_2$, 2.0 µL of 2.5 mM dNTP mix (PCR Grade), 0.25 µL of 100 pmol/µL universal primers (forward: 5'-GAG TTT GAT CCT GCT CAG-3' and reverse: 5'-GTT ACC TTG TTA CGA CTT-3'), 0.5 µL of 5 U/mL Taq polymerase (Lucigen, USA) and 5 µL of genomic DNA as template (50-200 ng). Gradient PCR consisted of 35 cycles at 94 °C for 2 min, 35 cycles at 94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 1 min prior to 72 °C for 10 min.

Amplification of DNA samples was done by using protocol used by Nikunjkumar (2012) and conducted with some modifications. Amplified PCR product were separated by 1.0% agarose gel electrophoresis. Electricity of 75 to 80 V was applied for the gel electrophoresis analysis and observed using molecular imager Gel Doc (Bio Rad, USA) system.

The PCR product was purified with HiYield™ Gel/PCR DNA Mini Kit (Real Biotech, Taiwan) (Kumar *et al*., 2016). The agarose gel containing PCR products was excised until 300 mg and transferred into a 1.5 mL microcentrifuge tube. Detergent-Free (DF) buffer was added to dissolve the gel and eluted with elution buffer with final volume of 40 µL. The purified PCR product was analysed by First BASE Laboratories Sdn. Bhd. Sequences derived from the forward and reverse primers of PCR were aligned using BioEdit 7.2.4 (Hall, 1999) and ClustalW (Thompson *et al*., 1994) software. The aligned partial 16S rRNA gene sequence was compared with genes from Basic Local Alignment Search Tool (BLAST), NCBI Genbank (Johnson *et al*., 2008).

Statistical analysis

All data shown as the means ± standard deviation (SD) of four replicates for laboratory analysis and three replicates for pot experiments. Analysis of Variance (ANOVA) was used to verify the statistical difference between the treatment at $p<0.05$ and comparison of multiple treatment levels with the control, using Duncan's Multiple Range Test (DMRT). Statistical analysis was performed at 5% level using the Statistical Analysis System Package (SAS Institute, ver. 8.2).

RESULTS

Isolation and screening of bacterial isolates through *in vitro* study

In this study, 122 bacterial isolates were taken from eight healthy cocoa tree root samples. All the isolates were designated according to the sample location and classification based on the ability to fix nitrogen, solubilize phosphorus and potassium. In PGPR, plant growth promoting traits work as multiple mechanisms, direct or/and indirect for the plant growth and increased yield known as multi-traits PGPR. A total of 122 isolates were screened for nitrogen fixation on Nfb medium, phosphorus solubilization on Pikovskaya agar medium and potassium solubilization on modified Aleksandrov agar medium (Table 2). The nitrogen-fixing bacteria were preliminary screened on nitrogen-free solid malate agar medium containing Bromothymol blue as an indicator

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Table 2: Description of the bacterial isolates and result on nitrogen fixation, phosphorus and potassium solubilization reaction.

Sample number	Sample locations	No. of isolates	Isolate codes	No. of isolates show positive on nitrogen fixation, phosphorus and potassium solubilization
Sample 1	Bukit Kerayong, Selangor	18	AE1, AE2, AE3, AE4, AE5, AE6, AE7, AE8, AE9 AR1, AR2, AR3, AR4, AR5, AR6, AR7, AR8, AR9	5 (27.8%)
Sample 2	CRDC Jengka, Pahang	17	BE1, BE2, BE3, BE4, BE5, BE6, BE7, BE8 BR 1, BR2, BR3, BR4, BR5, BR6, BR7, BR8, BR9	5 (29.4%)
Sample 3	Serian, Sarawak	11	CE1, CE2, CE3, CE4 CR1, CR2, CR3, CR4, CR5, CR6, CR7	6 (54.5%)
Sample 4	Beseri, Perlis	18	DE1, DE2, DE3, DE4, DE5, DE6, DE7, DE8, DE9 DR1, DR2, DR3, DR4, DR5, DR6, DR7, DR8, DR9	9 (50.0%)
Sample 5	CRDC Tawau, Sabah	13	EE1, EE2, EE3, EE4, EE5, EE6 ER1, ER2, ER3, ER4, ER5, ER6, ER7	6 (46.2%)
Sample 6	Tanah Merah, Kelantan	14	FE1, FE2, FE3, FE4, FE5, FE6, FE7 FR1, FR2, FR3, FR4, FR5, FR6, FR7	3 (21.4%)
Sample 7	Batu Pahat, Johor	14	GE1, GE2, GE3, GE4, GE5, GE6, GE7 GR1, GR2, GR3, GR4, GR5, GR6, GR7	7 (50.0%)
Sample 8	CRDC Bagan Datuk, Perak	17	HE1, HE2, HE3, HE4, HE5, HE6, HE7, HE8, HE9, HE10 HR1, HR2, HR3, HR4, HR5, HR6, HR7	6 (35.3%)

