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ISOLATION, SCREENING, AND CHARACTERIZATION OF RHIZOSPHERIC AND ENDOPHYTIC BACTERIA FOR DIFFERENT PLANT GROWTH PROMOTION (PGP) ACTIVITIES

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ABSTRACT - *Plant growth-promoting rhizobacteria (PGPR) is a group of bacteria that colonize plant roots and enhance plant growth by a broad diversity of mechanisms while endophytic bacteria are defined as bacteria detected inside surface-sterilized plants or extracted from inside plants and having no visibly harmful effects on the plants. PGPR and endophytic bacteria comprise a heterogeneous genera of Pseudomonas, Burkholderia, Enterobacter, Rhizobium, Serratia, Arthrobacter, Flavobacterium, Azospirillum, Bacillus, Erwinia, and Acinetobacter and have been reported to enhance the plant nutrition. In the present study, five bacterial isolates were collected from cocoa environment. The objectives of the study were to determine the capabilities of PGPR isolated from cocoa environment and evaluate their efficiency to enhance the growth of cocoa seedlings under greenhouse conditions. Isolation and in vitro screening were done for different plant growth promotion activities i.e. nitrogen fixation, phosphate solubilization, ammonia production, ACC-deaminase activity and catalase activity. All five isolates showed several traits of nitrogen fixation, and phosphorus solubilization. All isolates were further screened for other PGP traits like catalase activity, ACC deaminase activity and ammonia production. In all PGP trait tests, all isolates showed the most prominent results for in vitro tests and suggested to further tested in vivo for growth promotion of cocoa seedlings under greenhouse conditions.*

Keywords: Isolation, Screening, Characterization, Rhizospheric and Endophytic Bacteria

INTRODUCTION

Intensive cropping systems with high input of inorganic fertilizers frequently lead to nonsustainability in production and also give a serious risk to soil health. Prolonged use of inorganic fertilizer causes water eutrophication, soil acidification, groundwater contamination, and atmospheric contamination. In addition, the usage of chemical fertilizers affect microbial biodiversity by limiting the amount of enzymes released by microbes through decreasing soil organic carbon, declining soil nitrogen content and breaking down the soil aggregates (Ozlu *et al*., 2019). Hence, the use of biofertilizer has been introduced to reduce the impact of chemical fertilizer use.

Biofertilizer are those substance contain living microorganism and it colonize in rhizosphere and endophytic in the plant root and increase supply of primary nutrient. In biofertilizer, the selective microorganism such as bacteria, fungi and algae were an important aspect to be included since it helps in nitrogen fixation, phosphorus and potassium solubilization. According to Aggani (2013), biofertilizer with the ability of nitrogen fixer and phosphate solubilizer able to fixes 20-40kg of nitrogen in one acre of soil. There are numerous species of soil bacteria colonize mainly in rhizosphere (known as plant growth promoting Rhizobacteria (PGPR) and endophytes.

Plant growth-promoting rhizobacteria (PGPR), are important bacteria in soil ecological

environment 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. PGPR is a heterogenous genera that comprise *Pseudomonas*, *Burkholderia*, *Enterobacter*, *Rhizobium*, *Serratia*, *Arthrobacter*, *Flavobacterium*, *Azospirillum*, *Bacillus*, *Klebsiella*, *Erwinia*, *Alcaligenes*, and *Acinetobacter* (Ahemad & Kibret, 2014). PGPR also enzymatically synthesize and modulate compounds, which assist 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 by antibiotics (Martinez *et al.,* 2010) or siderophore productions (Gupta *et al.,* 2015).

