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IDENTIFICATION OF PLANT GROWTH-PROMOTING COMPOUNDS IN LOCAL ISOLATED BACTERIA FROM CHILI PLANT ROOT

Nur Zulaikha Mohamad Faudzi[1] & Aslizah Mohd-Aris[2*]
[1,2] School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA Cawangan Negeri Sembilan, Kampus Kuala Pilah, 72000 Kuala Pilah, Negeri Sembilan, Malaysia
aslizah@uitm.edu.my

ABSTRACT

Plant Growth-Promoting Bacteria (PGPB) has been established as a cultivation strategy that manipulates biocontrol mechanisms within the agriculture industry. However, a detailed understanding of PGPB in the local setting is still somewhat understudied. This consequently limits the commercial value of PGPB. The objectives of this research converge towards identifying plant growth-promoting compounds and studying the growth of plants following a treatment using two potential PGPBs that were previously isolated from local chili plant roots (strains A4 and A8). Three elements were identified in this study; indole-3-acetic acid (IAA) production, phosphate solubilization, and biological nitrogen fixation (BNF). The ability to produce IAA was quantified using Salkowski's reagent. Additionally, the ability of both strains to grow on Pikovskaya's agar and yeast extract agar describes the capacity for phosphate solubilization and biological nitrogen fixation, respectively. The influence of both strains in promoting chili plant growth was assessed from a pot treatment. Essentially, it was found that both strains showed positive results in the compound detection analysis. Furthermore, the treatment using PGPB on chili plants showed an increase of 62.9 % in height and 57.1 % in number of leaves. In conclusion, A4 and A8 were strongly recommended for PGPB.

Keywords: Plant growth-promoting bacteria (PGPB); plant growth-promoting compounds; Indole-3-Acetic Acid (IAA); phosphate solubilization; biological nitrogen fixation (BNF)

Introduction

Agriculture is the connection point between each of the 17 Sustainable Development Goals (SDGs). Investments in agriculture can address not only hunger and malnutrition but also poverty, water and energy use, global warming, and unsustainable production and consumption. Furthermore, the predicted economic development in low- and middle-income nations is highly related to the increasing demand for agricultural commodities. Thus, to cater to this issue, agricultural output must be greatly enhanced. Regarding the current scenario, agriculture is the business sector that gives high revenue to the world, Malaysia included.

Agricultural product resources are in high demand owing to the ever-increasing human population worldwide. However, current practices in agriculture rely heavily on pesticides to promote crop growth and production. This includes protecting plants from weeds, fungi, or insects that are detrimental to crops. Despite being an essential part of farming, excessive and uncontrolled usage of pesticides is damaging the environment and poses adverse health effects to humans, especially farmers that encounter it daily (Poria et al, 2022; Mahmood et al., 2016). Although preserving crop quality is crucial for both farmers and consumers, it is far more critical to ensure that the food is safe to eat, and the accompanying agricultural process does not harm the people involved. As a result, it is strongly recommended that pesticides be handled with caution and that only a small amount be used.

Thus, an alternative and mitigative step to save the ecosystem from disruptions caused by the excessive use of dangerous chemicals is adopting PGPBs. These microorganisms were reported to have been used as substitutes for pesticides due to their characteristics as biofertilizers, biocontrol agents, and biopesticides (Kaushal et al., 2019). They are mostly found in the rhizosphere, whereby they help improve soil productivity by stimulating plant growth and suppressing plant pathogens. This not only promotes plant development but also helps plants cope with biotic and abiotic stressors (Ramakrishna et al., 2019). These microorganisms play important roles in accelerating the growth of plants and aid in soil bioremediation. This can be attributed to the ability of the microorganisms to secrete several hormones and metabolites, which further helps in nitrogen fixation and enhance the bioavailability of other nutrients through mineral solubilization (Poria et al, 2022).

This research identified plant growth-promoting compounds produced from two local strains of potential PGPBs (A4 and A8) as described in this article. Furthermore, the ability of the compounds to stimulate the chili plant was ascertained from observations of a pot experiment. In the future, results from this study may be used with the intention to prove the efficiency of testing PGPBs as biofertilizers. Future studies could also involve the evaluation of the effects of PGPB as a growth stimulants and its specific roles in boosting photosynthesis. It is anticipated that this would reassure farmers' confidence in practicing sustainable farming and contribute towards the commercialization of PGPB as an alternative to pesticides and fertilizers.