Figures in parenthesis represent the percentage values per location.

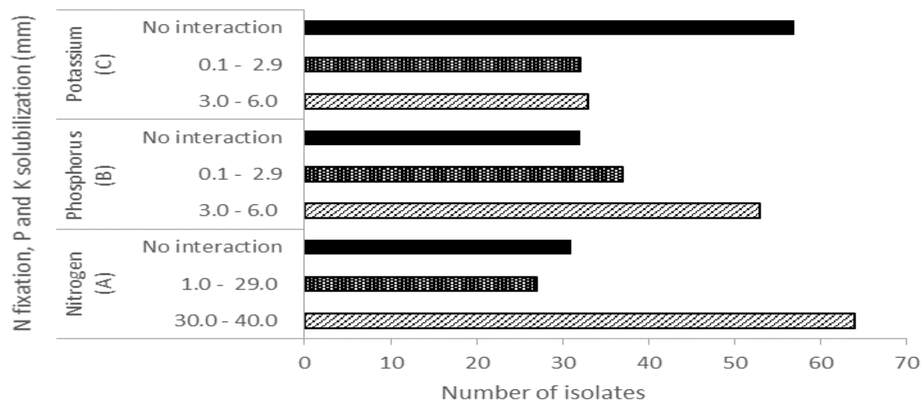

Figure 1: Interaction of isolates on (A) N free solid malate medium (Nfb), (B) Pikovskaya agar medium and (C) modified Aleksandrov agar medium.

which 64 bacterial isolates showed the development of a blue colour zone ranging from 30.0 to 40.0 mm, 27 bacterial isolates showed the development of a blue colour zone ranging from 1.0 to 29.0 mm, while 31 bacterial isolates showed no development of a blue colour zone (Figure 1).

For phosphorus solubilization activity, 53 bacterial isolates showed the development of sharp phosphate solubilization zones ranging from 3.0 to 6.0 mm, 37 bacterial isolates showed the development of phosphate solubilization zones ranging from 0.1 to 2.9 mm and 32 bacterial isolates did not show any halo zone development. Potassium-solubilizing bacteria were preliminary screened on modified Aleksandrov agar containing insoluble potassium, with muscovite mica as an indicator. Based on the development of a halo zone, 33 bacterial isolates showed potassium solubilization zones ranging from 3.0 to 6.0 mm, 32 bacterial isolates showed a halo zone ranging from 0.1 to 2.9 mm and 57 bacterial isolates did not show any development of halo zones. In summary, bacteria with the ability of nitrogen fixation, phosphorus and potassium solubilization and phytohormone production were the main targeted group to be used in the next experiment. There are 47 isolates were positive for nitrogen fixation, phosphorus and potassium solubilization ability test using agar plates. These 47 bacterial isolates were classified through their morphological colony characteristics similarity which is colour, elevation, margin and form (Table 3). In this study, 18 groups of isolates with the same morphology were observed and selected to be tested in the phytohormone production through colorimetric estimation (Table 4).

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Table 3: Morphological characteristics of eighteen groups of bacteria isolates from 47 isolates show positive interaction on nitrogen fixation, phosphorus and potassium solubilization.