Endophytes are diverse microbes (Strobel and Daisy, 2003) where it colonize living in internal tissues of plants without causing any immediate or negative effect to host plant (Long *et al.,* 2008). Similar with PGPR, endophytes can enhance plant growth non-leguminous crops and enhance host plant nutrition through nitrogen fixation, phosphate solubilization or siderophore production (Uribe *et al.,* 2010). Several genera and species have been identified such as *Azospirillum brasilense* and *Azospirillum amazonense* (Weber *et al.,* 1999), *Bacillus* spp. (Harish *et al.,* 2008), *Bulkholderia* spp. (Ting *et al.,* 2008), *Citrobacter* spp.(Martinez *et al.,* 2003), *Enterobacter* spp.(Martinez *et al.,* 2003), *Herbaspirillum* spp.(Weber *et al.,* 1999, 2001), *Klebsiella spp*.(Rosenblueth *et al.,* 2004), *Pseudomonas* spp. (Harish *et al.,* 2008), *Rhizobium* spp.(Martinez *et al.,* 2003), and *Serratia* spp (Ting *et al.,* 2008).

This study is designed to screen PGPR and endophytes associated with healthy cocoa tree root by using in vitro methods. Bacterial isolates showing the maximum PGP traits in the in vitro study were further tested in an in vivo pot study under greenhouse conditions.

MATERIALS AND METHODS

Soil and leaves sampling

Healthy cocoa tree roots and leaves samples were collected from the rhizosphere and endosphere of cocoa tree root and leaves growing at different locations in Malaysia. Intact root systems were dug out and the root samples were cut, meanwhile leaves samples were selected and carefully placed in plastic bags, and stored at 4 °C. Eight root and leaves samples were collected for the isolation of rhizospheric and endophytic bacteria.

Isolation of Rhizospheric and endophytic bacteria

This experiment was carried out at the Microbiology and Physiology Laboratory of Cocoa Research and Development Centre, Jengka, Pahang. One gram of fresh and whitish cocoa root tips and leaves was washed twice and transferred into McCartney bottles containing 15 mL sterile distilled water. The whitish cocoa root tips and leaves were then taken out and the surface was sterilized by soaking in 95% ethanol for 10 s, 1% sodium hypochlorite for 2 min, and washed with sterile distilled water six times. The roots and leaves were cut into small pieces using a sterilized blade.

A series of dilution up to 10^{-9} were prepared for both roots and leaves sample. 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, ammonia production, catalase activity and ACC-deaminase activity.

In vitro assessment of PGP traits of isolated Rhizospheric and Endophytic

a) Nitrogen fixation test

For nitrogen fixation test, nitrogen-free solid malate (Nfb) medium was prepared (Dobereiner & 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 vitamin solution (mg/100 mL distilled water), the components were biotin (10) and pyridoxine (20). After all components had been mixed, pH was adjusted to 6.8 by 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 color changes were recorded. The change of media color from pale green to blue indicated the nitrogen fixation process carried out by the bacteria; the color change resulted from the increase of pH due to the formation of ammonia and nitrates.

b) Phosphate solubilization test

Bacteria with the ability to solubilize phosphorus were tested on Pikovskaya agar (Pikovskaya, 1948). The medium was prepared by 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 clear halo zone indicated the bacteria's ability to solubilize phosphate.

c) Ammonia production

All the bacterial isolates were tested for ammonia production as described by Cappuccino and Sherman (1992). Overnight bacterial cultures were inoculated in 10 mL peptone broth and incubated at 30 ± 0.1 °C for 48 h in incubator shaker. After the incubation period, 0.5 mL of Nessler's reagent was added and development of faint yellow to dark brown color was observed and recorded as an indicator of ammonia production.

d) 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 by using a sterile toothpick. Effervescence indicated catalase activity.

e) 1-aminocyclopropane-1-carboxylate (ACC)-deaminase activity by germinating seed bioassay

The effect of bacterial isolates on root elongation was studied by germinating seed bioassay as described by Dey *et al.*, 2004 and Belimov (2002). Seed surface of *Cucumis sativus* and *Ipomoea reptans* L., were sterilized with 20% NaOCl for 3 min and washes with sterilize distill water for three times. All seeds were allowed to germinate at 25°C in 1% of water agar plates for 48h. Germinated seeds were individually dipped for 1 h in 20 ml bacterial cultures grown for 48h in NB and transferred to wet filter paper in petri dishes. The Petri dishes were incubated in dark at 30±0.1 °c with three replications for each treatment. The seedlings treated with uninoculated NB were served as control. The root length of both seedlings were measured after 5 days of incubation.

