DETECTION OF INDOLE-3-ACETIC ACID (IAA) PRODUCTION BY ENDOPHYTIC BACTERIA ISOLATED FROM COCOA PLANTS AND THEIR GROWTH PROMOTING EFFECTS ON CHILI SEEDLINGS

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ABSTRACT – *Microorganisms known as endophytes grow and colonize the tissue of plants without causing harm to their hosts. Endophytes and plant interactions are said to be symbiotic because both parties gain benefits from this partnership. The host plant offers the microorganisms protective niches, while these microbes create metabolites that promote nutrient absorption, altering the growth and biomass accumulation of the plant. Cocoa plants have been shown to contain endophytes that may promote the wellbeing of the plants. This research targets on detection of Indole-3-Acetic Acid (IAA) production by previously isolated endophytes from cocoa plants using Salkowski reagent. This study will also look at the effect of applying the isolated endophyte to evaluate endophytic bacterial beneficial effects on their plants hosts either directly or indirectly. The findings of this study will give advanced knowledge on the selected endophytic bacteria's capacity (PA and PD) to boost and encourage plant growth which may be related to their production of indole-3-acetic acid (IAA).*

***Keywords**:* Endophyte, bacteria, plant growth, indole-3-acetic acid, and Salkowski

INTRODUCTION

Malaysia is one of the Asean countries that generally has high consumption of chilies, where it is considered as one of the vital parts of the diet and cuisine. It was reported that the consumption figures may vary from 64.46 kg to 300,000 tonnes per year (Daros, 2019). Malaysia primarily imports fresh chilies from Thailand whereas dried chilies from India and China (Department of Statistics Malaysia, 2021). Although, chilies are produced locally, the high demands by food manufacturers and consumers however exceeds the capacity of the local production. This is primarily due to challenges such as low yields, high agricultural cost as well as problems arise from climate change. Measures that can mitigate these challenges are very much needed to increase the local productions of chilies, hence reduces our reliance on imports.

Endophytic bacteria colonize internal structures of plant host without causing disease or negative effects. They often produce bioactive substances such as plant growth hormones to increase plant growth (Khan *et al*., 2014). Endophytic bacteria create a core of attention due to increasing demand in bringing down the use of chemical fertilizers and pesticides, as effort in promoting environmental protection (Vale *et al*., 2010). In many cases, the symbiosis between plant and endophyte occurs in such a way that the plant protects and feeds the endophyte, while the endophyte, in return, produces plant growth hormones to enhance plant growth. Agricultural-based problems caused by the long-term use of chemical fertilizer products, not only pollutes environment, but also causes an imbalance in the proportions of various nutrients, the destruction of organic matter in the soil and a decrease in the structural integrity and properties of aggregates, leading to soil compaction, salinization and others (Wang *et al*, 2020).

Auxin is a substance that "transmits its effects from one area of the plant to another" and controls various plant development responses,

according to Charles Darwin dated 1880. The name "auxin" was first used by biochemists in the 1930s. This phrase comes from the Greek verb "auxein" which means "to grow" or "to increase". The auxin class of plant hormones, most frequently known as indole-3-acetic (IAA), controls several aspects of plant growth and development. As a result, "auxin" and "IAA" are sometimes used in the same sentence. Despite IAA's significance for plant development, little is known about how the biosynthetic pathways are shaped by natural selection and how IAA has evolved over time (Fu *et al*., 2015).

The most prevalent phytohormone of the auxin class, indole-3-acetic acid (IAA), is essential for plant growth and development processes such fruit formation, cell division, cell expansion, and cell differentiation. IAA homeostasis is crucial for preserving an optimal hormonal balance adequate for typical plant growth and development. However, excessive IAA concentrations may have an inhibitory influence on certain physiological processes in plants. IAA decreases plant development and seed germination when present at high concentrations (Park *et al*., 2015). The ethylene burst that occurs from the inhibition scenario results from aminocyclopropene-1-carboxylic acid synthase (ACC synthase) activity that is stimulated by high amounts of auxin accumulation.