Bacterial isolates	Label	Colony morphology on agar plates			
		Colour	Elevation	Margin	Form
AE6, BR7, DR6	B1	Red	Umbonate	Entire	Circular
AR2, CR7, DR2	B2	White	Umbonate	Lobate	Rhizoid
DR9, HE10	B3	Beige	Convex	Entire	Circular
BE2, CR5, EE5	B4	Yellow	Slightly raised	Entire	Circular
DR3, EE4	B5	Orange	Convex	Entire	Circular
DE3, ER5, GE7	B6	White	Convex	Entire	Circular
BR1, CR6, FE6, GE6	B7	Cream	Raised	Entire	Circular
DE1, FE5	B8	Beige	Raised	Entire	Circular
CR3, FR2	B9	Off-white	Convex	Entire	Irregular
AE1, DE6, GR3	B10	Cream	Umbonate	Entire	Irregular
BE3, ER3, GR6	B11	White	Convex	Entire	Punctiform
DE9, GR2	B12	Light yellow	Convex	Undulate	Filamentous
AR3, ER4, GR1	B13	Off-white	Convex	Entire	Circular
DE8, EE1, GR5, HE2	B14	Beige	Raised	Curled	Circular
BR8, HE8	B15	Beige	Raised	Entire	Filamentous
AR5, HR2	B16	Yellow	Pulvinate	Lobate	Irregular
CE4, HR3	B17	White	Raised	Entire	Circular
CR2, HR6	B18	Light yellow	Umbonate	Wavy	Irregular

Table 4: Classification of 18 bacterial isolates based on their ability to fix nitrogen, solubilize phosphorus and potassium.

No.	Isolate code	N fixation (mm)	P solubilization (mm)	K solubilization (mm)
1	BR7	39.65 ± 0.48^{a}	5.93 ± 0.10^{a}	6.00 ± 0.00^{a}
2	DR2	39.50 ± 0.58^{a}	5.98 ± 0.06^{a}	4.95 ± 0.13^{b}
3	DR9	35.00 ± 1.83^{b}	5.00 ± 0.33^{b}	4.00 ± 0.36^{c}
4	EE5	35.00 ± 0.82^{b}	5.98 ± 0.05^{a}	2.00 ± 0.36^{e}
5	EE4	39.50 ± 1.00^{a}	5.00 ± 0.32^{b}	4.00 ± 0.24^{c}
6	ER5	30.00 ± 3.74^{c}	4.00 ± 0.23^{c}	5.00 ± 0.18^{b}
7	FE6	40.00 ± 0.00^{a}	5.98 ± 0.05^{a}	5.00 ± 0.22^{b}
8	FE5	32.00 ± 1.41^{c}	3.00 ± 0.08^{d}	3.00 ± 0.47^{d}
9	FR2	39.75 ± 0.50^{a}	5.00 ± 0.08^{b}	2.00 ± 0.58^{e}
10	GR3	35.00 ± 4.08^{b}	6.00 ± 0.00^{a}	5.00 ± 0.22^{b}
11	GR6	39.50 ± 0.58^{a}	5.98 ± 0.05^{a}	4.00 ± 0.22^{c}
12	GR2	40.00 ± 0.00^{a}	4.98 ± 0.05^{b}	3.00 ± 0.18^{d}
13	GR1	31.00 ± 5.83^{c}	5.00 ± 0.00^{b}	5.00 ± 0.16^{b}
14	HE2	39.75 ± 0.50^{a}	5.95 ± 0.06^{a}	3.00 ± 0.42^{d}
15	HE8	39.50 ± 0.58^{a}	5.98 ± 0.05^{a}	2.00 ± 0.00^{e}
16	HR2	39.50 ± 0.58^{a}	6.00 ± 0.00^{a}	1.98 ± 0.17^{e}
17	HR3	40.00 ± 0.00^{a}	6.00 ± 0.00^{a}	3.00 ± 0.22^{d}
18	HR6	39.75 ± 0.50^{a}	6.00 ± 0.00^{a}	3.00 ± 0.29^{d}

Two-way ANOVA with Duncan's multiple range test was used to detect significant differences. Means denoted with letters are significantly different ($p \leq 0.05$).

