Malaysian Journal of Microbiology, Vol 18(3) 2022, pp. 315-321
DOI: http://dx.doi.org/10.21161/mjm.211365

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Isolation of bacteria with plant growth-promoting activities from a foliar biofertilizer

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Received 6 December 2021; Received in revised form 6 February 2022; Accepted 14 March 2022

ABSTRACT

Aims: Plant growth-promoting bacteria are the key components of a biofertilizer. This study was aimed to isolate and identify the predominant bacteria found in a foliar biofertilizer and characterizes the potential of the bacterial isolates as plant growth promoters.
Methodology and results: Potential bacteria with plant growth-promoting activities were isolated from a foliar biofertilizer on HiCrome™ *Bacillus* agar and Nutrient agar. Bacteria with unique colonial morphology were selected and categorized by Gram's differential staining. Subsequently, the bacterial isolates were being further characterized for plant growth-promoting potentials, such as the production of indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase and siderophore; as well as the ability of nitrogen fixation and phosphate/potassium solubilization. Based on the characterized traits, three bacterial isolates, namely M17, M22 and M52 showed great potential for being a plant growth promoter. Based on their 16S rRNA gene sequence analysis, M17, M22 and M52 were identified as *Leclercia adecarboxylata*, *Margalitia shackletonii* and *Lysinibacillus pakistanensis*, respectively.
Conclusion, significance and impact of study: Bacterial isolates exhibiting plant growth-promoting activities were successfully isolated from a biofertilizer and identified in this study. This finding provides an insight into the potential bacteria of a foliar fertilizer that may promote plant growth. Identification of these plant-growth promoters may help the scientists and agrochemical manufacturers to determine and disclose the key microorganisms of their biofertilizers, thereby contributing to the improvement of biofertilizers and promoting them as reliable alternatives to chemical fertilizers.

Keywords: Bacteria, chemical fertilizers, foliar biofertilizer, indole acetic acid, plant growth promoters

INTRODUCTION

Fertilizer has been introduced to the agriculture sector to supply additional nutrients to ensure optimal crop growth and productivity. Given the growing human population, it is expected that food demand will continue to escalate. Foliar and soil-applied fertilizers are widely used to boost crop growth and productivity (Canellas *et al.*, 2015). Soil-applied fertilizers, which usually come in solid form, provide the main and long-term nutritional needs of plants where nutrients are absorbed by the root system. Foliar fertilizers may come in solid or liquid form. Both forms are dissolved and diluted before direct application of nutrients through aerial plant parts by spraying, thus increasing the effectiveness of nutrient absorption at a reduced cost. For example, cotton mainly relies on its root system for nutrient uptake from soil. However, because of the poor nutrient uptake capability of the root system at the seedling stage and the reduced root activity in later stages, it is difficult to meet the nutrient needs solely based on the root system and foliar fertilization acts as a good way to keep up the nutrient supply in these two different stages (Niu *et al.*, 2020).

The components of foliar fertilizers can be changed according to the nutritional needs of the crop plant at the time of application. Microbial inoculants are one of the promising alternatives to replace and reduce chemical fertilizer input into the agroecosystem. The mixture of different microbial inoculant formulations with plant growth-promoting properties stimulates plant growth in a number of diverse mechanisms such as synthesis of hormones and other molecules, phosphate solubilization, biological nitrogen fixation and even biological control of pests. Fertilizers that contain living microorganisms or compounds derived from microorganisms such as fungi, bacteria and algae are called biofertilizers (Fernández *et al.*, 2013; Canellas *et al.*, 2015; Garcia-Gonzalez and Sommerfeld, 2016).

Some of these microorganisms that are added in biofertilizers are able to promote plant growth and development by secreting plant growth-promoting regulators (PGPR), including phytohormones such as

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(3) 2022, pp. 315-321
DOI: http://dx.doi.org/10.21161/mjm.211365

auxins and gibberellin, into the environments. For instance, certain members of the genera *Aeromonas*, *Agrobacterium*, *Alcaligenes*, *Azospirillum* and *Enterobacter* have been reported to be able to produce auxin while *Pseudomonas fluorescens* and *Rhizobium leguminosarum* were reported to produce cytokinin. *Bacillus* spp., such as *Bacillus circulans, B. firmus and B. globisporus*, were reported to stimulate root elongation, while *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. mycoides* and *B. sphaericus*, are known to elicit a significant reduction in disease incidence on a diversity of hosts (Bhattacharyya and Jha, 2012). Therefore, biofertilizers are promising alternatives for replacing and reducing chemical fertilizer input into the ecosystem. The beneficial properties of a biofertilizer largely depend on its microbial content. Hence, there is a need to isolate and identify microorganisms that confer plant growth beneficial properties. The bacterial isolates that contribute to plant growth and increase of crop production can be mixed and formulated to cope with different stages of plant growth and eventually help in the development of foliar biofertilizers (Lugtenberg, 2015; Shi *et al.*, 2017).

