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ISOLATION AND CHARACTERIZATION OF PLANT GROWTH-PROMOTING RHIZOBACTERIA (PGPR) AND THEIR EFFECTS ON GROWTH OF STRAWBERRY (*FRAGARIA ANANASSA* DUCH.)

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Abstract

The interaction between plant and microbes can contribute towards plant health and productivity. Plant growth-promoting rhizobacteria (PGPR) are the bacteria that can enhance plant growth performance and production. PGPR were isolated from the rhizosphere of strawberry plants and the beneficial properties were identified. Effects of bacterial isolates on strawberry plant also being observed. Eighty isolates were isolated from three different strawberry cultivars. Seven isolates were positive for the N_2-fixation. Eleven and twenty isolates showed solubilizing activity for potassium and phosphate, respectively. Phosphate solubilization efficiency ranged from 30.0 to 42.3%. Three isolates showed positive results for cellulase enzyme production. Meanwhile, the phytohormone (IAA) production of the isolates ranged from 2.001 to 42.414 µg/ml. Plants applied with bacterial isolates, namely STUPM01 (*Microbacterium oxydans)*, STUPM12 (*Bacillus cereus)*, STUPM20 *(Leclercia adecarboxylata*) and STUPM25 (*Pseudomonas umsongensis)* showed root and shoot growth enhancement compared to control. STUPM01 showed better performance compared to other isolates.

Introduction

Strawberry is the main crop planted in Cameron Highlands and it gives a significant contribution towards the local market as fresh fruit or as side product. Chemical fertilizers have been widely used in order to increase the production to meet the demand of the consumers. High fertilization application can increase the input cost but may lead to environmental pollution. PGPR inoculation will minimize the amount of chemical fertilizer that usually being applied. PGPR is a group of beneficial plant bacteria that are useful for stimulating plant growth and yield (Bhattacharyya and Jha 2012). The plant growth stimulating efficiency of bacterial inoculants is affected by nutritional condition. The bacterial inoculation has better effect on plant growth in nutrient deficient soil than in nutrient rich soil (Egamberdiyeva 2007). PGPR has the potential to replace the chemical fertilizer, pesticides and supplements. PGPR enhance the nutrient status of host plants through biological nitrogen fixation, increasing the availability of nutrients in the rhizosphere, inducing increases in root surface area, enhancing other beneficial symbioses of the host and with the combination of other modes of action (Kevin Vessey 2003). These microbes are able to convert nutritionally important elements from unavailable to available form (Kevin Vessey 2003). The aims of this study were: (i) to isolate plant growth-promoting rhizobacteria from different variety of strawberry plants; (ii) to characterize the beneficial traits from the isolates; and (iii) to determine the effects of rhizobacteria application on plants.

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Material and Methods

Strawberry plants of different cultivars were collected from different farms in Cameron Highlands, Pahang, Malaysia. Three gram of fresh and whitish strawberry roots with its adhering soil were transferred into conical flask containing 99 ml sterilized distilled water. A series of ten-fold dilutions of the suspension up to 10^{-8} were prepared. 0.1 ml of each dilution was spread onto Tryptic Soy Agar (TSA) media and incubated for 24 hrs at 30°C. After 24 hrs, plates were observed for different types of bacteria based on colony morphology. Different bacteria were isolated and sub-cultured for several times to obtain pure isolates.

All pure isolates were tested for nitrogen fixation ability on N-free solid malate agar (NFA). After incubation, N_2 fixation activity can be observed through color change from pale green to blue. This color change is due to increase in pH attributed to the formation of ammonium and nitrates from the atmospheric nitrogen fixation (Dobereiner and Day 1975). The phosphate solubilizing activity was tested on Pikovskaya agar which incubated for 24 hrs to one week at 30°C (Pikovskaya 1948). Phosphate solubilizing bacteria was determined by the formation of halo zone around the colony. The efficiency was measured by dividing the growth diameter with solubilization diameter. Aleksandrov agar medium was used to determine the ability of bacteria to solubilize potassium (Hu *et al.* 2006). The potassium solubilization ability was derived by the formation of halo zone around the colony as the bacteria are able to release K from potassium aluminium silicate. To determine the phytohormone production, one loopful of bacteria inoculated into 100 ml tryptic soy broth (TSB) and was shaken for 24 hrs. L-tryptophan was added as precursor. The bacterial culture was transferred into the new TSB and was centrifuged at 7000 rpm for 7 min. Salkowsky reagent was added into cuvette contained the supernatant and allowed to react for 25 minutes. Development of pink color indicated the IAA production. The absorbance of the solution was measured using spectrophotometer at 535 nm and compared with the standard curve to get the IAA concentration (Gordon and Weber 1951). Cellulase enzyme activity was done on carboxymethylcellulose (CMC) agar medium. A sterile paper disc was dipped into the bacteria culture and transferred onto the CMC agar. After incubation, the plate was flooded with Congo red solution for 15 min. Then de-staining with salt solution for another 15 min. The unstained areas indicate the cellulase activity where the CMC has been broken down by the bacterial strain (Kasana *et al*. 2008).

