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Short Communication

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Functional characteristics of plant growth-promoting rhizobacteria from *Acacia mangium* L.

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ABSTRACT

Nodulation, a pivotal developmental process in nitrogen-fixing plant-microbe associations, was investigated in *Acacia mangium*. This study aimed to isolate and characterise root nodule bacteria for plant growth-promoting traits. Ten bacterial isolates underwent screening, revealing similar morphologies and growth rates. All isolates exhibited positive indole acetic acid production, while PI1A and PI2A demonstrated phosphorus and potassium solubilisation. Four isolates, namely PI1A, PI2A, PI3A, and PDG1A, showed positive nitrogen fixation. PI1A emerged as the most promising strain, significantly enhancing ($p<0.05$) mung bean seedling root fresh weight at 210.37±5.22 mg and root dry weight at 112.67±3.28 mg as compared to the control at 139.27±5.71 mg and 65.40±3.70 mg, respectively. Based on fatty acid analysis, isolate PI1A was identified as belonging to the species *Photorhabdus luminescens*. In conclusion, *P. luminescens* isolate PI1A exhibits significant potential candidate in a biofertiliser and bio-stimulant formulation for sustainable agriculture.

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INTRODUCTION

One of Southeast Asia's fastest-growing tropical trees, *Acacia mangium* originates from Papua, Western Irian Jaya, the Maluku islands in Indonesia, Papua New Guinea, and northeastern Queensland in Australia (Maheshwar *et al.*, 2013). Renowned for its excellent wood quality traits, this tree serves multiple purposes, including furniture, particleboard, plywood, veneer, fence posts, firewood, charcoal, and pulp and paper making. Its high-quality pulp traits result in substantial pulp yields and paper with commendable optical, physical, and surface properties.

A. *mangium*, a productive nitrogen-fixing legume tree, hosts root nodule-forming bacteria that convert atmospheric nitrogen into inorganic forms for the plant's use. Commonly associated genera with root nodules include Rhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium, Allorhizobium, and Sinorhizobium (Ngom *et al.*, 2003). However, research by Saïdi *et al.* (2013) found other genera in root nodules, like Agrobacterium, Bacillus, and Pseudomonas, which may help plant growth in a roundabout way (Bumunang *et al.*, 2014).

Beneduzi *et al.* (2012) have classified root nodule bacteria into beneficial, harmful, and neutral groups based on their effects on plant growth. Plant growth-promoting rhizobacteria (PGPR), a group of bacteria, perform tasks such as fixing nitrogen in the air, dissolving phosphate and potassium, and producing indole-3-acetic acid (IAA). These activities play a crucial role in enhancing plant growth and development. Root nodule bacteria and their associated plants engage in numerous complex interactions. These interactions affect many living things in the soil and help move nutrients around and keep crops growing (Chandler *et al.*, 2008).

Root nodule bacteria provide supplementary advantages, such as the synthesis of plant growth regulators and defence against phytopathogens. Their functions encompass the processes of heavy metal detoxification, pesticide degradation and tolerance, salinity tolerance, and the biological regulation of phytopathogens and insects. The objective of this study was to extract root nodule bacteria from A. *mangium* trees obtained from various places inside the Universiti Putra Malaysia Sarawak. These bacteria can be characterised using tests to detect indole-3-acetic acid (IAA) and determine their ability to fix nitrogen, solubilise phosphate and potassium. In addition, the present

study performed an extensive examination of the bacteria found in the root nodules by analysing the total fatty acid methyl esters (FAME) using gas chromatography.

This study is valuable in advancing our understanding of the diverse and beneficial roles of root nodule bacteria associated with A. *mangium*, with potential implications for sustainable agriculture practices.