Although commercially available biofertilizers often claim to possess microorganisms with beneficial properties, the identity of the microorganisms are not disclosed, or the biochemical activities of these microorganisms that promote plant growth are not provided. In this study, we aim to isolate predominant bacteria from a commercial foliar biofertilizer and characterize them as potential plant growth promoters through their ability to produce indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase and siderophore, fix nitrogen and solubilize phosphate and potassium.

MATERIALS AND METHODS

Isolation of bacteria

INO Nature "PK" Bio Foliar liquid fertilizer was subjected to serial dilutions by using 0.85% (w/v) NaCl solution. Subsequently, 100 µL each of the 1,000× and 10,000× dilutions were spread-plated onto Nutrient agar (NA) (Merck, United States) and HiCrome™ *Bacillus* agar (HiMedia, India).

Quantitative estimation of IAA production

All isolates were screened for IAA production as described by Gordon and Weber (1951) with slight modifications. Ten microlitres of overnight bacteria culture grown in Nutrient Broth (NB) (Merck, United States) was transferred into 5 mL of fresh NB supplemented with L-tryptophan (1 mg/mL) (Wako Pure Chemical, Japan) and incubated at 28 °C for 2 days with agitation at 220 rpm. The bacterial culture was then spun down at 4,000× *g*, room temperature, for 10 min. Later, 100 µL of the supernatant was mixed with an equal volume of Salkowski's reagent (30 mL 95-97% sulfuric acid; 50 mL sterile distilled water; 1 mL 0.5 M $FeCl_3$) and the mixture was then kept in the dark for 30 min. The development of pink or red color indicates IAA production and its absorbance was measured at 535 nm using a spectrophotometer. The concentration of IAA produced by the bacterial isolates was determined by extrapolating the standard curve which was prepared by using commercial IAA (Duchefa Biochemie, Netherlands) at different concentrations (1, 5, 10, 25, 50 and 100 µg/mL). 1-5 µg/mL, 5.1-10 µg/mL and ≥10 µg/mL of detected IAA concentrations were classified as weakly positive ("+"), moderately positive ("++") and strongly positive ("+++"), respectively.

Quantitative estimation of ACC deaminase production

All isolates were screened for ACC deaminase production as described by Glick (2014) and Li *et al.* (2011) with slight modifications. Five microlitres of overnight bacteria culture grown in NB was spun down and washed once with a commercial nitrogen-free medium, Jensen's broth (HiMedia, India). Subsequently, the bacterial pellet was resuspended in 3 mL of Jensen's broth supplemented with 3 mM ACC, which serves as the sole nitrogen source. The culture was incubated at 28 °C for 24 h with agitation at 220 rpm. The bacterial culture was then spun down at 8,000× *g* for 5 min, room temperature and the supernatant was subjected to 10× dilution with Jensen's broth. Then, 60 µL of the diluted supernatant was mixed with 120 µL of ninhydrin reagent, followed by placing it in boiling water for 30 min. The absorbance was measured at 570 nm using a spectrophotometer. The ninhydrin reagent was prepared according to Li *et al*. (2011). The concentration of remaining ACC was compared by extrapolating the standard curve prepared by using commercial ACC (Sigma-Aldrich, USA) at different concentrations (0.01, 0.025, 0.05, 0.1, 0.5, 1.0 and 2.0 mM). Detected ACC concentrations of ≥1 mM, 0.5-0.99 mM, 0.25-0.49 mM and 0.01-0.24 mM were classified as negative ("-"), weakly positive ("+"), moderately positive ("++") and strongly positive ("+++"), respectively.

Phosphate solubilization activity

Bacterial isolates which were positive for either IAA production or ACC deaminase production tests were subsequently screened for inorganic phosphate-solubilizing activity as described by Sukweenadhi *et al.* (2015). Fresh bacterial cultures grown on NA were adjusted to OD_{600} of 0.1 with 0.85% (w/v) NaCl solution. Then, 10 µL of the respective bacterial culture was spotted on Pikovskaya agar (Himedia, India). The plates were incubated at 28 °C for 7 days. The formation of a clear zone in the surrounding bacterial colony was scored as positive in phosphate solubilization activity.