Indole-3-acetic acid (IAA) production varied among the different species and strains. From the experiments, IAA production started at 24 h for 15 bacterial isolates while three bacterial isolates showed production after 48 h of incubation (Figure 2). EE4 showed high production after 24 h and decreased slowly, while ten bacterial isolates, namely DR2, DR9, EE5, ER5, FE5, FR2, GR2, HE2, HR2 and HR6, reached maximum production at 48 h when the bacterial isolates reached a stationary phase of growth and slowly decreased. Seven bacterial isolates, namely BR7, FE6, GR3, GR6, GR1, HE8 and HR3 showed increasing trends in IAA production after 72 h of incubation. Catalase activity was detected in ten bacterial isolates. Bacterial strains with catalase activity are highly resistant to environmental, mechanical and chemical stress. In this study, seven bacterial isolates (label as BR7, FE6, GR3, GR6, GR1, HE8 and HR3) were the most efficient PGPR which fixed nitrogen, solubilized insoluble phosphorus and potassium, and showed increasing trend in producing IAA and catalase (Table 5).

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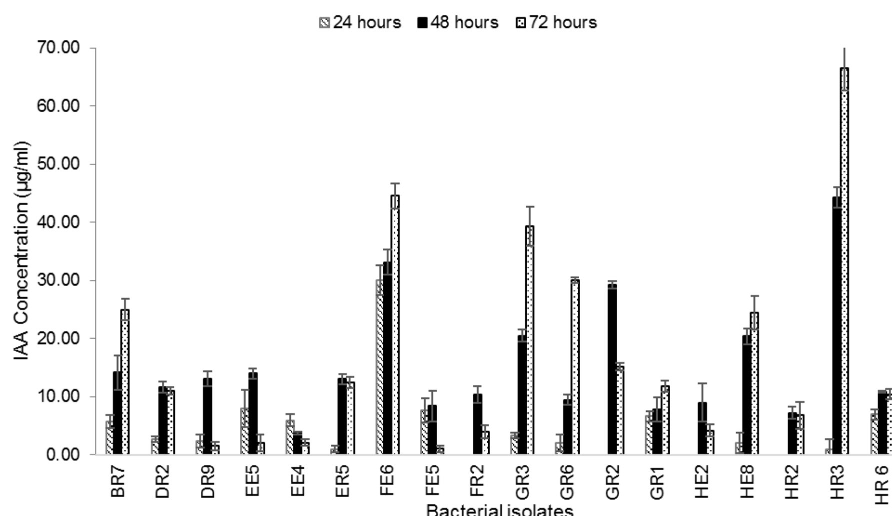

Figure 2: Quantitative estimation of IAA production by bacterial isolates in the medium supplemented with 5 mL of L-tryptophan at 24 h, 48 h and 72 h.

Table 5: Bacterial isolates showing different plant growth promotion activities.

No.	Isolate code	N fixation	P solubilization	K solubilization	IAA production (µg/mL)	Catalase activity
1	BR7	++	++	++	24.91	+
2	DR2	++	++	++	10.99	-
3	DR9	++	++	++	1.46	+
4	EE5	++	++	+	2.03	+
5	EE4	++	++	++	2.03	-
6	ER5	++	++	++	12.36	-
7	FE6	++	++	++	44.54	+
8	FE5	++	++	++	1.00	-
9	FR2	++	++	+	3.97	-
10	GR3	++	++	++	39.28	+
11	GR6	++	++	++	30.02	+
12	GR2	++	++	++	15.16	+
13	GR1	++	++	++	11.74	+
14	HE2	++	++	++	4.13	-
15	HE8	++	++	+	24.46	+
16	HR2	++	++	+	6.73	-
17	HR3	++	++	++	66.48	+
18	HR6	++	++	++	10.39	-

N fixation: 1.00-29.00 mm (+), 30.00-40.00 mm (++); P and K solubilization 0.10-2.90 mm (+), 3.00-6.00 mm (++)
Catalase activity: (+) indicates positive result, (-) indicates negative result

Therefore, these isolates were further tested for application in greenhouse conditions.

Evaluation of efficient PGPR isolates for plant growth under greenhouse conditions

The effects of seven selected bacterial isolates, BR7, FE6, GR3, GR6, GR1, HE8 and HR3 on the growth performance of cocoa seedlings were evaluated in a greenhouse experiment. The effects of treatments on 6-month-old seedling girth are shown in Table 6. After 120 days of inoculation, treatment using BR7 isolates with 2.5 g of inorganic fertilizer showed a greater plant girth (4.84 mm) and was significantly different compared to the control treatment (C2) using 2.5 g inorganic fertilizer only (increased 21%). This was followed by the application of HR3 isolates with 2.5 g of inorganic fertilizer with 4.47 mm growth, which was increased 11.75% from the initial girth of the control treatment (2.5 g of inorganic fertilizer). Both isolates gave a significant increase in plant girth compared to control with non-fertilizer application (C1).