Literature Review

Plant growth-promoting bacteria (PGPB)

Plants deal with various abiotic stresses due to their sessile nature. Polluted environments, along with additional abiotic factors such as excessive salinity and temperature fluctuations, significantly impact plant growth (Ullah et al., 2021). Appropriate utilization of plant-microbe interaction is a great way to overcome these problems without adding to it. These microorganisms can be found in the soil region—where the root system has a strong effect on microorganism-mediated activities—known as the rhizosphere (Saeed et al., 2021). In this region, the plant-microbe interaction can be categorized into three categories, which are neutral, negative, and positive (Bhattacharyya & Jha, 2012).

Neutral microbes or those that are commensals do not pose any threat, nor does it benefit the plant. However, there are microbes that are commensal for several months and proceed to be pathogenic (Beattie, 2006). Pathogenic microorganisms create phytochemicals that have a damaging effect on the plant (Bhattacharyya & Jha, 2012). In this situation, the same bacterium that was beneficial to the plant when it was commensal has now turned against it. Here is where PGPB becomes favorable. PGPB encompasses a diverse collection of microorganisms. PGPB can boost plant development and protect it against diseases and

abiotic challenges (de Souza et al., 2015). In addition, to develop a sustainable and eco-friendly agricultural practice, crop inoculation with microorganisms with biofertilizer capacity would improve soil fertility, enhance microbial metabolic activity, and increase crop yield (Wang et al., 2021).

Mechanisms of plant growth promoting bacteria (PGPB)

In a report, Pathania et al. (2020) highlighted Pseudomonas, Aeromonas, Klebsiella, Azoarcus, Enterobacter, Azospirillum, Clostridium, Azotobacter, Arthrobacter, Rhizobium, Gluconacetobacter, Bacillus, and Serratia, which were widely demonstrated within the PGPB genera. This could mean that the mechanism of action may differ among species and strains (de Souza et al., 2015). Many studies have discovered that the mechanisms of PGPB may be separated into direct and indirect processes in general. Direct mechanisms occur inside the plant and involve the plant's metabolism (Kumar et al., 2020). Auxin, cytokinin, gibberellin, nitrogen fixation, phosphorous solubilization, and iron uptake by bacterial siderophores are among them. Indirect mechanisms, on the other hand, are mechanisms that occur outside of the plant and prevent one or more plant pathogens from functioning. ACC deaminase, antibiotics, cell wall-destroying enzymes, competition, hydrogen cyanide, induced systemic resistance, quorum quenching, and siderophores are all examples of this (Olanrewaju et al., 2017).

Direct mechanism

Direct mechanisms are defined as the use of bacterial characteristics that directly promote plant growth. Among them are the production of auxin, ACC deaminase, nitrogen fixation, phosphorous solubilization, and iron sequestration by bacterial siderophores. In addition, de Souza et al. (2015) classified PGPB traits into specific functions, which are: (i) tolerance towards abiotic stress through the action of ACC deaminase, (ii) defense against pathogens by the presence of competitive traits such as siderophore production, and (iii) increase of fertility and plant growth through BNF, IAA production, and phosphate solubilization around roots.

Auxin

Auxin is recognized as a plant growth hormone because of its significance in plant differentiation in response to gravity and light stimuli (Bhattacharya, 2019). It also affects cell elongation by altering cell wall plasticity. Auxin also promotes the division of cambium cells and the differentiation of secondary xylem in plant stems. One of the most studied auxins is known as indole-3-acetic-acid (IAA). IAA is a plant hormone that may alter every aspect of plant growth, including cell enlargement and division, tissue differentiation, and light and gravity responses (Johnston-Monje et al., 2019). Furthermore, research by de Souza et al. (2015) has revealed that plant-associated bacteria exhibit a heightened production of indolic compounds compared to their counterparts in bulk soil. It is worth noting that the presence of precursors in root exudates is necessary for IAA synthesis.