Potassium solubilization activity

Fresh bacterial cultures grown on NA were adjusted to OD_{600} of 0.1 with 0.85% (w/v) NaCl solution. Then, 10

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(3) 2022, pp. 315-321
DOI: http://dx.doi.org/10.21161/mjm.211365

Table 1: Summary of colony morphology of bacterial isolates.

Isolates	Colony Morphology						
	Shape	Size	Surface	Color	Elevation	Margin	Gram staining
M7	Round	Medium	Glistening	Creamy-white	Raised	Even	Positive, rod
M17	Round	Medium	Glistening	Pale yellow	Flat	Even	Negative, rod
M19	Round	Small	Glistening	White	Raised	Even	Positive, round
M22	Round	Medium	Glistening	White	Flat	Even	Positive, rod
M35	Round	Medium	Glistening	Creamy-white	Flat	Even	Positive, rod
M38	Round	Medium	Glistening	White	Flat	Even	Negative, rod
M42	Round	Medium	Glistening	White	Flat	Even	Positive, rod
M44	Round	Medium	Glistening	White	Flat	Even	Positive, rod
M52	Irregular	Very large	Rough	Slightly transparent	Flat	Wavy	Positive, rod
M72	Irregular	Medium	Wrinkled	White	Flat	Curled	Positive, rod
M74	Round	Medium	Glistening	White	Flat	Even	Positive, rod
M77	Round	Medium	Glistening	White	Flat	Even	Positive, rod

µL of the respective bacterial suspension was spotted onto Aleksandrov agar (Himedia, India). The plates were then incubated at 28 °C for 7 days. The formation of a clear zone at the surrounding of the bacterial colony was scored as positive in potassium solubilization activity.

Nitrogen fixation activity

Nitrogen-free Jensen's medium (Himedia, India) was used in detecting the nitrogen fixation ability of the isolated bacteria via a method described by Jain *et al.* (2016). Firstly, fresh bacterial cultures grown on NA were adjusted to OD_{600} of 0.1 with 0.85% (w/v) NaCl solution. Next, a loopful of the bacterial suspension was streaked on Jensen's agar and incubated at 28 °C for 7 days. Growth observed on the nitrogen-free Jensen's agar after the incubation period indicates a positive result for nitrogen fixation ability.

Siderophore production assay

The method used for this assay was described by Schwyn and Neilands (1987) with slight modifications. Fresh bacterial cultures grown on NA were adjusted to OD_{600} of 0.1 with 0.85% (w/v) NaCl solution. Then, 10 µL of the respective bacterial suspension was spot onto Chrome Azurol S (CAS) agar and incubated at 28 °C for 48 h. The CAS agar was prepared according to the recipe taken from Louden *et al.* (2011). When the bacteria utilized iron, which is present in the blue-colored CAS agar, the surrounding of the bacteria colony turns orange, thus indicating the presence of siderophore production.

Identification of bacteria isolates by 16S rDNA sequencing

The bacterial genomic DNA was extracted using the GF-1 Bacterial DNA Extraction kit (Vivantis, Malaysia). The extracted genomic DNA was stored at –20 °C and used as a template for 16S rRNA PCR amplification. The 16S rRNA gene amplification using a pair of universal primers, 27F (5'-AGAGTTTGATCCTGGTCAG-3') and 1492R (5'-GTTACCTTGTTACGACTT-3'). The PCR amplification was performed using MyTaq DNA polymerase (Bioline, United Kingdom). The PCR was performed with a standard 3-step cycling profile; an initial denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 15 sec, annealing at 55 °C for 15 sec and extension at 72 °C for 10 sec and after the cycle, a final extension step at 72 °C for 2 min 30 sec. The PCR products were purified using the FavorPrep™ Gel/PCR Purification kit (Favorgen, Taiwan). Purified PCR products were outsourced for DNA sequencing (Apical Scientific, Malaysia). Sequences obtained were analyzed using BLASTn program to search for the nucleotide sequences homologous to the 16S rRNA gene.