IAA can be made by both plants and microbes, including bacteria and fungi (Limtong & Koowadjanakul, 2012). Recently, there has been an increase in interest in the function of microbial IAA in plant-microbe interactions. The interaction between plants and phytopathogenic bacteria has been extensively studied. These bacteria can impede plant growth by upsetting the auxin balance in plants and result in tumors and galls. Additionally, because IAA influences the gene expression of numerous microbes, numerous studies have demonstrated that it functions as a signalling molecule in microorganisms (Yuan *et al*., 2008).

The present study will address research findings to evaluate endophytic bacteria beneficial effects on their plants hosts either directly or indirectly. This finding of this study will give advanced knowledge on endophytic bacteria's capacity to boots and encourage plant growth which may be related to their production of indole-3-acetic acid (IAA).

MATERIALS AND METHODS

Culture preparation.

Nutrient broth (Oxoid, USA) was used as the basic culture medium for the cultivation of the endophytic bacterial isolates PA and PD respectively. An amount of 1.3 g of the nutrient broth formulated powder was dissolved in 100 mL of distilled water by heating and stirring of the mixture until all traces of the formulated powder is homogenized. When needed, 15 mg of tryptophan was added to the mixture to obtain a percentage of 15% (w/v). The broth medium was then sterilized by autoclave at 121°C at 15 psi for 15 minutes. Subsequently, the sterilized medium was allowed to cool down to room temperature before inoculation with desired bacterial isolates.

Salkowski reagent preparation.

The Salkowski reagent was prepared by dissolving 0.811 g of anhydrous $FeCl_3$ in 10 mL of distilled water to obtain a 0.5 M solution. Subsequently, 1 mL of the 0.5 M $FeCl_3$ solution was added to 49 ml of 35% perchloric acid. The solution was thoroughly mixed and stored in a dark brown bottle at room temperature.

Indole quantification using Salkowski Reagent

A volume of 1.5 ml of the sample culture broth was taken out and put in a microcentrifuge tube after 24 hours. A microcentrifuge tube was used to centrifuge the sample at 16,278 g for 5 min. As much as 1 ml of supernatant was carefully transferred to a fresh test tube. Equal volume of Salkowski reagent consisting of 0.5 M ferric chloride ($FeCl_3$) and 35% perchloric acid ($HClO_4$), was added to the supernatant and mixed by gentle vortex. Subsequently, the mixture ~~will be~~ allowed to react at 30°C for 30 minutes in the dark. Next, the color intensity of the mixture was analyzed using 536 nm. Salkowski reagent was used as a blank for this analysis.

To determine the concentration of IAA produced, a standard IAA calibration plot with a range 10-100 $g.ml^{-1}$ was compared to the optical density of the test sample. The procedure was repeated at regular intervals until the optical density showed trends of decline which would indicate that no more IAA was being produced.

Endophytic bacteria as a trigger factor for growth of seeds

Endophytic bacteria were applied to seedlings by soaking the chili seeds for 2 hours before planting. The chili seeds that have been treated with endophytic bacteria were planted in a mixture of soil media, sand, and manure with a ratio of 2:1:1. When required, spraying a suspension of endophytic bacteria (10^8-10^9 CFU mL^{-1}) when the seedlings were 6 weeks after planting was performed until the bacterial suspension dripped off the leaves of the chili seedlings. Observations were made on the parameters of growth of chili seedlings such as plant height and the number of leaves after 10 weeks post treatment.