Four isolates (STUPM01, STUPM12, STUPM20 and STUPM25) with the best traits were selected for genetic identification using molecular techniques involving DNA extraction and amplication using PCR. Universal primers (16sF and 16sR) were used for forward and reverse DNA sequencing reaction of purified PCR amplicon. They were identified as *Microbacterium oxydans, Bacillus cereus*, *Leclercia adecarboxylata* and *Pseudomonas umsongensis.* These selected bacteria were screened on plant to observe their performances. The roots of runners were submerged in the suspensions containing approximately 10^6cfu/ml for 30 min. Root of control plant was submerged in distilled water. Treatments were as follows: (T_1) Control, (T_2) STUPM01, (T_3) STUPM12, (T_4) STUPM20 and (T_5) STUPM25. Non-inoculated treatment served as control and all treatments were supplied with 70% from recommended rate of strawberry fertilizer. There were 5 treatments with 3 replications (5 plants per replicate) arranged in randomized complete block design (RCBD). All data were analyzed for variance using the Statistical Analysis System (SAS) and the treatment means separation was determined using LSD Test.

Results and Discussion

Growth of microorganisms is affected by several factors, namely varietal differences and cultural practices, including fertilization. Plant growth-promoting rhizobacteria (PGPR) were

successfully isolated from the strawberry plants collected from different farms in Cameron Highlands. Eighty strains were isolated from three different strawberry cultivars. Cultivar Camarosa had 20 isolates, Festival35 and Chandler25 (Table 1). Seven isolates were positive for the biological N_2-fixation test (Table 2). Some of the isolates showed solubilizing activity for potassium and phosphate test respectively, where P solubilization efficiency ranged from 30.0 to 42.3%. Three isolates showed positive results for cellulase production and five isolates can produce siderophore. Meanwhile the phytohormone (IAA) production of the isolates ranged from 2.00 to 42.41 µg/ml.

Table 1. Number of bacteria isolates of different strawberry cultivars collected from Cameron Highlands.

Cultivars	Classification	Isolates	Percentage (%)
Camarosa	Rhizospheric	15	75
	Endophytic	5	25
Total isolates		20	100
Festival	Rhizospheric	25	71
	Endophytic	10	29
Total isolates		35	100
Chandler	Rhizospheric	14	56
	Endophytic	11	44
Total isolates		25	100
Total isolates collected		80	-

Table 2. The beneficial characteristics of some isolated bacterial strains.

Isolates	N_2 fixing ability	Phosphate solubilizing ability (%)	Potassium solubilizing ability	Cellulase enzyme production	Siderophore production	Phytohormone production (µg/ml)
STUPM01	+	33.33	+	+	+	42.41
STUPM02	+	-	-	-	-	20.24
STUPM12	+	42.3	+	+	+	2.50
STUPM18	+	-	-	-	+	2.00
STUPM19	+	-	-	-	-	5.12
STUPM20	+	30.0	+	+	+	3.22
STUPM25	+	35.12	+	-	+	9.64

+ = Positive, – = Negative.

Nitrogen is the most important nutrient for plant growth. Atmospheric nitrogen can be made available to the plants through symbiotic relationship with N_2-fixing bacteria (Saikia and Jain 2007). Other nutrients which are important for plant include potassium (K) and phosphorus (P) during the process of photosynthesis. Potassium regulates the opening and closing of stomata and therefore regulates CO_2 uptake (Crops 1998). Phosphorus also involved in other plant processes such as photosynthesis, energy transfer, and nutrient mobilization (Odeniyi 2009). Cellulase produced by the isolates enables the cellulose in the cell wall to be broken down for the endophytic to penetrate (De Boer *et al.* 2005). Meanwhile IAA production could induce root elongation, cell division and root extension (Perrot-Rechenmann 2010).