RESULTS

A total of 210 bacterial colonies were obtained in the preliminary isolation. They were grouped into 35 isolate types based on the colony morphological characteristics, such as form, size, surface, color, elevation and margin of the bacterial colony formed. One representative isolate from each of the 35 bacterial types were further characterized. Among the 35 bacterial isolates, 25 bacterial isolates were Gram-positive (three coccus-shaped and 22 rod-shaped bacteria) and 10 isolates were Gram-negative rod-shaped bacteria (Table 1). Among these 35 bacterial isolates, 12 isolates that produced IAA and/or ACC deaminase were subjected to further *in vitro* plant growth-promoting characterization such as siderophore production, nitrogen fixation and phosphate/potassium solubilization.

For IAA production, after 48 h incubation, isolate M17 produced the highest amount of IAA (22.63 ± 0.67 µg/mL), followed by 8.75 ± 0.80 µg/mL produced by isolates M52 and for M74 and M22 are 7.97 ± 0.43 µg/mL and 3.52 ± 0.44 µg/mL, respectively. Isolate M44 produced 2.25 ± 0.15 µg/mL, while M38 and M72 produced 1.79 ± 0.14 µg/mL and 1.46 ± 0.23 µg/mL, respectively. For isolates M7, M19, M35, M42 and M77,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(3) 2022, pp. 315-321
DOI: http://dx.doi.org/10.21161/mjm.211365

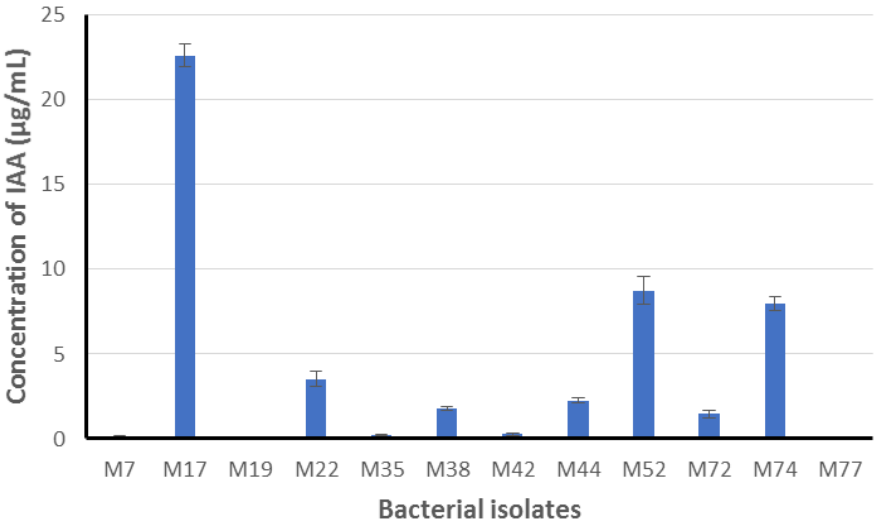

Figure 1: IAA production of bacterial isolates. Bars indicate the means obtained from 3 replicates and the error bars are the respective standard deviations. Detected IAA concentrations were classified as weakly positive (1-5 µg/mL), moderately positive (5.1-10 µg/mL) and strongly positive (≥10 µg/mL).

Table 2: Summary of plant growth-promoting activities of bacterial isolates.

Isolates	Plant growth-promoting properties					
	IAA production	ACC deaminase production	Nitrogen fixation	Phosphate solubilization	Potassium solubilization	Siderophore production
M7	-	++	+	-	-	-
M17	+++	-	+	+	+	+
M19	-	++	+	-	-	-
M22	+	++	-	+	+	-
M35	-	+++	+	-	-	-
M38	+	-	+	-	-	-
M42	-	++	+	-	-	-
M44	+	-	+	-	-	-
M52	++	++	+	+	+	-
M72	+	-	+	-	-	-
M74	++	-	+	-	-	-
M77	-	+++	+	-	-	-

* "+" = weak positive; "++" = moderate positive; "+++" = strong positive.

they produced less than 1 µg/mL of IAA and thus categorized as non-producers of IAA (Figure 1, Table 2).

For the ACC deaminase production test, after 24 h incubation, bacteria M7, M19, M22, M35, M42, M52 and M77 showed positive results for the test. In this test, higher ACC deaminase activity is indicated by the lesser amount of ACC remaining in the supernatant. Isolates M17, M38, M44, M72 and M74 were categorized as non-producers of ACC deaminase due to more than 1 mM of ACC were present in the supernatant after 24 h incubation (Figure 2, Table 2).