RESULTS AND DISCUSSIONS

Endophytic bacteria PA and PD which were originally isolated from cocoa plants showed promising results on IAA production. The lowest IAA production by isolate PA was 4.417 µg/ml when supplemented with 10 µg/ml L-tryptophan while the highest IAA production by isolate PA was 92.958 µg/ml when supplemented with 100 µg/ml L-tryptophan (Table 1). On the other hand, the lowest IAA production by isolate PD was 5.923 µg/ml when supplemented with 10 µg/ml L-tryptophan, while the highest IAA production by isolate PD was 96.438 µg/ml when supplemented with 100 µg/ml L-tryptophan (Table 1). In all cases, the amount of IAA production is directly in correlation to the amount of L-tryptophan supplemented because L-tryptophan plays a role as the key precursor to IAA production particularly in both tryptophan-dependent pathway (Khiangam et al., 2023, Akter, Juraimi and Saud, 2021). In bacteria, the indole-3-pyruvic acid (IPyA) pathway is substancially important for IAA biosynthesis, where the indole-3-pyruvate decarboxylase enzyme encoded by the ipdC gene plays a crucial role in this process by converting IPyA to IAA (Zhang *et al*., 2021).

The effects of submerging the chili seeds in the endophytic bacteria PA and PD respectively when compared to the control, showed significant differences in terms of their heights (Figure 1). The heights of 10-week-old seedlings that have been treated by submersion of seeds in the endophytic bacterial isolates PA and PD were found to be 13.100 ± 0.3162 cm and 14.225 ± 0.4425 cm respectively compared to the control which was 10.002 ± 0.4761 cm. This significant difference in height confirms that the endophytes do exhibit growth promoting effects on the chilli seedlings. When chili seedlings that have been treated by submersion of seeds where further treated by spraying the endophytic bacterial isolates PA and PD after 6 weeks of germination, the heights at 10 weeks were found to be 16.925 ± 0.5852 cm and 18.075 ± 0.5123 cm respectively. This is even more significantly higher than the control 10.002 ± 0.4761 cm. When comparing between PA and PD, it is clear that PD exhibit significantly better growth promoting effects on chili seedlings in terms of the height of plants. This finding is in trend with the findings of research done using soybean seeds, whereby it was found that seeds that were coated with endophytic bacteria *Stenotrophomonas* sp. at 10^6 CFU/mL showed higher rate of germination and higher speed of germination compared to those that were not coated (Kangsopa and Atnaseo, 2022). It was also suggested that IAA produced by the endophyte are responsible for these positive effects.

Unfortunately, the effects of submerging the chili seeds in the endophytic bacteria PA and PD respectively when compared to the control, showed no significant differences in terms of their number of leaves. The number of leaves of the 10-week-old seedlings that have been treated by submersion of seeds in the endophytic bacterial isolates PA and PD were found to be 7.00 ± 0.816 and 7.00 ± 1.414 respectively compared to the control which was 5.75 ± 0.500. Even though the number of leaves seemed to be higher with treatment, but statistically, they are not significantly different. Nevertheless, when chili seedlings that have been treated by seeds submersion where further treated by spraying of the endophytic bacterial isolates PA and PD after 6 weeks of germination, the number of leaves at 10 weeks were found to be 9.50 ±1.291 and 9.25 ± 1.258 respectively. This is significantly higher than the number of leaves for the control group which was 5.75 ± 0.500. Hence, we can safely say that the treatment of chili seedling by spraying of endophytes after successful germinations with PA and PD respectively also showed significant differences when compared to the control group. However, when comparing between PA and PD treatments relatively, in terms of number of leaves of the chili seedling, we found that they are not significantly different with each other (Figure 1). Hence, we can conclude that the growth promoting effects of PA and PD spray treatments are almost equally effective in promoting the growth of the chili seedlings.

Endophytes have been known to play a crucial role in plant growth promotion by both directly and indirectly influencing plant development. They can improve nutrient uptake, produce plant hormones, and enhance plant tolerance to biotic and abiotic stresses, ultimately leading to increased growth and yield (Solanki *et al*., 2023; Ali *et al.*, 2024). These results are in consistence with other studies which has been shown to prove that some endophytes have the ability to promote plant growth by producing plant growth hormone such as IAA (Etesami, Alikhani and Hosseini, 2015; Adeleke, Babalola, and Glick, 2021; Burragoni and Jeon, 2021).