MATERIALS AND METHODS

Nodule samples were collected from *Acacia mangium* roots at three different locations around Universiti Putra Malaysia Sarawak: Pusat Islam, the football field, and the staff residential area. Selected plant roots containing nodules were cut and transported to the laboratory for further examination. One gram of nodules was removed from the roots and washed thoroughly four to five times using sterile distilled water. The nodules then underwent surface sterilisation by immersing them in 5.25% sodium hypochlorite for 5 minutes. Subsequently, the nodules were immersed in 95% ethanol for 3 minutes, followed by six washes with sterile distilled water. The surface-sterilized nodules were then crushed using a clean mortar and pestle. A saline solution (0.9%) was added to create a turbid suspension. Next, 100 µL of the dilution was transferred onto yeast extract mannitol agar (YEMA) by spreading it for isolation and selection.

The YEMA medium contained (g/L): yeast extract, 1 g; mannitol, 10 g; dipotassium phosphate (K_2HPO_4), 0.5 g; magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), 0.2 g; sodium chloride (NaCl), 0.1 g; calcium carbonate ($CaCO_3$), 1 g; and Bacto agar, 15 g. A few drops of bromomethyl blue were added as a pH indicator. The inoculated YEMA plates were incubated at 28-30 °C for 24-72 hours to allow culture growth, considering the possibility of inoculating slow-growing rhizobacteria. After culture growth, the streaking process was performed until pure cultures were obtained from each plate.

Agar was prepared using Jensen's medium semi-solid agar. Jensen's medium contained (g/L): sucrose, 20 g; dipotassium phosphate, 1 g; magnesium sulfate, 0.5 g; sodium chloride, 0.5 g; ferrous sulfate ($FeSO_4$), 0.1 g; sodium molybdate, 0.005 g; calcium carbonate ($CaCO_3$), 2 g; and Bacto agar, 2 g. The medium was autoclaved at 121 °C for 15 min before being poured into sterilised test tubes. A loopful of each pure culture was stabbed into the medium and incubated for three days at 28-30 °C. Positive results were indicated by the formation of pellicles on the medium, suggesting the fixation of atmospheric nitrogen.

The National Botanical Research Institute's phosphate (NBRIP) agar medium was made by adding the following ingredients (g/L): glucose (10 g), tricalcium phosphate (5 g), magnesium chloride hexahydrate (5 g), magnesium sulphate heptahydrate (0.25 g), potassium chloride (0.2 g), ammonium sulphate (0.1 g), and Bacto agar (15 g). The medium was autoclaved at 121 °C for 15 minutes before being transferred to sterile Petri dishes. To facilitate inoculation, each Petri dish was divided into four parts. A loopful of each pure culture was applied to the surface of the NBRIP medium. The isolates were cultured for five days at 28-30 degrees Celsius. The creation of a halo zone or clean zone around the colonies indicated a successful outcome.

Aleksandrov agar medium was prepared by adding the following (g/L): glucose, 5 g; magnesium sulfate heptahydrate, 0.005 g; ferric chloride ($FeCl_3$), 0.1 g; calcium carbonate ($CaCO_3$), 2 g; calcium phosphate ($Ca_3(PO4)_2$), 2 g; mica powder, 3 g; and Bacto agar, 20 g. The medium was autoclaved at 121 °C for 15 min before being poured into sterilised petri dishes. A loopful of each pure culture was placed on the surface of the Aleksandrov medium. The isolates were incubated at 28-30 °C for five days. The formation of a halo zone or clear zone around the colonies indicated a positive result.

Bacteria were grown in a 100 mL conical flask containing 50 mL of sterilised nutrient broth. The liquid culture was incubated at 28-30 °C at 100 rpm for 48 h. After incubation, the culture liquid was centrifuged at 4000 rpm for 10 min. Two mL of the supernatant were transferred into sterilised test tubes. Next, four mL of Salkowski reagent was added to the test tube. Salkowski's reagent consisted of 1 mL of $FeCl_3$ and 50 mL of concentrated sulfuric acid. Finally, 1 mL of orthophosphoric acid was added to the test tube. The culture was then incubated at 28-30 °C in a dark environment for 30 min. A positive result was indicated by a colour change from yellow to pink, signifying the production of indole-3-acetic acid (IAA).

