



Selecting the most effective plant growth-promoting bacteria from oil palm (*Elaeis guineensis* Jacq) roots

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ABSTRACT

A total of 30 bacterial isolates were isolated from soil, rhizoplane, and internal tissue of oil palm roots. The isolates were qualitatively tested for their potential to fix N₂, solubilize inorganic P and K, and produce phytohormone indole-3-acetic acid. Of the 30 isolates, six isolates were able to exhibit multiple beneficial traits. All six isolates were then identified based on fatty acid methyl esters profile as *Escherichia coli* strain EX2, *Serratia* sp. strain EN1, *Pantoea ananatis* strain EN3, *Bacillus* sp. strain EN5, *Pantoea ananatis* strain EN8 and *Pantoea* sp. strain EN9. Subsequently, all shortlisted isolates were evaluated for plant growth-promoting potential by using shallot as a test plant. The plant test showed no significant difference ($p>0.05$) between inoculated and uninoculated plants except for *Pantoea* sp. strain EN9 inoculation which increased significantly ($p<0.05$) total root length over uninoculated control. Host specificity and IAA capacity of the isolates may be among the important factors affecting their effectiveness in plant growth promotion.

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Introduction

Chemical fertilizer is widely used in agricultural crop production. However, improper use can cause negative effects to environment such as soil acidity and groundwater pollution. Use of certain fertilizer products can cause addition of heavy metals in plant and soil (Gall *et al.*, 2015). In recent years, various studies have been conducted to overcome the negative impact of chemical fertilizer to the environment (Gupta *et al.*, 2015). One of the approaches is using plant growth-promoting rhizobacteria (PGPR). PGPR which act as biofertilizer and biostimulant is safer for the environment and cost-effective as compared to chemical fertilizer. Srivastava and Singh (2017) stated that PGPR technology is a method used to dismiss the use of chemical fertilizers and pesticides by utilizing the microorganisms in soil. PGPR is referred to the bacteria found in soil that attach to roots of plant or rhizosphere. There are growing number of isolates, collected from various host plants. PGPR can be

isolated from agricultural crops such as wheat (Majeed *et al.*, 2015), rice (Tan *et al.*, 2014), and oil palm (Azlin *et al.*, 2005). Various successful application of PGPR on agricultural crops such as wheat (Kumar *et al.*, 2017), maize (Sood *et al.*, 2017), rice (Singh *et al.*, 2016), bell pepper (Tariq *et al.*, 2014), and oil palm (Zakry *et al.*, 2012) were reported. PGPR exhibit several beneficial mechanisms such as biological nitrogen fixation, P, and K solubilisation and growth hormone IAA production (Zakry *et al.*, 2010). Isolation of PGPR is a continuous process with aim to search for new strains of superior activity in plant growth promotion. Therefore, present study was aimed to isolate, and select for the most promising isolates in promoting plant growth.

Materials and Methods

Root samples were collected from the Oil Palm Plantation (3°12'20.36" N, 113°3'57.41" E) of Taman Pertanian Universiti, Universiti Putra Malaysia Bintulu Sarawak

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Campus, Bintulu, Sarawak, Malaysia. The soil and roots of the oil palm were collected and stored in sterile plastic bags. The plastic bags were placed in an ice box and brought to the Microbiology Laboratory for further analysis which was conducted within 48 hours.

To collect isolates from rhizospheric soil, the adhered soil from roots was removed and 10 g of soil was weighed. Then, the soil was mixed with normal saline solution (0.9% NaCl) that makes up 100 ml into 150 ml conical flask. The solution was shaken using orbital shaker for an hour at 80 rpm. One ml from the solution was pipette into falcon tube containing 9 ml of 0.9% NaCl solution. It was serially diluted up to 10^{-8} then 100 μ l from each dilution was plated onto fresh nutrient agar medium. The inoculated plate was incubated at $28\pm 2^{\circ}\text{C}$ for up to 48 hours.

For collection of isolates from root surface, the roots were washed thoroughly using sterile distilled water two to three times. The roots were cut into small pieces (2 to 3 cm). 10 g of the roots were weighed and mixed with 90 ml of 0.9% NaCl solution. The solution was shaken using orbital shaker for 30 minutes at 80 rpm. One ml from the solution was pipette into 9 ml of 0.9% NaCl solution in falcon tube. It was serially diluted and pipette onto fresh nutrient agar medium.