Table 1: IAA production capability test on PA and PD strain

ISOLATE PA WITH VARIOUS CONC. OF TRYPTOPHAN (ug/ml)	Control	10	20	30	40	50	60	70	80	90	100
ABSORBANCE (536nm)	0.062	0.106	0.312	0.587	0.726	1.010	1.273	1.435	1.754	2.068	2.231
IAA CONCENTRATION (µg/ml)	2.583	4.417	13	24.458	30.25	42.083	53.042	59.792	73.083	86.167	92.958
ISOLATES PD WITH VARIOUS CONC. OF TTRYPTOPHAN (ug/ml)	Control	10	20	30	40	50	60	70	80	90	100
ABSORBANCE (536 nm)	0.052	0.138	0.354	0.573	0.845	1.196	1.379	1.542	1.739	1.981	2.247
IAA CONCENTRATION (µg/ml)	2.232	5.923	15.193	24.592	36.266	51.33	59.185	66.18	74.635	85.021	96.438

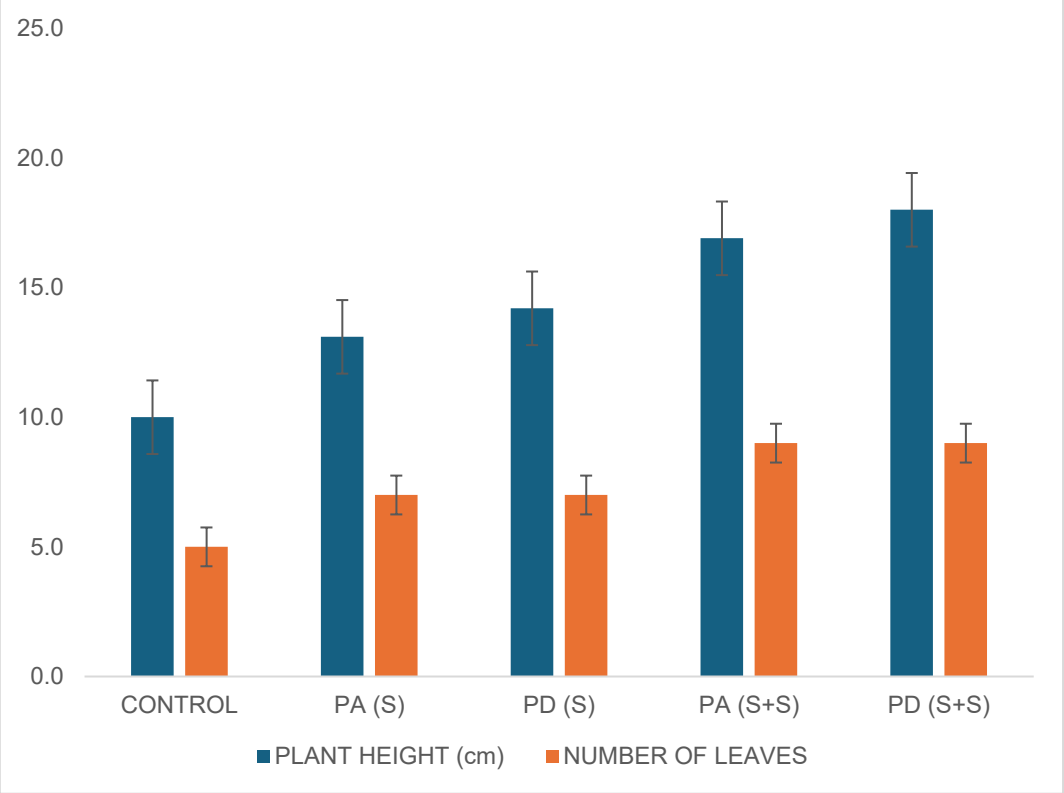

Figure 1: Differences of Plant Height and Number of Leaves; PA isolates seed submersion (S), PD isolates seed submersion (S), PA isolates seed submersion and spray (S+S) and PD isolates seed submersion and spray (S+S)

CONCLUSIONS

In conclusion, this study discovered that previously isolated endophytic bacteria can produce IAA and that seed immersion treatment with endophytic bacterial isolate suspension has a positive impact on the growth and development of chili plants. The findings of this study show that endophytic bacteria can improve phytoremediation and plant growth by producing indole-3-acetic acid (IAA). As a suggestion, this study could be improved by including more endophyte isolate samples for screening and quantification to determine the ability of isolated endophytic bacteria to produce IAA.