Mung bean (*Vigna radiata*) was chosen as the test plant to determine plant growth-promoting potential after inoculation. Mung beans were germinated by direct seeding onto sterilised growth pouches containing one seed each. The growth pouch consisted of two layers of paper towels and was watered daily. The seeds were allowed to grow for approximately two weeks until the specialised seed leaves (cotyledons) sprouted. After two weeks, about 40 mL of hydroponic solution was added to the growth pouch to provide nutrients for the plant's growth. The plants were observed daily and left for one week before bacterial inoculation.

The liquid inoculation of bacteria was prepared by adding a loopful of pure culture into 10 mL of sterilised yeast extract mannitol broth (YEMB). The inoculated broth was incubated at 28-30 °C at 100 rpm for 24 h. After incubation, 90 mL of 0.9% saline solution was added to the 10 mL of liquid culture to dilute it to a 10% culture concentration before injection or addition to the plants. Two mL of the 10% culture concentration was added to the growth pouches after one week of hydroponic solution application. The mung bean plants, together with their individual treatments, were produced in a laboratory setting using a completely randomised design. The plants' growth was observed daily for 45 days. Fresh root weight, root dry weight, shoot fresh weight, shoot dry weight, and the number of nodulations were recorded for data collection.

GC FAME analysis using a MIDI bacterial identification system began with bacterial culturing. Sample preparation requires

24 hour-old isolates. Five critical processes were needed to prepare GC-ready extract:

1. Harvesting: Bacteria or isolates were inserted at the bottom of a clean culture tube.
2. Saponification: Each cell tube received 1 mL of reagent 1 (45 g sodium hydroxide, 150 mL methanol, 150 mL distilled water). The tubes were sealed with Teflon caps, vortexed, then boiled at 100 °C for 5 min. Following another 5-10 s of vortexing, the tubes were heated for 25 min in the 100 °C water bath.
3. Methylation: Each tube received 2 mL of reagent 2 (325 mL hydrochloric acid, 275 mL methyl alcohol). Following a brief vortex, the tubes were heated in an 80 °C water bath for 10 min and cooled quickly.
4. Extraction: The cooled tubes received 1.25 mL of reagent 3 (200 mL hexane, 200 mL methyl tert-butyl ether). Unicapped tubes were manually rotated for 20 min to establish a layer, and the aqueous phase at the bottom was pipetted out and discarded.
5. Base Wash: The tubes' organic phase received 3 mL of reagent 4 (10.8 g sodium hydroxide, 900 mL distilled water). Recapped, manually rotated, and tumbled tubes for 10 min. Two-thirds of the organic phase was pipetted into a GC vial for analysis.

The data from plant assays were collected to assess the growth response of plants after inoculation. The data was processed for variance and then compared using the Duncan test to identify any significant differences across treatments at a significance level of 5%.

RESULTS AND DISCUSSION

A total of 10 isolates were successfully recovered from root nodules of wildly grown A. *mangium* at Universiti Putra Malaysia Bintulu Campus, Sarawak. The isolates were all observed as fast-growing bacteria after considering that they could emerge within 24 hours of incubation. Ten isolates were obtained from nodules of A. *mangium* and subjected to screening for nitrogen fixation, phosphorus (P) and potassium (K) solubilization, and indole acetic acid (IAA) production. Figure 1 illustrates the results of N fixation, P and K solubilization, and IAA production tests conducted on the bacterial isolates from A. *mangium* nodules.

In terms of nitrogen-fixing activity, four out of the ten isolates demonstrated the ability to grow on an N-free medium, indicating their N-fixing potential. Regarding P solubilization, only two isolates, namely PI1A and PI2A, exhibited positive results, while the remaining isolates were negative. Similar results were observed for K solubilization. Remarkably, all ten isolates demonstrated the capacity to produce IAA. These findings highlight the diverse functional characteristics of the bacterial isolates from A. *mangium* nodules, with implications for their potential roles in plant-microbe interactions and nutrient cycling in the rhizosphere.