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Table 6: Effect of bacterial isolates with inorganic fertilizer on plant girth, plant height, leaves number and root length of cocoa seedlings.

Bacterial isolates	Plant girth (mm)	Plant height (cm)	Leaves number	Root length (cm)
BR7	4.84 ± 0.38[a]	28.01 ± 0.55[a]	26.33 ± 4.04[ab]	68.67 ± 6.43[ab]
FE6	3.96 ± 0.37[bc]	29.51 ± 1.08[a]	27.33 ± 3.06[a]	70.67 ± 8.60[a]
GR3	3.89 ± 0.25[c]	24.50 ± 4.62[a]	25.67 ± 2.52[ab]	56.33 ± 5.51[ab]
GR6	3.94 ± 0.13[bc]	28.20 ± 3.33[a]	26.00 ± 2.65[ab]	56.83 ± 9.72[ab]
GR1	3.95 ± 0.21[bc]	26.01 ± 3.79[a]	23.67 ± 2.89[ab]	53.63 ± 8.29[b]
HE8	4.35 ± 0.05[abc]	26.82 ± 1.99[a]	23.67 ± 1.53[ab]	66.03 ± 9.95[ab]
HR3	4.47 ± 0.15[ab]	25.98 ± 2.37[a]	22.00 ± 3.61[b]	55.67 ± 6.98[ab]
C1	2.71 ± 0.06[d]	15.73 ± 3.50[b]	16.33 ± 2.31[c]	56.33 ± 7.23[ab]
C2	4.00 ± 0.48[bc]	25.51 ± 3.50[a]	23.33 + 2.08[ab]	64.55 ± 8.69[ab]

Two-way ANOVA with Duncan's Multiple Range Test was used to detect significant differences. Means denoted with different letters are significantly different ($p \leq 0.05$).

Table 7: Effect of bacterial isolates on fresh weight and dry weight of plant components.

No.	Treatment	Root (g)	Stem (g)	Total fresh weight (g)	Total dry weight (g)
1	BR7	9.39 ± 4.00[ab]	14.42 ± 5.67[a]	41.56 ± 15.39[ab]	15.71 ± 4.27[a]
2	FE6	11.49 ± 3.35[ab]	17.51 ± 5.16[a]	47.89 ± 11.62[a]	18.38 ± 1.41[a]
3	GR3	12.23 ± 6.62[a]	16.07 ± 7.00[a]	47.35 ± 23.89[a]	17.88 ± 7.82[a]
4	GR6	10.79 ± 2.71[ab]	14.74 ± 4.58[a]	39.52 ± 13.34[ab]	17.08 ± 3.20[a]
5	GR1	9.14 ± 3.34[ab]	12.26 ± 4.61[a]	34.35 ± 13.02[ab]	12.97 ± 1.40[ab]
6	HE8	8.76 ± 5.06[ab]	11.81 ± 2.67[a]	36.79 ± 7.19[ab]	14.17 ± 2.93[ab]
7	HR3	8.71 ± 2.02[ab]	14.05 ± 6.32[a]	36.73 ± 8.63[ab]	12.92 ± 3.63[ab]
8	C1	5.24 ± 2.22[b]	8.39 ± 4.71[a]	19.27 ± 7.55[b]	7.57 ± 3.23[b]
9	C2	9.53 ± 1.64[ab]	16.57 ± 2.42[a]	42.82 ± 4.42[ab]	14.69 ± 0.65[ab]

Two-way ANOVA with Duncan's Multiple Range Test was used to detect significant differences. Means denoted with different letters are significantly different ($p \leq 0.05$).