A nitrogen fixation test was carried out using Jensen's agar. All bacterial isolates except M22 were able to grow on Jensen's agar which indicates their ability to utilize nitrogen in the air as their nitrogen source. As for both phosphate and potassium solubilizing tests, only isolates M17, M22 and M52 showed weak activity when they were tested on Pikovskaya agar and Aleksandrov agar, respectively. Lastly, only strain M17 showed a positive result in the siderophore production assay.

From the series of tests, isolates M17, M22 and M52 showed positive results for more than two tests which indicated that they each carry more than 1 beneficial property. Both strain M17 and M53 showed positive results on five out of six tests, whereas isolate M22 gave positive results on four out of six tests. These strains were then selected for bacterial identification. The identification of these bacterial isolates was performed via 16S rRNA gene sequencing. The sequencing results were compared with the database in NCBI GenBank. Isolates M17 and M22 were identified as *Leclercia adecarboxylata* and *Margalitia shackletonii*, respectively, with the sequence

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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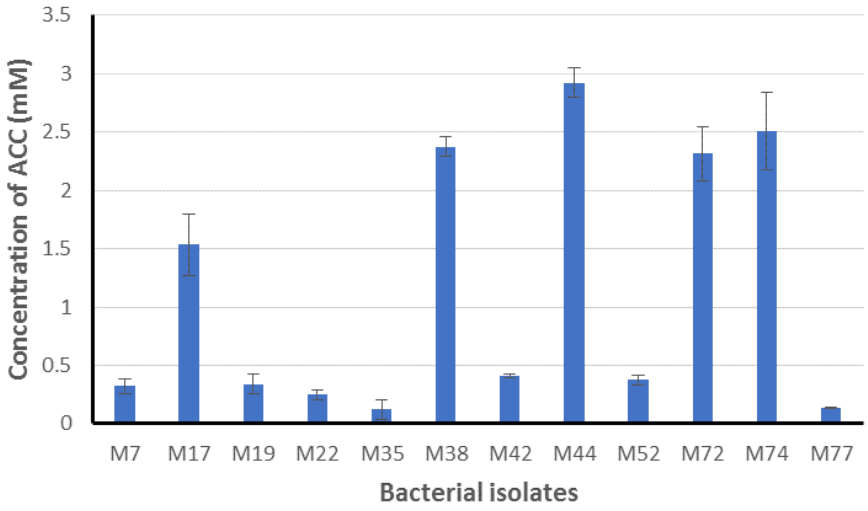

Figure 2: ACC deaminase activity of bacterial isolates. ACC deaminase activity is indicated by the decrease in ACC, the substrate of ACC deaminase, remaining in the culture medium. Bars indicate the means of 3 replicates and the error bars are the standard deviation of respective bacteria isolates. Detected ACC concentrations were classified as negative (≥1 mM), weak positive (0.5-0.99 mM), moderate positive (0.25-0.49 mM) and strong positive (0.01-0.24 mM).

identities of 99.37% to *L. adecarboxylata* EGTM31 and 99.65% to *M. shackletonii* LMG 18435. On the other hand, isolate M52 showed 99.86% identity to *Lysinibacillus pakistanensis* NCCP-54. The sequences were then deposited into GenBank and the accession numbers are OM212809, OM212810 and OM212811.

DISCUSSION

In this study, bacteria with seemingly unique colonial morphology, based on their different size, form, color and surface of the colonies formed, were isolated from the foliar biofertilizer. To isolate bacteria from the chosen foliar biofertilizer, the rich medium NA agar was used to provide an insight into the bacterial diversity and population of foliar biofertilizer. HiCrome™ *Bacillus* agar was also used simultaneously in isolation that provides certain selectivity towards *Bacillus* spp. The 35 bacterial isolates with distinct colony morphology obtained were first tested for the key plant growth-promoting activities, IAA and ACC deaminase production.

Plant growth-promoting bacteria (PGPB) use different ways to improve plant growth, for instance, producing plant growth phytohormones and facilitating nutrient uptake. Plant growth-promoting phytohormones include auxins, cytokinins and gibberellins. In contrast, reducing growth-inhibiting phytohormone, such as ethylene, by synthesizing ACC deaminase that reduces the ethylene level would enhance the effect of auxins (Glick, 2014). Some PGPB produce organic acid to solubilize the insoluble potassium and phosphate, making them available to plants for absorption (Khan *et al.*, 2010). PGPB may fix atmospheric nitrogen, converting it to ammonia, which is eventually converted in the form of nitrate and ammonium that can be absorbed by plants (Di Benedetto *et al*., 2017). Bacterial siderophores are known to chelate insoluble iron (III) (Fe^{3+}), forming soluble Fe^{3+} complexes that can be absorbed by plants (Ahmed and Holmström, 2014).