The present study confirmed that all isolates exhibited indole-3-acetic acid (IAA) production. This observation aligns with

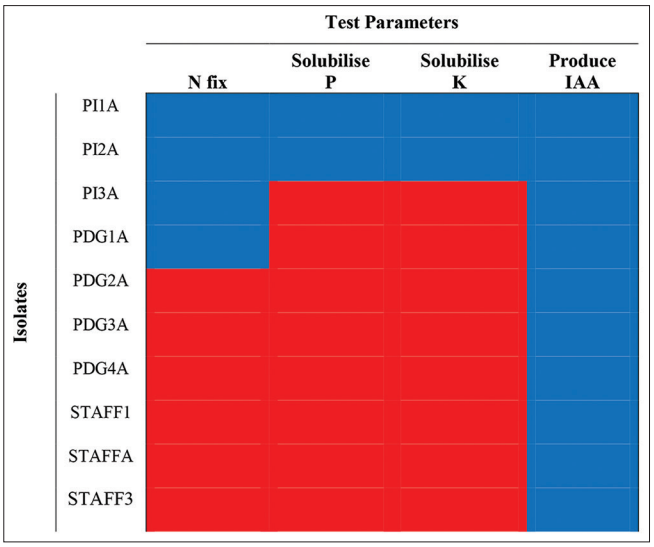

Figure 1: N fixation, P and K solubilization, and IAA tests were conducted on bacterial isolates from nodules of *Acacia mangium* L. The figure presents a colour-coded representation of the isolates, with a blue shade indicating positive results and a red shade indicating negative results for different properties

the review by Ganesh *et al.* (2024), which emphasized that IAA biosynthesis in plant growth promoting rhizobacteria (PGPR) is frequently associated with additional traits such as phosphate solubilization and nitrogen fixation. The present study found that adding tryptophan to culture media will result in higher IAA production. Linda *et al.* (2024) also reported that adding L-tryptophan into bacterial culture media increased IAA biosynthesis. Four isolates, namely PI1A, PI2A, PI3A and PDG1A, showed pellicle formation, indicating a positive reaction for nitrogen fixation activity. Isolates PI1A and PI2A were the only isolates showing positive phosphate and potassium solubilising activity (Figure 1).

Both formed colonies with a clear zone on agar plates, indicating their ability to solubilize phosphate and potassium. Previous studies revealed that the clear zone formed by solubilizing suspended tricalcium phosphate was due to the release of organic acids into the surrounding medium (Khan *et al.*, 2022; Mayadunna *et al.*, 2023). The present study demonstrated that only one isolate, PI1A, showed significantly improved ($p<0.05$) root growth of mung bean (*Vigna radiata*) but failed to enhance shoot growth over the uninoculated control. Isolate PI1A significantly increased ($p<0.05$) root fresh weight and root dry weight of mung bean seedlings at 210.37 ± 5.22 mg and 112.67 ± 3.28 mg, respectively, as compared to the uninoculated control at 139.27 ± 5.71 mg and 65.40 ± 3.70 mg, respectively (Figure 2). This phenomenon indicated that the isolate PI1A was able to colonise plant roots and deliver its beneficial properties such as nitrogen fixation, phosphate and potassium solubilization, and IAA production (Figure 1) for root growth.

Moreover, the host-specific adaptation or host-specific rhizobacteria that are only compatible with their original host plant, *Acacia mangium*, may explain why most isolates do not stimulate the growth of mung bean plants. Recent work shows