The application of FE6 with 2.5 g of inorganic fertilizer resulted in taller plant height (29.51 cm) compared with the control treatments of 2.5 g of inorganic fertilizer application (25.51 cm) and non-fertilizer (15.73 cm), which is an increase of 15.68% and 87.60%, respectively. This is followed by the application of GR6 isolates with 2.5 g of inorganic fertilizer with 28.20 cm growth, which is a 10.54% increase over the initial plant height of the control treatment. The order of treatments which caused an increase in leaf number is as follow: FE6 > BR 7 > GR6 > GR3 > GR1 > HE8 > C2 > HR3 > C1. Treatment with FE6 resulted in an increase of 17.14% in leaf number compared with the control treatment of 2.5 g of inorganic fertilizer. For root length, treatment with FE6 showed the highest increase (70.67 cm) which is a 9.48% increase over the control treatment of 2.5 g of inorganic fertilizer and a 25.46% increase over the non-fertilizer treatment. This is followed by the application of BR7, with 68.67 cm growth, which is a 6.38% increase over the application of 2.5 g inorganic fertilizer and 21.9% over the non-fertilizer treatment.

Evaluation of PGPR isolate efficiency for plant components under greenhouse conditions

The root fresh weight of cocoa seedlings inoculated with GR3 showed the highest increase (12.23 g) which is a 28.33% increase over the control treatment of 2.5 g of inorganic fertilizer, followed by FE6, GR6, C2, BR7, GR1, HR8, HR3 and lastly non-fertilizer application. Among the inoculated variants, the highest stem fresh weight was recorded in isolate FE6 (17.51 g), which is a 5.67% increase compared with the application of 2.5 g of inorganic fertilizer and a 108.7% increase compared to non-fertilizer application. As shown in Table 7, the bacterial isolate that exhibited the maximum increase in total weight compared with the control treatment (2.5 g of inorganic fertilizer) was FE6, with 47.89 g (fresh weight) and 18.38 g (dry weight). This is followed by GR3 with 47.35 g (fresh weight) and 17.88 g (dry weight).

Identification of potential bacteria

Sample of FE6 were sent to First BASE Laboratories for bacteria identification. Based on the 16S rRNA sequence analysis, the bacteria showed 99% similarity with *Leclercia adecarboxylata*.

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DISCUSSION

Plant growth-promoting rhizobacteria (PGPR) promotes plant growth through biological nitrogen fixation, phytohormone production, phosphate and potassium solubilization, and siderophore production (Pedraza, 2008). Many studies refer to the advantages and screening of PGPR and endophytes from crops such as rice, sugarcane, French beans and maize, but only a few studies refer to *Theobromae cacao*. In the present study, beneficial bacteria were isolated from the rhizosphere and endosphere of cocoa tree roots and screened for different plant growth promotion activities. For nitrogen fixation, 64 bacterial isolates showed blue colour zones of nitrogen fixation greater than 30.0 mm. Meanwhile, 53 bacterial isolates showed zones of phosphate solubilization greater than 3.0 mm. It has been reported that higher concentrations of phosphate solubilizing bacteria are commonly found in the rhizosphere soil as compared to non-rhizospheric soil (Reyes and Valduz, 2006). For potassium, 33 bacterial isolates showed halo zones greater than 3.0 mm. Based on the screening analysis, 18 bacterial isolates showed the highest reaction in nitrogen fixation, phosphorus solubilization and potassium solubilization. Phosphate solubilization is basically due to the production of microbial metabolites, including organic acids, which reduce the pH of the culture media (Shahid *et al*., 2012). The presence of a phosphate solubilizing microbial population in the soil of cocoa plantations may be considered as a positive indicator for utilising the microbes as biofertilizer for cocoa growth and production. Similarly, nitrogen fixers present in the soil of cocoa plantations can be beneficial in improving the nitrogen fixation of cocoa trees.

Another important trait of PGPR was the production of IAA. IAA is the most active and best-known plant hormone of the auxin group (Kenneth *et al*., 2019). All 18 isolates were positive for IAA production in a range of 0.96-66.48 µg/mL, but from these, seven isolates namely BR7, FE6, GR3, GR6, GR1, HE8 and HR3 were selected as potential IAA producers. This indicated a substantial variability among rhizosphere and endophytic cocoa roots for IAA production. The potential for bacterial isolates to produce IAA indicates their capacity to be used as growth regulators. These results were in line with previous studies, where the PGPR from the rhizosphere of wheat was shown to produce 0.27-77.98 µg/mL of IAA (Majeed *et al*., 2015). Most studies showed that IAA-producing organisms are Gram-negative (Datta and Basu, 2000). However, a number of studies have shown the Gram-positive strains belonging to *Bacillus* strains are capable of producing IAA (Mohite, 2013). The amount of IAA detected in the present study (with and without tryptophan) was relatively higher, indicating that the cocoa root contains bacteria with the characteristics to enhance the growth of plants. All isolates were further tested for catalase production. Basically, bacterial isolates with the capability of producing catalase are highly resistant to environmental, mechanical and chemical stress. A number of studies suggested that PGPR is able to increase crop yield, enhance crop growth and seed emergence and contribute to the protection of plants against pathogens and diseases (Herman *et al*., 2008). In the present study, seven bacterial isolates showed the ability to fix nitrogen, solubilize phosphorus, solubilize potassium and produce IAA, ammonia and catalase, which suggests that these bacterial species possess a potent ability to act as PGPR and therefore all seven bacteria were further tested in pot experiments.