The selected bacteria isolates were identified through molecular method using the 16S rDNA partial gene sequencing (Table 3). Bacterial strain of STUPM 01, STUPM 12, STUPM 20 and STUPM 25 were identified as *Microbacterium oxydans, Bacillus cereus, Leclercia adecarboxylata* and *Pseudomonas umsongensis.*

Table 3. Molecular identification of bacteria strains.

Isolates	16s rDNA fragment length (bp)	Closest relatives in NCBI	NCBI acession number	Similarity (%)
STUPM01	1411	*Microbacterium oxydans* Strain DSM 20578	NR 044931.1	99
STUPM12	1408	*Bacillus cereus* Strain ATCC 14579	NR 074540.1	99
STUPM20	1430	*Leclercia adecarboxylata* Strain NBRC 102595	NR 114154.1	98
STUPM25	1422	*Pseudomonas umsongensis* Strain Ps 3-10	NR 025227.1	99

These isolates were selected for subsequent test to observe the effects on strawberry. Higher root length obtained in control, STUPM01, and STUPM12. STUPM01 showed higher root surface area and number of root tips. Root diameter and root volume showed significant different in STUPM20, and STUPM25 compared to control (Table 4).

Table 4. Effects of different isolates on root volume, surface area, diameter, length and number of tips.

Treatment	Plant roots				
	Volume (cm^3)	Surface area (cm^2)	Diameter (mm)	Length (cm)	Number of tips
Control	3.45 c	262.49 d	0.52 c	1570.32 ab	6537 bc
STUPM01	7.11 b	508.14 a	1.08 a	1819.44 a	9588 a
STUPM12	8.49 b	450.54 b	0.77 b	1761.48 a	7931 ab
STUPM20	11.43a	414.06 b	1.16 a	1208.82 bc	5546 c
STUPM25	11.14 a	318.08 c	1.19 a	1048.69 c	4640 c

The mean values marked with the same letters are not significantly different at $p \leq 0.05$. All inoculated treatments were given with 70% fertilizer rate. Control (100% fertilizer) was not inoculated with bacteria.

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This showed that inoculated plants with reduced rate of fertilizer were able to enhance the roots growth as compared to control plant. However, amongst the isolates, STUPM01 has contributed more in root development where it showed higher root length, surface area, diameter and number of root tips. Similar to leaf surface area and dry weight, STUPM01 inoculated plant showed significant different compared to other treatment. On the other hand control and STUPM20 had the lowest leaf surface area. Control showed the lowest in term of dry weight. These bacteria helped in atmospheric nitrogen fixation where nitrogen is a component of

chlorophyll and important for photosynthesis (Hawkesford *et al*. 2011).Bacteria can survive around the root with the presence of root exudates which is a source of nutrients and the root area is an ideal place for it growth (Nadeem *et al.* 2014). Plant roots of bacterial inoculated plants were better than control due to the production of indole acetic acid (IAA) which regulates root development and give an efficient nutrient uptake lead to the improvement of shoot growth (Perrot-Rechenmann 2010). Similar finding also reported with microbial inoculation in apricot, mulberry, blueberry and apple (Esitken *et al.* 2006).

Table 5. Effects of different isolates on leaf surface area, fresh weight and dry weight.

Treatment	Shoot growth	
	Leaf surface area (cm^2/plant)	Leaf dry weight (g/plant)
Control	296.62c	2.98d
STUPM01	456.70a	3.88a
STUPM12	227.81d	2.99dc
STUPM20	301.69c	3.37b
STUPM25	342.86b	3.34bc

From this study beneficial bacteria namely STUPM01 (*Microbacterium oxydans)*, STUPM12 (*Bacillus cereus)*, STUPM20 *(Leclercia adecarboxylata*) and STUPM25 (*Pseudomonas umsongensis)* have been successfully isolated from strawberry root. These bacteria lived around the root hold multiple traits such as the ability to fix N_2, solubilize P and K, produce cellulase enzyme, siderophore and phytohormone. It has been demonstrated that with bacterial application definitely enhanced roots and shoots growth with reduced fertilizer condition. All bacterial inoculated plant treatment have higher root volume, surface area and diameter as the bacteria produced IAA which promotes the roots growth. In addition, STUPM01 treated plant has the highest leaf surface area and dry weight. The results suggested that fertilizer can be reduced with the application of plant growth-promoting rhizobacteria on strawberry plant.

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