RESULTS

Five bacteria were isolated from healthy cocoa root and leaves tree. All bacterial isolates were screened for nitrogen fixation on Nfb medium agar, of which all isolates showed the development of blue color zone ranging from 3.2 to 4.0 cm at the end of 120 hours. In the first 12 hours and 72 hour, there were no significant difference among the bacteria and there was a significant difference in 24, 36, 48, 60, 84, 96, 108 and 120 hours. Based on data recorded (Table 1), at the end of 120 hours, three bacterial isolates showed the highest colour zone which is B1, B7 and B11 meanwhile BL and UL showed lower colour zone as compared to other bacteria.

Table 1. Interaction of isolates on N free solid malate medium (Nfb) in every 12 hours for 5 days.

Trtmt	12 hours	24 hours	36 hours	48 hours	60 hours
B1	0.05±0.06a	0.45±0.06a	0.90±0.00a	1.45±0.06a	1.88±0.05a
B7	0.18±0.10a	0.35±0.13ab	0.83±0.10a	1.23±0.17a	1.65±0.25a
B11	0.13±0.05a	0.25±0.06b	0.48±0.05b	0.75±0.06b	1.18±0.05b
BL	0.15±0.06a	0.35±0.13ab	0.85±0.10a	1.30±0.27a	1.73±0.22a
UL	0.13±0.05a	0.20±0.00b	0.55±0.13b	0.88±0.29b	1.23±0.13b
CV	57.04	29.65	13.59	18.13	11.03
FV	1.03ns	2.60*	8.90*	5.12*	8.12*

Trtmt	72 hours	84 hours	96 hours	108 hours	120 hours
B1	2.18±0.05a	2.75±0.06a	3.08±0.05a	3.58±0.05a	4.00±0.00a
B7	2.05±0.24a	2.48±0.17ab	2.98±0.21a	3.50±0.08a	4.00±0.00a
B11	1.73±0.22b	2.18±0.10b	2.78±0.21ab	3.38±0.10a	4.00±0.00a
BL	2.05±0.17a	2.28±0.29b	2.48±0.36bc	2.88±0.36b	3.20±0.40b
UL	1.50±0.14b	1.85±0.17c	2.13±0.25c	2.80±0.28b	3.48±0.36b
CV	57.04	8.09	8.92	6.34	6.55
FV	1.03ns	7.62*	6.37*	7.22*	5.75*

Phosphorus solubilizing bacteria were preliminary screened on modified Pikovskaya agar containing insoluble tricalcium phosphate, as an indicator. All bacterial isolates showed the development of halo zone ranging from 3.3mm to 7.0 mm at the end of 120 hours. Based on the data recorded (Table 2), all treatments show a significant difference in 24, 48, 72, 96 and 120 hours. At the end of 120 hours, three bacterial isolates which are B1, B7, and BL showed highest halo zone meanwhile B11 and UL showed low halo zone.

Table 2: Interaction of isolates on Pikovskaya agar medium in every 24 hours for 5 days.

Trtmt	24 hours	48 hours	72 hours	96 hours	120 hours
B1	0.00±0.00b	0.20±0.00c	0.30±0.00c	0.48±0.12c	0.60±0.05ab
B7	0.30±0.00a	0.55±0.06a	0.70±0.00a	0.70±0.00a	0.70±0.00a
B11	0.00±0.00b	0.10±0.12cd	0.25±0.06cd	0.40±0.08c	0.45±0.06bc
BL	0.28±0.05a	0.43±0.10b	0.55±0.10b	0.58±0.11b	0.60±0.14ab
UL	0.00±0.00b	0.00±0.00d	0.20±0.00d	0.28±0.10d	0.33±0.13c
CV	19.44	31.21	13.69	19.56	17.73
FV	114.14**	18.80**	35.43**	13.70**	5.67*

All the bacterial isolates were tested for ammonia production as described by Cappuccino and Sherman (1992). Ammonia production has been reported to indirectly influence plant growth. All the five isolates were able to produce ammonia. Bacterial strains with catalase activity are highly resistant to environmental, mechanical, and chemical stress. Based on the data recorded (Table 3), catalase activity was detected in all bacterial isolates. All isolates were further tested with ACC deaminase test.