Phosphate solubilization

Following nitrogen, phosphorus becomes the second most vital nutrient for plant growth. It plays a pivotal role in essential plant functions such as energy transfer, photosynthesis, nutrient transport, genetic inheritance, and sugar metabolism (Satyaprakash et al., 2017). When phosphorus is insufficient, carbohydrate utilization lags, even as photosynthesis generates carbohydrates, leading to glucose buildup and the emergence of dark green leaves (Sharma et al., 2011). Phosphorus is easily mobilized in plants; when a deficiency arises, it is

translocated from older tissues to active meristematic tissues, causing foliar deficiency symptoms to show on the plant's older section (Sharma et al., 2011).

Despite being an essential nutrient for plants, phosphorus often exists in insoluble form and is not readily available for plant uptake. This is because plants can only absorb phosphorus in two soluble forms, which are monobasic (H_2PO^{4-}) and dibasic (HPO_4^{2-}) (Satyaprakash et al., 2017). Phosphorus is known to be the least mobile nutrient element, causing the use of pesticides to tackle phosphorus deficiency to be ineffective as the soluble inorganic phosphorus is quickly immobilized, making it unavailable to plants (Novo et al., 2018). Alternatively, the use of PGPB with phosphate solubilizing ability has been considered, as researchers found evidence that these microorganisms promote plant growth by converting phosphate to a form that is more accessible for plants (Olanrewaju et al., 2017).

Methodology

Growth maintenance and screening for plant growth-promoting compounds

Maintenance of bacterial growth

Two previously isolated bacteria designated as A4 and A8 were used as test bacteria (Ab Rani & Mohd-Aris, 2022). Both isolates were maintained in nutrient agar (NA) and nutrient broth (NB). General incubation was performed in an incubator at 37 ºC overnight (Shin Saeng Scientific Co. Ltd, Korea). Gram staining was performed following common protocol. Observation of staining was done using a compound light microscope (Olympus CX31, Olympus Corporation, Japan).

Screening for indole-3-acetic acid (IAA)

Salkowski's reagent was prepared following the formulation described by El-Sherbini et al. (2022) to screen the presence of IAA compounds. The reagents consist of 0.3g $FeCl_3$ diluted in 12mL H_2SO_4. One mL of Salkowski's reagent was mixed with 1 mL of supernatant of each test sample and incubated in the dark at 30 ^oC for 30 minutes. Changes in color were observed and recorded after 30 minutes. The presence of IAA is justified if the initial yellow hue of Salkowski's reagent shifts to red or pink due to the formation of an IAA complex with Fe^{3+} reduction.

Screening for nitrogen fixing ability

Biological nitrogen-fixing ability was screened using a method described by Verma et al. (2019). Yeast extract agar was prepared by mixing 0.45 g yeast extract powder with 2.25 g agar powder in 150 mL distilled water prior to being poured and solidified in petri dishes. Test strains were streaked onto the yeast extract agar, followed by incubation at 37 ^oC for 24 hours. The medium showed a translucent initial condition, and the presence of growth on the agar was an indication of nitrogen-fixing ability.

Screening for phosphate-solubilizing trait

Phosphate solubilizing characteristic was screened using Pikovskaya's agar as a screening medium for phosphate-solubilizing traits following the formulation described by Sharma et al. (2013). The medium consists of a mixture of 0.25 g yeast extract powder, 5 g glucose, 0.25 g tricalcium phosphate, 0.25 g ammonium sulfate, 0.1 g potassium chloride, 0.05 g magnesium sulfate, 0.05 mg manganese sulfate, 0.05 mg ferrous sulfate, and 7.5 g agar powder in 500

mL distilled water. After heating, mixing, autoclaving, and cooling, the mixture was poured into petri dishes prior to inoculation with the test strains. The test strains were streaked onto the agar and incubated at 37 ^{o}C for 6 days. Formation of halo zones around the colonies was observed as an indication of phosphate solubilization.

Pot experiment

Preparation of bacterial root treatment (BRT) solution

Root treatment was carried out following the method described by Ren et al. (2019). A total of 200 mL of NB medium supplemented with 1g of *L*-tryptophan was prepared in a 250 mL Schott bottle. The medium was divided into two conical flasks. 100 µL inoculum of pure A4 and A8 was added into each conical flask. The culture was incubated at 30 ºC with 120 rpm shaking. All incubation steps were performed in the GFL Shaking Incubator 3032 (GFL Gesellschaft für Labortechnik, Germany). The incubation period varied for each strain depending on IAA production ability. Strain A4 was incubated for 96 hours, while A8 was incubated for 24 hours.