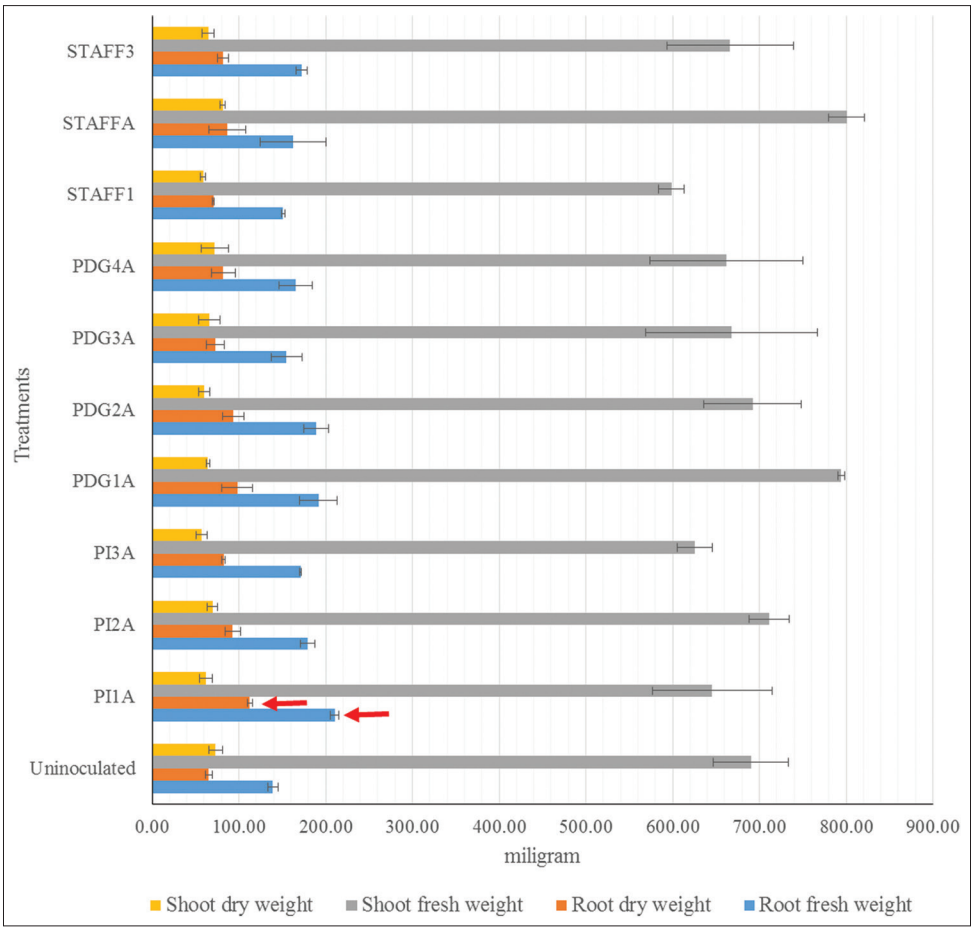

Figure 2: The impact of bacterial inoculation on the growth of mung bean (*Vigna radiata*) plants. Red arrows indicate significant differences compared to the uninoculated control (Duncan test, $p < 0.05$)

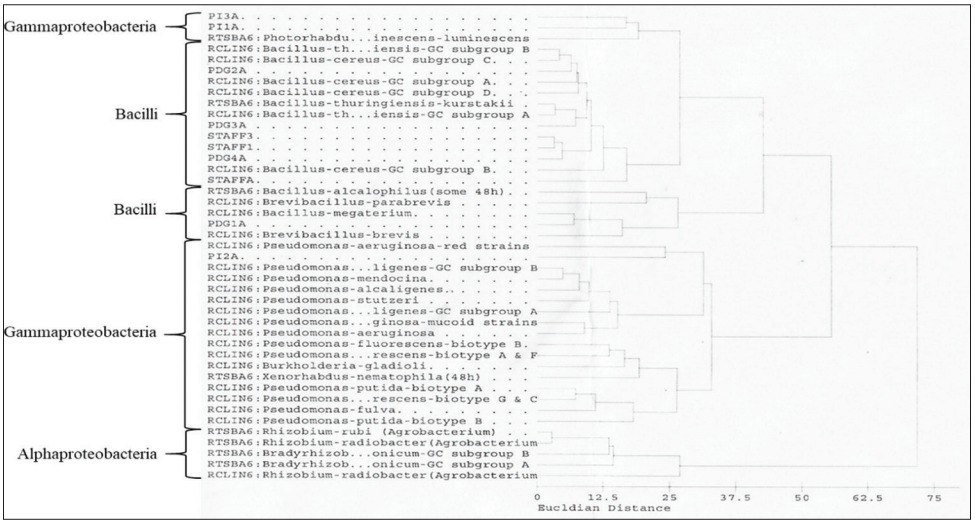

Figure 3: Dendrogram of fatty acid relationship among ten isolates recovered from root nodule of *Acacia mangium*. The isolates and references were clustered in three different phyla namely Alphaproteobacteria, Bacilli, and Gammaproteobacteria