For endophyte isolation, the roots were soaked in 70% ethanol for 3 minutes to disinfect them. They were then washed thoroughly using sterile distilled water before soaked in NaOCl for 10 minutes. Next, the roots were washed thoroughly again using sterile distilled water for two to three times. 10g of the roots were weighed and placed into mortar. The roots were macerated using mortar and pestle with few drops of 0.9% NaCl solution. 1 g of the macerated tissue was placed into 9 ml of 0.9% NaCl solution. The serial dilution was prepared up to 10^{-7} . Then 0.1 ml of each dilution pipette and plated on fresh nutrient agar medium. The plate was incubated at $28\pm 2^{\circ}\text{C}$ for up to 48 hours.

Screening was conducted qualitatively to determine Nitrogen (N)-fixing activity, qualitative assay for phosphorus (P)-solubilizing bacteria, potassium (K)-solubilisation activity and Indole Acetic Acid (IAA)-producing activity.

Jensen's medium was used for detection and cultivation of N-fixing bacteria. Jensen's medium containing 20 g of sucrose, 1 g of dipotassium phosphate, 0.5 g of magnesium sulphate, 0.5 g of sodium chloride, 0.1 g of ferrous sulphate, 0.005 g of sodium molybdate, 2 g of calcium carbonate and 2 g of bacteriological agar. The procedure was carried out in accordance with Ram *et al.* (2017). The isolates were considered as N-fixing bacteria when pellicle formed.

National Botanical Research Institute's Phosphate (NBRIP) growth medium was prepared by weighing 10 g of glucose, 5 g of tricalcium phosphate, 5 g of magnesium chloride hexahydrate, 0.25 g of magnesium sulphate heptahydrate, 0.2 g of potassium chloride, 0.1 g of ammonium sulphate and 15 g of bacteriological agar. The isolates were characterized as P-solubilizing bacteria based on the clear zone formation (Liu *et al.*, 2015).

Aleksandrow Medium (AM) was prepared by weighing 5 g of glucose, 0.005 g of magnesium sulphate, 0.1 g of ferric chloride, 2 g of calcium carbonate, 3 g of mica powder, 2 g of calcium phosphate and 20 g of bacteriological agar. The isolates were characterized as K-solubilizing bacteria based on the clear zone formation (Verma *et al.*, 2016).

Determination of indole acetic acid producing ability was conducted according to Zakry *et al.* (2010). One loop-full of respective bacterial culture was inoculated into 50 ml of nutrient broth using sterile inoculating loop then incubated at 28°C for four days. After incubation, 30 ml of bacterial culture was transferred into 50 ml sterile falcon tube. It was centrifuged at 4000 rpm for 10 minutes to separate the supernatant from bacterial cell. Then 2 ml from supernatant was pipette into sterile test tube continued by 4 ml of Salkowski reagent and two drops of 85% orthophosphoric acid. Salkowski reagent was prepared by mixing 50 ml of 35% sulphuric acid (H_2SO_4) and one ml of 0.5M iron trichloride (FeCl_3). The test tube was then incubated in dark for 30 minutes. The isolates were characterized as IAA-producing bacteria based on changes of the solution into pink colour.

Selection from the total collection was made based on isolate that exhibit multiple traits or three or more with positive traits. Shortlisted PGPR isolates with multiple traits were characterized morphologically. Colony morphology was characterized based on colour, shape, size, and texture. Cell morphology was identified through Gram staining for determination of cell arrangement, cell shape and Gram reaction.

The shortlisted isolates from prior screening of PGPR were selected for gas chromatography analysis to determine the fatty acid methyl esters profile (GC-FAME). GC-FAME began by harvesting 40 mg of the bacteria cells from nutrient agar medium plate using sterile inoculating loop. The bacteria cell was placed in a sterile test tube. The procedure then involved saponification, methylation, and extraction of fatty acid of cells prior to GC-FAME analysis.

Shallot (*Allium ascalonicum*) was used as a test plant for inoculation with the shortlisted PGPR isolates. The shallots were cleaned by removing one or two outer scales. The clean shallots were cut horizontally into half before surface sterilized by soaking into 70% ethanol around 30 seconds. Then, it was soaked in 10% NaOCl

for 30 minutes. Next, rinsed thoroughly using sterile distilled water for five times. The base of the shallots was dipped for 5 minutes into the broth culture of the selected PGPR isolates (10^8 CFU per ml). The shallots were then transferred to disposable clear plastic cups containing sterilized white sand. There were four replications of shallots for each treatment and one shallot for one replication. Shallots treated with fresh sterile nutrient broth served as control. The experiment was assigned in completely randomized design under ambient temperature of laboratory ($28 \pm 2^\circ\text{C}$). After 17 days, the number of roots, total length of roots, shoot length and dry weight of roots and shoots were recorded.