Table 3. Bacterial isolates showing different plant growth promotion activities.

No.	Isolate code	N fixation	P solubilization	Ammonia production	Catalase activity
1	B1	++	++	+	+
2	B7	++	++	+	+
3	B11	++	+	+	+
4	BL	+	++	+	+
5	UL	+	+	+	+

Five isolates positively affected the germination of *Cucumis sativus* and *Ipomoea reptans* L. seeds. Highest root elongation for *Ipomoea reptans*, L was recorded when seeds were pre-treated with B1 isolate (Figure 1). Bacterial isolates B11, UL, B7 and BL also showed better ability to increase the length of root as compared to control. For *Cucumis sativus*, B1 showed highest root elongation, followed by BL and B11. Seeds pre-treated with UL and B7 showed low root elongation as compared to control treatment.

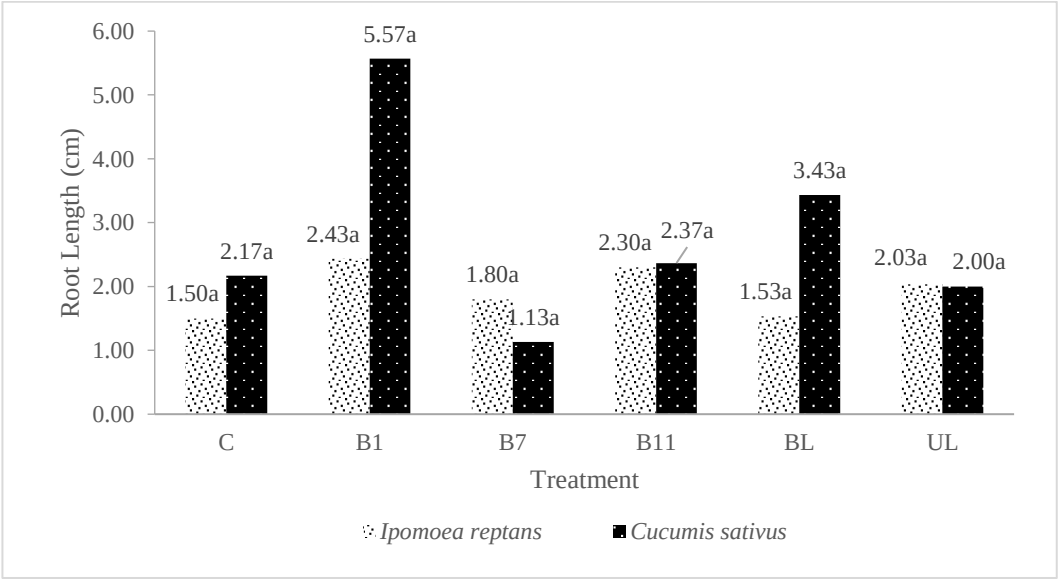

Figure 1. Comparison of Ipomoea reptans L. and Cucumis sativus for root elongation

Figure 2 shows all plant growth promotion activities, starting from nitrogen fixation, phosphorus solubilization, ammonia production, catalese activity and ACC-deaminese activity.

