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ISOLATION AND CHARACTERIZATION OF BACTERIAL COMMUNITY IN ROOT OF CHILI PLANT AS POTENTIAL PLANT GROWTH-PROMOTING BACTERIA

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Abstract

Agricultural sector is a significant contribution to the growth of Malaysia's economic sector, and its sustainability should be maintained. Dependency on the application of synthetic chemical fertilizers and pesticides required special attention due to their negative effect on extensive usage. Attention to the search for alternative strategies is urgently needed. The plant growth-promoting bacteria (PGPB) showed great potential as biofertilizers and biocontrol of pathogens. This research aims to isolate and identify the potential PGPB from the local chili plant's roots. Isolation and characterization of the potential isolates of PGPB were performed using standard microbiology procedures. A total of 10 isolates were successfully obtained during the isolation stage. The isolates were designated as strain A1 to strain A10. Based on Gram staining, strains A1, A5, A6, A8, A9, and A10 were found to be Gram-positive. Strains A2, A3, and A4 were found to be Gram-negative. However, strain A7 was not able to be retrieved after a series of restreaking procedures, perhaps due to symbiosis factors. Besides, strains A5 and A10 were found to be unmatched by any PGPB strains. Further biochemical analysis proposed that strains A1, A3, A6, and A8 belong to *Bacillus* sp., strains A2 and A4 belong to *Pseudomonas* sp., and strain A9 proposed to be *Brevibacillus* sp. In conclusion, it was strongly suggested that these nine isolates belong to PGPB due to the strain identification resembling a common PGPB strain. In the future, studies on the ability of the isolates to act as bioprotectants and biofertilizers are recommended.

Keywords: Plant growth-promoting bacteria (PGPB), root of chili plant, morphology characterization, biochemical analysis.

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Introduction

Excessive fertilization application might cause heavy metal deposition, water eutrophication, and nitrate accumulation in the environment (Innes, 2013). These circumstances also can cause air pollution since the nitrogen and sulphur containing gases can lead to greenhouse effect issues (Savci, 2012). About 1,500 indoor food poisoning cases are related to plant consumption were recorded by the American Association of Poison Control Centers in 2014. The number rose to almost 5,000 cases, including issues created by outdoor and unknown fertilizers. Most of the patients who suffered the poison effects were recorded to be under the age of five years old (Gabbey, 2017).

Although part of pesticide and non-edible use on the plants is safe, some precautions need to be taken when handling and storing the plant food. Thus, pesticide and synthetic fertilizer should be applied in a safe margin for human and animal consumption. Alternatively, the PGPB can also be used to replace chemical fertilizers as it shows the potential to function as biofertilizers, biocontrol agents, or biopesticides. Microorganisms are beneficial by promoting growth and controlling plant diseases, as well as reducing environmental pollution (More, 2020; Bonaccio et al., 2019). A study by Yousaf et al. (2017) showed that nitrogen fertilizer is essential in rice production under most conditions because nitrogen acts as the primary factor limiting plant growth. Nitrogen-fixing bacteria in the PGPB can control atmospheric nitrogen, enhancing plant growth and reducing plant diseases. Therefore, PGPB is

commonly used as inoculants to promote growth and crops yield, which provides an attractive way for pesticides and chemical fertilizers to be supplemented (Ji et al., 2014; Saharan & Nehra, 2011; Stefan et al., 2008).

The PGPB was identified to colonize several plant species. It is present predominantly through a variety of direct and indirect mechanisms in many environments to promote and enhance plant growth. The number of PGPB that enhance plant growth has been reported, with a tremendous increase in species identification like *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Enterobacter*, *Arthrobacter*, *Bacillus*, and *Serratia* (Ngalimat et al., 2021; Ji et al., 2014; Gururani et al., 2013). Therefore, in this study, an attempt was carried out to isolate the potential PGPB from the local chili plant's roots. The potential PGPB isolates were further identified and characterized based on morphological characteristics (both microscopic and macroscopic identification) and biochemical analysis. Based on the results obtained from this study, a significant contribution is aimed to instill awareness of the presence of potential PGPB and further refute or support the research regarding PGPB from local isolates.