Data on plant test were analysed statistically using the Statistical Analysis System (SAS version 9.4). Data were subject to analysis of variance (ANOVA) and mean values between treatments were compared using Duncan's New Multiple Range test at p value of 0.05.

Results and Discussion

The 30 isolates were coded and sequentially numbered as RHI (rhizospheric soil), EX (rhizoplane) and EN (endophyte) (Table 1). The isolates were tested for their biological nitrogen fixation, phosphate and potassium solubilisation and IAA production abilities. The isolates with at least three multiple traits were shortlisted for further testing in plant test. The present study showed that EX2, EN1, EN3, EN5, EN8 and EN9 exhibited multiple plant growth-promoting traits where most of them were endophytic rhizobacteria or rhizobacteria isolates that recovered from interior root tissue of oil palm. The collection of multiple traits from this study was at 20% of the total collection which considerably higher than

Govindasamy *et al.* (2017) who found that only 5 bacterial isolates from a total of 280 isolates (1.8%) from sorghum (*Sorghum bicolor*) rhizosphere were having multiple traits (more than three plant growth promotion traits).

Colony and cell morphology were identified, and the results were shown in Table 2. A total of six PGPR isolates with multitraits were analysed by GC-FAME (Table 2). Isolate EX2 was identified as *Escherichia coli* while EN1 as *Serratia* sp. EN3 and EN8 were identified as *Pantoea ananatis*, EN5 as *Bacillus* sp. and, EN9 was identified as *Pantoea* sp. Bacterial isolates which were identified as *Bacillus*, *Serratia*, and *Pantoea* were among common and widely reported genera belong to plant growth-promoting rhizobacteria group (Martínez *et al.*, 2018; Aziz, 2015; Susilowati *et al.*, 2015). *E. coli* strain EX2 which much related to clinical strain or human pathogen was rarely found as plant growth-promoting rhizobacterium species especially when exhibits multiple beneficial traits. Golubov *et al.* (2016) reported that *E. coli* can interact and even infect plants as demonstrated in transcriptomic profiling of *Arabidopsis thaliana* plants and human pathogen *Escherichia coli* strain O157-H7. Further clinical test on potential pathogenic PGPR strain usually needed before it can be formulated as an inoculum for sustainable crop production. Besides that, some reports suggest to totally avoid the use of potential pathogenic strains especially risk 2 group or higher (Vilchez *et al.*, 2017; WHO, 2015) and making this as one of the criteria during screening and selection process for the most promising plant growth-promoting rhizobacteria strains.

Table 1. Qualitative screening of PGPR isolates from oil palm (*Elaeis guineensis* Jacq.) for biological nitrogen fixation, phosphate and potassium solubilisation and IAA production

Beneficial traits	Isolates
Fix nitrogen	RHI1, RHI3, RHI7, EX3, EX9, EN5, EN9
Solubilise phosphate	EX2, EX6, EN1, EN2, EN3, EN5, EN8, EN9
Solubilise potassium	RHI5, RHI6, RHI8, EX2, EX7, EN1, EN3, EN5, EN8, EN9
Produce indole acetic acid	RHI1, RHI2, RHI6, RHI7, RHI8, RHI9, EX1, EX2, EX6, EX9, EN1, EN2, EN3, EN4, EN7, EN8, EN9, EN10

Note: RHI = isolates from rhizospheric soil; EX = isolates from rhizoplane; EN = isolates from internal root tissue

Table 2. Colony, cell morphology and identity of PGPR isolated from oil palm (*Elaeis guineensis*)