After incubation, the medium growth was centrifuged at 3000 rpm for 10 minutes to separate the supernatant. The centrifugation was done using Centurion K240 centrifuge (Centurion Scientific Ltd, UK). Then, the cell pellet was resuspended in 20 mL of distilled water and vortexed to obtain a homogenous suspension. Initial measurements and the number of leaves of each chili plantlet were recorded before they were gently uprooted. Excess soils were removed, and the roots were rinsed with sterile distilled water prior to being submerged into the test bacteria root treatment solution.

Application of bacterial root treatment (BRT) solution

The roots were submerged in the root treatment for 30 minutes before being replanted in a similar pot. Measurements and number of leaves were recorded every week. Any notable changes were observed and recorded. The plant roots were treated with the BRT twice, the first treatment occurred on the first week, while the second took place on the fourth week of the experiment.

Plant growth conditions

The chili plants used in this study were obtained from a local nursery. Three sets of treatment were performed: control (Group C), plant treatment with Strain A4 (A4), and plant treatment with Strain A8 (A8), and were all executed in triplicates. After the application of BRT, the plants were maintained and observed for any changes. The pots were placed equidistant from each other in an open space with a temperature of 28 – 30 ºC. Water was supplied twice a day, with no additional fertilizer given. The measurements and number of leaves were recorded biweekly, and any notable changes were recorded. Average increment of plant height and number of leaves were calculated using the following formula:

$$Average\ increment\ = \frac{Average\ final\ measurement - Average\ initial\ measurement}{Average\ final\ measurement} \times 100$$

Results and Discussion

Confirmation of gram staining

Gram staining showed both bacteria having a rod shape, with A4 belonging to gram-negative bacterium while A8 is gram-positive. Figure 1 depicts the gram staining results for both strains.

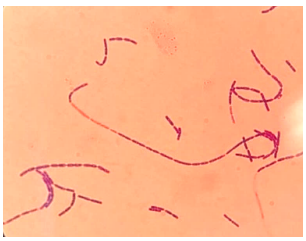
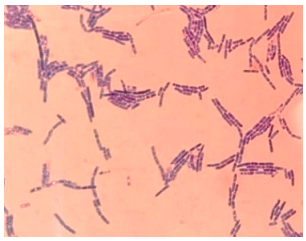
(a)  (b) 

Figure 1. The Gram staining of (a) A4 and (b) A8; observed under light microscope (×1000 magnification)

Screening for plant growth-promoting compounds

Table 1 shows both strains displaying positive results for IAA production, nitrogen-fixing ability, and phosphate-solubilizing characteristics. These results indicate that both strains are recommendable candidates as potential PGPB.

Table 1. Plant Growth Promoting Compounds Produced from A4 and A8

Strain	IAA production		Nitrogen fixing ability		Phosphate-solubilizing characteristics	
	Final color	Result	Halo zone	Result	Growth	Result
A4	Red	+	1cm	+	Present	+
A8	Brownish red	+	1cm	+	Present	+

Note: (+) indicates a positive result

From observations of the IAA analysis, each bacterial strain exhibited a unique capacity to produce IAA. Strain A4 was able to produce IAA with an incubation period of 72 hours. Meanwhile, A8 showed IAA production after only 24 hours of incubation. These durations indicate the periods of maximum IAA production, followed by a gradual decrease thereafter (Wagi & Ahmed, 2019). Based on positive BNF tests, both strains demonstrated the ability to produce nitrogenase enzymes and mediate the conversion process of atmospheric nitrogen (N_2) into ammonium. This characteristic is important for nitrogen fixation by making it readily absorbed by the roots (de Souza et al., 2015).

The positive formation of halo zones resulting from phosphate solubilizing activity showed that both strains were able to produce phosphomonoesterase (PMEase) enzymes. Both strains showed 1-cm halo zone diameter and were thereby considered to have a similar level in terms of efficiency in solubilizing phosphate. As described by Widawati et al. (2022), PMEase is a vital element for solubilizing phosphate. Indeed, these phosphate solubilization properties were concomitant to the significant characteristics of PGPB (Ngalimat et al., 2021).