In the pot experiments, inoculation with PGPR strains significantly promoted the growth of cocoa seedlings. Other studies have shown that the application of beneficial bacterial isolates increased component yield of maize (Nezarat and Gholami, 2009), groundnut (Goswami *et al*., 2013), strawberries (Laili *et al*., 2017) and faba beans (Bechtaoui *et al*., 2020). In this study, the inoculation of PGPR strains increased all parameters as determined in the field experiment. In all parameters, treatment with FE6 showed a higher increase in plant height, leaf number, root length, fresh weight of stem and total fresh and dry weight. This shows that the bacterial isolates used to inoculate plants with reduced amounts of fertilizer were able to enhance the plant growth compared to the control treatment.

The 16S rRNA sequence analysis from the NCBI database of bacterial strain FE6 was most closely related to *Leclercia adecarboxylata* (99% similarity). *Leclercia adecarboxylata* is commonly characterized as an oxidase-negative, facultative anaerobic, Gram-negative *Bacillus* and a member of Enterobacteriaceae family (Shah *et al*., 2011). In previous studies, most members of Enterobacteriaceae were characterized as human pathogens. Nonetheless, it has been reported to cause sepsis, cellulitis, endocarditis and cholecystitis in immunocompromised patients with polymicrobial infections (Ying *et al*., 2019) and safety concerns for human health remain. However, in recent studies, *Leclercia adecarboxylata* that has been successfully isolated and screened from cocoa tree roots showed various plant growth promoting activities. The members of Enterobacteriaceae played significant roles in plant growth promotion (Ahemad and Khan, 2010; Kisiel and Kepczynska, 2016; Mehmood *et al*., 2018), but recently only a few reports have been reported for the genus *Leclercia*. The plant growth promoting activity of *Leclercia* sp. FE6 was reported in this study by nitrogen fixation, phosphate solubilization, potassium solubilization and plant growth promoting activities. FE6 also solubilized the inorganic phosphate to organic, which was confirmed by the clear halo zone on Pikovskaya's medium. According to Naveed *et al*. (2014), a 1.2 kb partial sequence of *Leclercia* sp. *gdh* gene was sequenced as similar to the *gdh* gene of Enterobacteriaceae. The *gdh* gene in Enterobacteriaceae members plays important role in sugar metabolism (conversion of glucose into gluconic acid). In the present study, the growth promoting effect of *Leclercia* sp. FE6 was observed in cocoa seedlings. Results showed significant differences ($p≤0.05$) in cocoa seedling height, total numbers of leaves, root length, stem fresh weight, total fresh and dry weight in comparison with

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the control. This result is in line with Naveed *et al.* (2014), who reported that *Phaseolus vulgaris* inoculated with *Leclercia* sp. showed significantly greater shoot and root length, by 10%.

CONCLUSION

In the present study, inoculation of a beneficial bacteria species, FE6 (*Leclercia adecarboxylata*) resulted in better growth performance of cocoa seedlings. These bacteria live around the roots of cocoa seedlings and hold multiple traits such as the ability to fix nitrogen, solubilize phosphorus and potassium, produce IAA and catalase. Based on the greenhouse experiments, these bacteria enhanced plant height, leaf number, root length, stem weight, and total fresh and dry weight with reduced fertilizer conditions. The results suggest that this bacterial species can be used as biofertilizer and the use of the fertilizer can be reduced with the application of plant growth-promoting rhizobacteria on cocoa plants.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest regarding this work.

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