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REFERENCES

Adeleke B.S., Babalola O.O., and Glick B.R. (2021). Plant growth-promoting root-colonizing bacterial endophytes. *Rhizosphere*: 100433.

Akter S., Juraimi A.S. and Saud H.M. (2021). Production and optimization of indole acetic acid by Pseudomonas spp. Isolated from rice phyllosphere. *Bangladesh Phytopathological Society* **37(1)**: 1-9.

Ali M.A., Ahmed T., Ibrahim E., Rizwan M. Chong K.P. and Yong J.W.H. (2024). A review on mechanisms and prospects of endophytic bacteria in biocontrol of plant pathogenic fungi and their plant growth-promoting activities. *Heliyon* **10(11)**: e31573.

Burragoni S.G. and Jeon J. (2021). Applications of endophytic microbes in agriculture, biotechnology, medicine, and beyond. *Microbiological Research* **245**: 126691.

Daros, R. (2019). "Grow your own food." *The Malaysian Reserve.* https://themalaysianreserve.com/2019/08/15/grow-your-own-food/

Department of Statistics Malaysia. (2021). *Supply and utilization accounts selected agricultural commodities Malaysia 2016-2020.* DOSM. https://dev.dosm.gov.my/portal-main/release-content/supply-and-utilizationaccounts-selected-agricultural-commoditiesmalaysia-2016-2020.

Etesami H., Alikhani H.A., and Hosseini H.M. (2015). Indole-3-acetic acid (IAA) production trait, a useful screening to select endophytic and rhizosphere competent bacteria for rice growth promoting agents. *MethodsX*: 72-78.

Kangsopa J. and Atnaseo C. (2022). Seed coating application of endophytic and rhizosphere bacteria for germination enhancement and seedling growth promotion in soybeans. *International Journal of Agricultural Technology* **18(1)**: 215 – 230.

Khan, A.L., Waqas, M., Kang, S.M., Al-Harrasi, A., Hussain, J., Al-Rawahi, A., Al-Khiziri, S., Ullah, I., Ali, L., Jung, H.Y. and Lee, I.J. (2014). Bacterial endophyte *Sphingomonas* sp. LK11 produces gibberellins and IAA and promotes tomato plant growth. *Journal of Microbiology* **52 (8)**: 689-695.

Khiangam S., Meetum P. Chaingmai P.N. and Tanasupawat S. (2023). Identification and Optimisation of Indole-3-Acetic Acid Production of Endophytic Bacteria and Their Effects on Plant Growth. *Tropical Life Sciences Research* **34(1)**: 219-239.

Solanki M. K., Solanki A.C., Singh A., Kashyap B.K., Rai S., and Malviya M.K. (2023). Chapter 1 - Microbial endophytes' association and application in plant health: an overview. In M.K. Solanki, M.K. Yadaf, B.P. Singh and V.K. Gupta (Eds) *Microbial Endophytes and Plant Growth: Beneficial Interactions and Applications.* pp: 3-25.

Vale, M., Seldin, L., Araujo, F.F. and Lima, R. (2010). Plant growth promoting rhizobacteria: fundamentals and applications. *In: Maheswari D.K. (ed). Plant growth and health promoting bacteria. Springer, Berlin*, 21-43.

Wang, J., Li, R., Zhang, H., Wei, G., and Li, Z. (2020). Beneficial bacteria nutrient and promote wheat growth under conditions of reduced fertilizer application. *BMC Microbiology* **20(30)**: 1-12.

Zhang, B.X., Li P.S., Wang Y.Y., Wang J.J., Liu X.L., Wang X.Y. and Hu X.M. (2021). Characterization and synthesis of indole-3-acetic acid in plant growth promoting *Enterobacter* sp.*RSV Advances*, **11**: 31601-31607.