4.3 *Effect of plant growth treated with test strains*

It was observed that both strains demonstrated an increment in height and number of leaves, with no notable physical changes after eight weeks of treatment. Figure 2 shows the result of the average height and number of leaves from the A4 and A8 treatments, as well as in control plants (C). Data were derived from the mean values of the triplicate test.

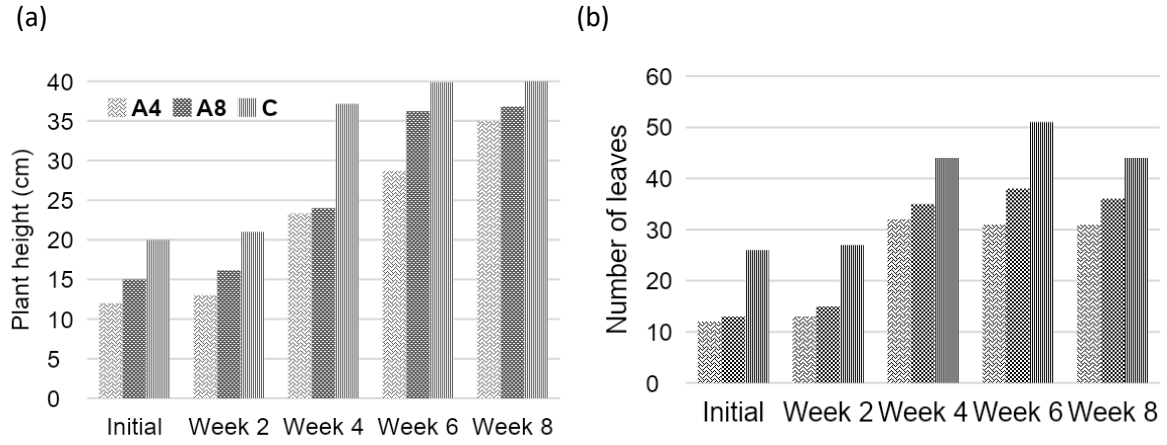


Figure 2: Effect of treatment towards plant growth height (a) and number of leaves production (b)

From the findings, A4 and A8 inoculation to the plant roots have demonstrated impactful results towards chili plant height. Treated plants exhibited larger increments compared to control (untreated) plants. Strain A4 and A8 treatments resulted in average increases of 62.9 % and 56 % for the height of the plant, respectively. Whereas the untreated control showed only a 47.9 % increment in the height of the plant.

In the pot experiment, the treated Group A4 and A8 showed a higher average (a) ase in number of leaves compared to those of Group C (control). The highest increase in the number of leaves was recorded by Group A4, where it gained 26 leaves on the fourth week of observation. In the eighth week, Group C showed a major decline in the number of leaves. This might be due to the rotting of leaves. Besides, they also exhibited yellowing of leaves and slightly slower growth. This condition is known as chlorosis and can be caused by iron deficiency in plants. Surprisingly, none of the treated plants showed any damage to their leaves.

Results obtained from the plant growth-promoting compound were found in good agreement with the pot experiment results. This suggested that the presence of a test compound was found to support the plant growth. The synthesis of indole-3-acetic acid (IAA), nitrogen fixation, and phosphate solubilization were deemed to contribute to the enhancement of plant development. This result conforms with that of Fu et al. (2015), who suggested that IAA plays a crucial role in leaf morphogenesis and vascular network development. Moreover, increasing phosphate solubility may also increase phosphate uptake and indirectly influence the biological nitrogen fixation ability of microorganisms (Janati et al., 2021).

Conclusion and Recommendations

In conclusion, Strain A4 and A8 have shown positive plant growth-promoting traits. Both strains were found to exhibit the production of IAA, fix nitrogen, and produce a compound to aid in phosphate solubilization. The presence of the test compound was inferred to have a relation

towards the growth enhancement of treated plants compared to control. Strain A4 showed good results with a three-fold increase in plant height, while Strain A8 showed a three-fold gain in leaf production. For further studies, it is highly recommended to evaluate the effects of PGPB as a growth stimulant and its specific roles in boosting photosynthesis. Studies pertaining to the long-term effects of PGPB treatment to confirm its potential to be applied in large-scale agriculture are also strongly encouraged.

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