Methods

Sample Collection and Preparation of the Plant Roots for the Isolation

Several methods were employed to isolate and characterize the plant growth-promoting bacteria from the local chili plant's root. The root samples were obtained from a locally grown chili plant purchased at the Seremban nursery. The isolation stage was focused on the internal area of chili roots to obtain the endophytic bacteria, following methods described by Ambrosini and Passaglia (2017). The root sample was washed in running tap water to remove the rhizospheric soil, and the root was dried with a paper towel. The disinfection procedure of the roots was done twice to make sure the roots were clean from the soil. The clean roots were soaked in 70% (v/v) of ethanol, at least 5 cm above to cover the roots. This procedure was performed under laminar flow and performed using a sterile scalpel.

Isolation of Plant Growth-Promoting Bacteria

The roots were transferred into a beaker containing the household bleach, which is a sodium hypochlorite solution diluted in sterilized water in a 1:1 ratio. The roots were incubated for 2 minutes with intermittent stirring using a glass rod or mixing manually. Then, the roots were placed in another beaker and washed in distilled water 5 times by mixing manually with adequate volume to cover the material. After the disinfection steps, the excess water was immediately removed using filter paper. The roots were transferred into a sterile Petri dish and followed isolation steps (Ambrosini and Passaglia, 2017).

The chili plant roots were cut transversely with a scalpel on a glass plate, approximately into small pieces of 0.5 to 1 cm. Some of the root pieces were placed, in triplicate, in a Petri dish containing the nutrient agar medium. This step is to verify the efficiency of the disinfection procedure carried out during the process and preparation stage of the root. The Petri dish containing the processed root was incubated at 30^{o}C for 48 hours. The absence of growing microorganisms on the plate showed the disinfection steps were efficient.

Serial dilution and streak plate technique was used based on general methods described by Ambrosini and Passaglia (2017). A total of 1 g of roots segments were added into a 50 ml Erlenmeyer flask containing 9 ml of sterile saline solution to prepare initial bacterial suspension (10^{0}). The incubation steps for liquid growth were carried out for 24 hours at 30^{o}C while shaking at 180 rpm. While for the serial dilution, a total of 8 test tubes (10^{-1} to 10^{-8} dilution) were prepared from the initial bacterial suspension with 9 ml of sterile distilled water were done following general procedures.

Afterward, an aliquot of 0.1 ml of the suspension was spread on the nutrient agar medium plates in triplicate for 10^{-4} to 10^{-8} dilution. The nutrient agar with the isolates was incubated at 30^{o}C for 24 hours. After 24 hours onwards, the typical and clear bacterial colonies were counted for each Petri dish, and characteristics of the colonies were observed as described in the morphology characterization of isolates.

The well-isolated single bacteria colony was picked and restreaked to the fresh nutrient agar medium and was incubated again for 3 days at room temperature as strain A1 to strain A10 (Kushwaha et al., 2013).

Morphology Characterization of Isolates

Morphology characterization of the potential PGPB isolates was performed using macroscopic and microscopic characterizations. Macroscopic characterization of the colonies was observed based on the growth, the form of the colony, and each colony's colour on the agar plate (Tankeshwar, 2021). At the same time, the colony characteristics were determined by performing Gram staining techniques to distinguish between Gram-positive and Gram-negative bacteria for microscopic characterization (Kushwaha et al., 2013). The *E. coli* was used as a reference for this analysis.

Biochemical Characterization of Isolates

The biochemical characterization was performed to test the identification of bacterial species based on their biochemical activities as previously described. The tests including the methyl-red test (Kushwaha et al., 2013), indole test (Kushwaha et al., 2013), hydrogen sulphide production test (Kushwaha et al., 2013), catalase test (Kushwaha et al., 2013), and ammonia production test (Agbodjato et al., 2015). The *E. coli* was used as a reference for this analysis.