IAA plays a key role in regulating organogenesis and other cellular responses. IAA induces root development and loosens the root cell wall to alleviate and increase the root exudates, which allow plants to have better access to nutrients (Ahmed *et al.*, 2017). In the present study, isolate M17 (*L. adecarboxylata*) produced 22.63 µg/mL after 48 h of incubation, which is comparable to the IAA production of 2.6 µg/mL and 9.815 µg/mL by *L. adecarboxylata* reported by Snak *et al*. (2021) and Kang *et al*. (2019), respectively. On the other hand, isolates M52 (*L. pakistanensis*) and M74 produced 8.75 µg/mL and 7.97 µg/mL of IAA, respectively. Previously, *L. pakistanensis* have been reported to produce IAA of 10.571 µg/mL thus, supporting the finding of this study (Lelapalli *et al*., 2021).

Ethylene is a key phytohormone that involves in plant growth and development, but it also causes detrimental effects, such as leaf abscission, leaf senescence and flower wilting. ACC is the precursor of ethylene. ACC deaminase, cleaves ACC into ammonia and α-ketobutyrate, thus inhibiting the biosynthesis of ethylene, ameliorating the detrimental effects of ethylene (Glick, 2014). Studies showed that ACC deaminase producing bacterial strains promote plant growth under stressed soil. For instance, an ACC deaminase-producing *Pseudomonas putida* UW4 strain maintained and promoted plant growth in the saline environment (Yan *et al.*, 2014). In this study, both isolates M35 and M77 produced the highest ACC deaminase activity. As for M22, this isolate produced the third-highest ACC deaminase activity.

Nitrogen, phosphate, potassium and iron are important nutrients for plant growth. Only three bacterial

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

isolates, namely M17, M22 and M52 showed weak positivity for both phosphate- and potassium-solubilizing activities. In addition, M17 also showed siderophore producing activity. Isolate M17 (*L. adecarboxylata*), which is a Gram-negative bacterium belonging to the Enterobacteriaceae family, has been reported to possess many plant growth-promoting traits. Other than IAA production, these bacteria also can fix nitrogen, solubilize phosphate/potassium and also produce siderophore that chelates iron and make it available to the plant. These traits could protect the plants to overcome stress and facilitate plant growth for better crop production (Snak *et al*., 2021).

Lysinibacillus pakistanensis, is a rod-shaped, aerobic and Gram-positive bacterium (Ahmed *et al*., 2014). This bacterium has been shown to possess a few plant growth-promoting traits and thus has good potential as a plant growth-promoting bacterium. Based on a study conducted by Jinal *et al*. (2021), the plant growth-promoting properties that are possessed by *L. pakistanensis* include IAA and siderophore producing activities, potassium solubilizing activity and it is resistant to up to 100 mg/100 mL. The ability to resist Zn was tested in maize plants and was shown to be able to enhance root length, shoot length and biomass content.

In this study, isolate M22 (*M. shackletonii*) is a Gram-positive spore-forming bacterium which possesses IAA-producing and ACC deaminase activities. Some of the plant growth-promoting activities of *M. shackletonii* have been previously reported (Jikare and Chavan, 2013). In addition, the strains isolated from the rhizosphere of groundnut also reported producing siderophore and solubilized phosphate on Pikovskaya medium. Based on the beneficial properties determined, the three bacterial isolates obtained in this study may be part of the key component microorganisms in the foliar biofertilizer.

CONCLUSION

In conclusion, in this study, three bacterial strains possess useful traits, such as IAA, ACC deaminase and siderophore production, nitrogen fixation and phosphate- and potassium-solubilization activities that may improve plant growth were isolated. Bacterial strains, *L. adecarboxylata*, *M. shackletonii* and *L. pakistanensis* are known to possess plant growth-promoting activities, thus, partly corroborating some of the beneficial properties attributed the foliar biofertilizer. The findings of this study may provide clues to biofertilizer manufacturers to identify those plant-beneficial bacteria for formulating a better biofertilizer, thereby maintaining the consistency and improving their quality. These efforts may help in making biofertilizers reliable alternatives to chemical fertilizers.

ACKNOWLEDGEMENTS

This research was funded by the Universiti Tunku Abdul Rahman-INO Nature Sdn. Bhd. collaborative research projects vote no. 8043/002 and 8043/001.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538