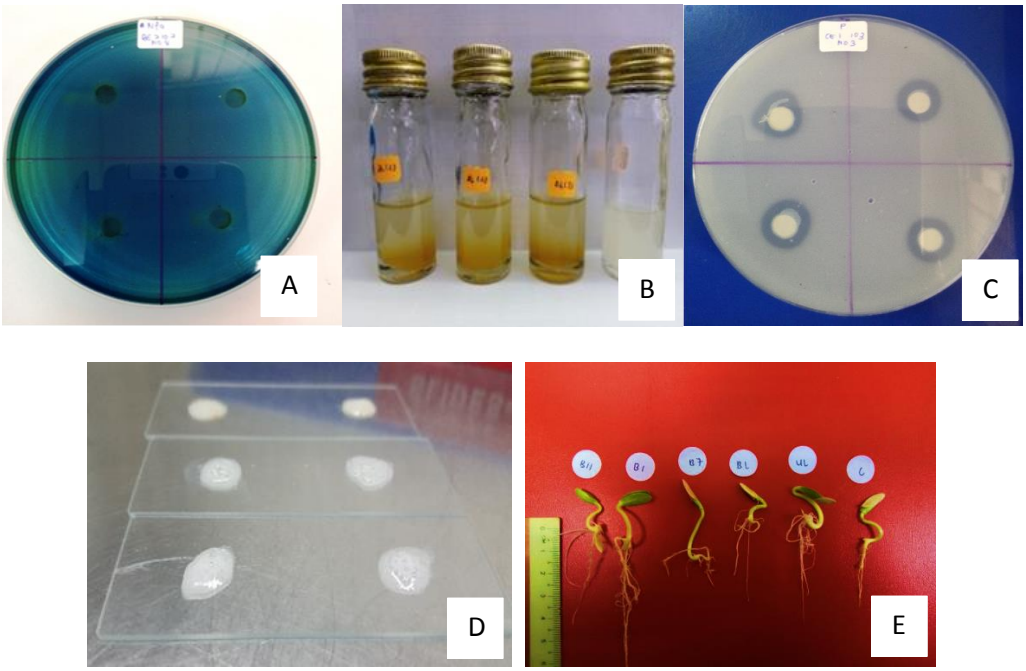

Figure 2. Plant growth promotion activities. (A) Nitrogen fixation; (B) Phosphorus solubilization; (C) Ammonia production; (D) Catalase activity; (E) ACC-Deaminase activity (root elongation)

DISCUSSION

PGPR and endophytes colonize roots of plant and promote plant growth and development through a variety of mechanisms. The mechanism of PGPR and endophytes reaction is not fully understood; however, several mechanisms such as suppression of deleterious organisms, activation of phosphate solubilization, promotion of nutrient uptake and productions of phytohormones are thought to be in charge in plant growth promotion (Kumar *et al.*, 2012). There are many paper related on the advantages and screening of PGPR and endophytes from crops such as rice, sugarcane, French beans and maize, but few in *Theobroma cacao*.

In current study, beneficial bacteria isolated from rhizosphere and endophytic cocoa root and leaves tree. Isolated bacteria were screened for different plant growth promotion activities. All bacterial isolates showed more than 3.0 cm blue color zone of nitrogen fixation. The isolates of B1 (*Serratia* sp.), B7 (*Leclercia* sp.), and B11 (*Staphylococcus* sp.) showed highest nitrogen fixation (4.0 mm) in Nfb agar medium. Meanwhile, all bacterial isolates showed more than 0.3 cm zone of phosphate solubilization. The isolates of B7 (*Leclercia* sp.) showed highest phosphorus solubilization zone (0.7cm) in Pikovskaya agar. 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)

Another important trait of PGPR and endophytes bacteria was production of ammonia. Ammonia was indirectly enhances the plant growth. In the study, all bacteria are capable in producing ammonia and catalase. Basically, bacterial isolates with capability in producing catalase are highly resistant to environment, mechanical and chemical stress. A number of studies suggested that PGPR and endophytes bacteria, able to increase crop yield, enhances crop growth, seed emergence, and contribute to the protection of plants against pathogens and disease (Herman *et al.*, 2008).

In present study, isolate B1 (*Serratia* sp.) significantly increased root length of

Ipomoea reptans and *Cucumis sativus* as indicator of high ACC deaminase activity. A possible explanation is the ability of 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 productions (Hayat *et al.*, 2010). In the current study, five bacterial isolates showed the ability to fix nitrogen, solubilize phosphorus, and produce ammonia, catalase, and ACC Deaminase activity, which suggest that these bacterial species possess potent ability to act as PGPR.

CONCLUSIONS

PGPR and Endophytic bacteria of cocoa tree in Malaysia were isolated and identified as *Serratia*, *Leclercia*, *Staphylococcus* and *Bacillus* species. *Leclercia adecarboxylata* was the most efficient in nitrogen fixation and phosphate solubilization respectively meanwhile *Serratia marcescens* resulted in better root elongation, therefore both bacteria can be proposed as potential microbes to be incorporated in biofertilizer production.

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