Result and Discussion

The root of the chili plant used was weighing 1.3409 g, has small fruit, and characteristically growing and pointing upwards (Figure 1).

Figure 1. Sample root of the chili plant used for the isolation stage.

A total of 10 isolates were identified and further characterized after a series of streak plating methods. The isolates were designated as strain A1 to strain A10. Table 1 summarizes the morphological characteristics of the isolates based on margin, elevation, colour, form, size, and opacity. Based on the observation, the isolates showed a distinct morphology characteristic. Based on microscopic characterization, strains A2, A3, and A4 were found to be Gram-negative. While strains A1, A5, A6, A8, A9, and A10 were found as Gram-positive. The shape characteristics range from rod-shaped strains A2, A3, A4, A6, A8, A9, and A10 and cocci, which are strains A1 and A5. Unfortunately, the growth of strain A7 was found unstable, which might be due to the symbiosis effect. Thus, strain A7 was eliminated as a potential pure isolate candidate for PGPB in this study.

Table 1. Morphological and microscopic characteristics of the isolates

Strain	Macroscopic Characteristics						Microscopic Characteristics	
	Margin	Elevation	Colour	Form	Size (mm)	Opacity	Gram staining	Shape
A1	Entire	Raised	White	Circular	8 mm	Opaque	+	Spherical
A2	Curled	Raised	Yellowish	Circular	6 mm	Opaque	-	Rod
A3	Undulate	Umbonate	White	Irregular	35 mm	Clear	-	Rod
A4	Curled & Filiform	Raised	Yellowish	Circular	10 mm	Opaque	-	Rod
A5	Entire	Raised	Yellowish	Circular	4 mm	Clear	+	Spherical
A6	Curled	Flat	White	Irregular	15 mm	Opaque	+	Rod
A7	Curled	Raised	Yellowish	Circular	5 mm	Clear	n. a.	n. a.
A8	Curled	Flat	White	Irregular	20 mm	Opaque	+	Rod
A9	Entire	Raised	Yellowish	Circular	8 mm	Opaque	+	Rod
A10	Curled	Raised	White	Irregular	12 mm	Clear	+	Rod

Table 2 describes the results observed for biochemical analysis. Based on the methyl-red test, three out of nine isolates can produce and maintain stable acid at the end of the glucose fermentation product (strains A3, A6, and A8). There were no isolates that were able to break tryptophan into indole and alpha-aminopropionic acid by hydrolytic activity, and only strain A10 was able to reduce the sulphur. All isolates were able to produce catalase, and four isolates (strains A1, A3, A6, and A8) were found positive for ammonia production.

Table 2. Results of biochemical analysis of the isolates

Type of test	Strain								
	A1	A2	A3	A4	A5	A6	A8	A9	A10
Methyl-red test	-	-	+	-	-	+	+	-	-
Indole test	-	-	-	-	-	-	-	-	-
Catalase test	+	+	+	+	+	+	+	+	+
Hydrogen sulphide production test	-	-	-	-	-	-	-	-	+
Ammonia production test	+	-	+	-	-	+	+	-	-

Based on all the results obtained, it is proposed that strains A1, A2, A3, A4, A6, A8, and A9 were commonly found as PGPB (Table 3). However, strains A5 and A10 were not found to be matched to any of the previously isolated PGPB. Thus, it is strongly suggested to further study the characterization of strains A5 and A10 as it could also be a promising candidate for PGPB.

Based on the results found, all isolates obtained in this study were believed to be potential PGPB candidates. The results were found to be in good agreement with previously documented species that have been reported as PGPB. According to Tank and Saraf (2010), dominant genera in saline soils are *Bacillus* spp. and *Pseudomonas* sp. The *Bacillus subtilis* also commonly be found and inhabited in the soil, plant roots, and aquatic environment (Martinez, 2013). In a study by Sgroy et al. (2009), *B. subtilis* were reported as PGPB and able to produce phytohormones such as indoleacetic acid (IAA), zeatin, abscisic acid (ABA), and gibberellic acid (GA3). These compounds were found to promote plant growth. Besides, the *B. subtilis* strain enhanced the growth and increased the productivity of the ribonucleases

of tomato plants (Veselova et al., 2022).