that inoculation with rice-associated bacteria can remodel native root endophyte and rhizosphere communities, and that PGPR inoculants can promote citrus growth. For example, early inoculation of a rice seed-borne endophyte significantly altered bacterial community diversity and assembly across rice root and rhizosphere compartments over development, evidencing microbiome restructuring by rice-derived strains (Wang *et al.*, 2023). In this study, isolate PI1A may be considered a non-specific host bacterium due to its effective growth promotion of a non-host plant, the mung bean.

Gas chromatography of fatty acid methyl esters (FAME) is one of the practical tools to identify bacteria that are important in clinical and industrial settings (Li *et al.*, 2020; Zhao *et al.*, 2020). The study's findings showed that the dendrogram of fatty acid relationships between bacteria in Acacia mangium root nodules can be broken down into two groups: bacilli and gammaproteobacteria. Of the ten isolates, seven belonged to bacilli. PI3A and PI1A demonstrated a close association with gammaproteobacteria within a nearby Bacilli cluster. The current study has identified PDG2A as *Bacillus cereus* due to its affiliation with a similar cluster. The strains STAFF (1), STAFF (2), STAFF (3), and PDG (4) shared similarities with *Bacillus cereus* and *Bacillus thuringiensis-kursakii*. The PDG (1) strain exhibited a relatively distinct relationship with the clusters of PDG (2), PDG (3), PDG (4), STAFF (1), STAFF (2), and STAFF (3). The PDG (1) strain shared similarities with *Bacillus megaterium* and *Brevibacillus brevis*. PI (2) had similarities with genera of *Pseudomonas*, such as *P. gladioli* and *P. aeruginosa*.

Based on a dendrogram analysis of the fatty acid methyl ester (FAME) profiles (Figure 3), it was found that isolate PI1A, which greatly enhanced mung bean growth, was from the *Photorhabdus luminescens* species. This identification was significant as *P. luminescens* is known for its dual role as both a symbiotic bacterium in nematodes and a pathogen for insects (Teoh *et al.*, 2021). However, Regaiolo *et al.* (2020) have recognized its potential as a PGPR due to its production of various secondary metabolites beneficial to plants. As presented in this study, Isolate PI1A of *P. luminescens* has many plant-growth enhancing properties, including the ability to fix nitrogen, dissolve phosphate, and produce indole acetic acid. This multifaceted potential underscores the versatility of *P. luminescens* strain PI1A as a biofertilizer candidate. Moreover, the presence of *P. luminescens* in root nodules of A. *mangium* emphasizes the diverse symbiotic relationships that this bacterium can establish, extending its ecological relevance beyond its traditional associations. This discovery contributes to the expanding knowledge of beneficial root nodule bacteria and their roles in promoting plant health and growth.

CONCLUSION

Ten bacterial isolates from A. *mangium* root nodules demonstrated abilities to solubilize phosphate and potassium, produce IAA, and fix atmospheric nitrogen. Isolates PI1A and PI2A exhibited multiple beneficial traits. The dendrogram analysis identified isolate PI1A as *P. luminescens*, a bacterium having plant growth-promoting traits such as nitrogen fixation, phosphate solubilization, and IAA production. The significant increase in mung bean root biomass highlights PI1A's potential as an effective biofertilizer. This discovery emphasizes the diverse symbiotic relationships and ecological relevance of *P. luminescens*, contributing to the broader understanding of beneficial root nodule bacteria in sustainable agriculture.

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