PGPR	Colony				Cell		GC-FAME identification		
	Colony colour	Shape	Size	Texture	Gram colour	Cell shape	Name	Sim index	Similar. (%)
EX2	Colourless	Circular	Small	Smooth	Pink	Bacilli	<i>Escherichia coli</i>	0.633	96.2
EN1	Red and white	Circular	Moderate	Smooth	Purple	Cocci	<i>Serratia</i> sp.	0.449	91.4
EN3	Yellow	Drop-shaped	Small	Shiny	Pink	Bacilli	<i>Pantoea ananatis</i>	0.568	98.2
EN5	White	Filamentous	Large	Rough	Purple	Bacilli	<i>Bacillus</i> sp.	0.156	54.8
EN8	Yellow	Circular	Small	Smooth	Purple	Bacilli	<i>Pantoea ananatis</i>	0.500	98.0
EN9	Yellow	Circular	Small	Smooth	Pink	Bacilli	<i>Pantoea</i> sp.	0.498	97.2

Table 3. Effect of PGPR inoculation on root number, total root length, shoot length, root dry weight, and shoot dry weight of shallot (*Allium ascalonicum*) plants

Treatments	Root number	Total root length (cm)	Shoot length (cm)	Root dry weight (mg/plant)	Shoot dry weight (mg/plant)
EX2	12.00 ^{bc} ± 5.03	38.07 ^{bcd} ± 17.04	2.17 ^a ± 0.67	26.03 ^{ab} ± 11.03	27.33 ^a ± 9.36
EN1	19.67 ^{abc} ± 9.94	72.47 ^{cd} ± 9.43	1.63 ^a ± 1.20	37.37 ^{ab} ± 17.78	50.60 ^a ± 43.90
EN3	7.00 ^c ± 2.65	15.17 ^d ± 5.36	0.67 ^a ± 0.07	12.83 ^b ± 3.94	12.50 ^a ± 0.81
EN5	26.33 ^{ab} ± 0.67	83.10 ^{ab} ± 10.74	3.40 ^a ± 2.50	49.17 ^{ab} ± 2.62	35.07 ^a ± 10.75
EN8	21.33 ^{abc} ± 1.86	63.57 ^{bc} ± 12.82	0.63 ^a ± 0.09	46.93 ^{ab} ± 8.52	42.27 ^a ± 33.46
EN9	32.00 ^a ± 2.31	120.13 ^a ± 12.28	3.87 ^a ± 1.33	61.47 ^a ± 7.97	60.30 ^a ± 24.10
Uninoculated	18.33 ^{abc} ± 5.21	51.73 ^{bcd} ± 24.08	1.83 ^a ± 1.48	36.83 ^{ab} ± 20.76	32.67 ^a ± 25.81

Note: Different letters within column indicate significant differences between means using Duncan's New Multiple Range test at P value 5%. ±: Standard error of mean

The single inoculation effect of PGPR on root number, total root length, shoot length and root and shoot dry weight were carried out and the data were tabulated in Table 3. There was no significant difference between treatments and control for root length, shoot length, root dry weight, and shoot dry weight. However, *Pantoea* sp. EN9 showed significant difference ($p < 0.05$) for total root length with 120.13 ± 12.28 cm higher than uninoculated control (51.73 ± 24.08 cm). An improvement in shallot roots by *Pantoea* sp. EN9 indicating that the strain was effectively colonising the roots and subsequently increase root length, suggesting its potential to be formulated as bioinoculant for sustainable cropping system.

Most of the rhizobacterial isolates did not promote the growth of shallots even though they were able to exhibit potential multiple traits. This phenomenon occurred probably due to the host specific characteristic that was exhibited by the certain rhizobacteria isolated from the oil palm roots. Failure of some rhizobacteria to promote plant growth inoculated to non-host plant has been reported by Thokchom et al. (2017) who found inoculation of mandarin by host strain enhanced plant growth compared to non-host strain. Moreover, the interaction between soil bacteria and plants can be either beneficial, harmful, or neutral depending on the species of the bacteria and types of plant (Turan et al., 2016) thus demonstrated variable plant responses to inoculation (Aziz et al., 2012). Even though only isolate EN9 indicates some promising effect on plant growth, other isolates demonstrated no deleterious effect upon plant growth (no significance difference at $p > 0.05$ over control). This phenomenon indicates that the collection of rhizobacterial isolates in the present study were not plant pathogenic and may be useful when applied to other crops.

Conclusion

30 bacterial isolates collected from oil palm roots showed multiple beneficial properties but most failed to show PGPR activities when inoculated to non-host shallot. Only isolate *Pantoea* sp. EN9 showed up better properties. Experiments are needed to test the PGPR

activities of the selected isolates through inoculation to their original host, oil palm.

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