Table 3: Proposed genus of the potential PGPB isolates obtained from this study

Strain	Proposed Genus
A1	*Bacillus subtilis*
A2	*Pseudomonas aeruginosa*
A3	*Bacillus amyloliquefaciens* subsp.
A4	*Pseudomonas aeruginosa*
A5	Unmatched to common reported PGPB
A6	*Bacillus* sp.
A8	*Bacillus* sp.
A9	*Brevibacillus borstelensis*
A10	Unmatched to common reported PGPB

In addition, *P. aeruginosa* is known for its nutritional and ecological flexibility and is able to create a biofilm that imparts resistance against root-secreted antibiotics upon root colonization (Walker et al., 2004). A study conducted by Korejo et al. (2019) stated that the presence of *P. aeruginosa* was found to be beneficial in reducing root rot disease and enhancing plant growth against plant pathogenic fungi and parasitic nematodes on *Salvadora* species. This can be proved by the reduction in the number of root-knot nematodes isolated from the roots and soil after treatment with *P. aeruginosa* (Shankar et al., 2011). The *Fusarium oxysporum, Fusarium solani,* and *Rhizoctonia solani*, also known as soilborne root-infecting fungi that cause root infection, were successfully inhibited after *P. aeruginosa* treatment (Ali Siddiqui and Ehteshamul-Haque, 2001).

Bacillus amyloliquefaciens is another species that has the potential to be commercialized as a biofertilizer. According to Chowdhury et al. (2015), 10 % of *B. amyloliquefaciens* genome is involved in synthesizing antibiotic metabolites that can function as a biocontrol. According to Abdallah et al. (2018), the *Bacillus amyloliquefaciens* species was clustered into two 'subspecies', which were the plant-associated *B. amyloliquefaciens* subsp. *plantarum* and the non-plant-associated *B. amyloliquefaciens* subsp. *amyloliquefaciens*. The *B. amyloliquefaciens* subsp. *plantarum* 32a bacteria remarkably stimulated the emergence and development of tomato seedlings, and the treated plants had longer roots, shoot lengths, and fresh weight. Strain 32a can enhance root development and improve the uptake of minerals and water by solubilizing phosphate and producing IAA (Abdallah et al., 2018). Research by Niazi et al. (2014) also reported the *B. amyloliquefaciens* subsp. *plantarum* strain UCMB5113 was able to promote plant growth, colonize the plant roots, and defend through unknown mechanisms. Furthermore, plants that have been reinforced by the *B. amyloliquefaciens* subsp. were found more resistant to biotic and abiotic stress conditions (Niazi et al., 2014).

In a study conducted by Nehra et al. (2016), *Brevibacillus* was also reported as a formidable plant growth-promoting rhizobacteria (PGPR). This can be seen in the significant usage of *B. brevis* to promote cotton crop growth and production. Various plant growth-promoting (PGP) characteristics detected phosphate solubilization, IAA production, acetylene reduction, and antifungal activity in *B. brevis* SVC (II) 14 on cotton plants. Moreover, *B. brevis* SVC (II) 14 enhanced the development and growth of the cotton plant, improving root function as evidenced by seed germination assay findings.

Conclusion

In conclusion, this study successfully isolated nine strains of isolates from the local roots of the chili plant, which potentially belong to PGPB. Seven strains out of nine were found to be common and shared a similar characteristic of previously reported PGPB. Strain A1 was found to resemble the *Bacillus subtilis*, strains A2 and A4 are proposed to be *Pseudomonas aeruginosa*, strain A3 is proposed to be *Bacillus amyloliquefaciens* subsp. While strains A6 and A8 are *Bacillus* sp., and strain A9 is *Brevibacillus borstelensis*. It is highly recommended to further analyze the capability of each isolate as PGPB prior to being used as biofertilizers. The identification based on 16S rRNA analysis is also

suggested to further identify the isolates.

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