



IAA (Indole-3-Acetic Acid) Production by ACC Deaminase-Producing Rhizobacteria Isolated from Peanut Rhizosphere

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Abstract: This study aimed to evaluate the indole-3-acetic acid (IAA)-producing capacity of rhizobacterial isolates obtained from the rhizosphere of peanut (*Arachis hypogaea* L.) and previously identified as ACC deaminase-producing bacteria under *in vitro* conditions. IAA production was assessed qualitatively and quantitatively. Qualitative detection was performed using the colorimetric Salkowski reagent assay, whereas quantitative determination was conducted spectrophotometrically by comparing sample absorbance values with an IAA standard curve. The results showed that all six rhizobacterial isolates, together with *Escherichia coli* used as a reference strain, exhibited IAA-producing activity, although the estimated IAA concentrations varied among isolates and incubation periods. Among the tested rhizobacteria, isolate I9 exhibited the highest IAA concentration, reaching 34.75 ppm after three days of incubation, and showed an overall increasing production trend during the incubation period. These findings demonstrate that the ACC deaminase-positive rhizobacterial isolates examined in this study also possess IAA-producing capacity, although their production profiles differ among isolates. The combined potential for IAA production and ACC deaminase activity suggests that these isolates warrant further evaluation as plant growth-promoting rhizobacteria (PGPR) for improving plant growth and development.

Keywords: ACC deaminase; IAA production dynamics; peanut; PGPR; rhizobacteria

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INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are root-associated bacteria that promote plant growth and productivity through several direct and indirect mechanisms, including nutrient acquisition and phytohormone production. These bacteria have been extensively studied as potential biological agents for improving crop growth and productivity. Their beneficial effects may involve diverse microbial populations rather than a single bacterial species, allowing several complementary mechanisms to contribute to plant growth and health (Wu et al., 2009). Direct plant growth-promoting mechanisms of PGPR include nitrogen fixation, phosphate and potassium solubilization, iron acquisition, indole-3-acetic acid (IAA) production, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Ratnaningsih et al., 2023). Several bacterial genera, including *Bacillus* sp. (Bal et al., 2013), *Azospirillum* sp., and *Azotobacter* sp. (Bhattacharyya & Jha, 2012), have been reported to produce IAA and possess ACC deaminase activity. These functional traits contribute to the potential role of PGPR in supporting plant growth, particularly under unfavorable environmental conditions.

Among the phytohormones produced by PGPR, IAA is particularly important because of its role in regulating root development, cell elongation, and other plant growth processes. Although plants synthesize IAA endogenously, IAA-producing rhizobacteria may provide an additional source of auxin in the rhizosphere and thereby influence plant development. Characterizing the IAA-producing capacity of rhizobacteria is therefore important for evaluating their potential contribution to plant

growth promotion. Bacterial IAA production is commonly associated with the availability of root-derived compounds, particularly tryptophan, which can serve as a precursor for bacterial IAA biosynthesis (Astriani & Murtiyaningsih, 2018). Most PGPR isolated from the rhizosphere have been reported to produce IAA (Patten & Glick, 1996).

The physiological effects of bacterial IAA are also closely related to ethylene metabolism in plants. Increased auxin levels can stimulate the synthesis of ACC, the immediate precursor of ethylene. Excessive ethylene accumulation, particularly under stressful conditions, may restrict plant growth and accelerate senescence. In this context, rhizobacteria possessing ACC deaminase activity can degrade ACC and thereby reduce the availability of the substrate required for ethylene biosynthesis. Consequently, rhizobacteria that both produce IAA and possess ACC deaminase activity may provide complementary functions, with IAA potentially supporting root development and ACC deaminase contributing to the regulation of stress-associated ethylene production (Zerrouk et al., 2019).

Despite the potential importance of this functional combination, information on indigenous rhizobacteria possessing both traits remains limited, particularly for isolates associated with peanut (*Arachis hypogaea* L.) rhizospheres in Indonesia. Previous studies on rhizobacterial isolates have frequently examined IAA production as an individual plant growth-promoting trait, whereas less attention has been given to the IAA-producing capacity of isolates previously characterized as ACC deaminase-positive. In addition, relatively little information is available on how IAA production by such isolates changes during incubation and whether these temporal patterns differ among isolates. This information is important for identifying isolates with promising physiological characteristics for further evaluation as PGPR.

Accordingly, this study hypothesized that ACC deaminase-positive rhizobacterial isolates from the peanut rhizosphere are also capable of producing IAA, although their production capacities and temporal patterns may differ among isolates. Therefore, this study aimed to qualitatively and quantitatively evaluate the IAA-producing capacity of selected rhizobacterial isolates previously characterized as ACC deaminase-positive and to describe variations in IAA production among isolates over the incubation period.

METHOD

Experimental Design

This study employed an exploratory experimental approach to screen six bacterial isolates previously identified as ACC deaminase-positive for their ability to produce indole-3-acetic acid (IAA). Qualitative and quantitative assessments of IAA production were conducted from April to November 2024. The IAA assays were performed as preliminary screening experiments without replication; therefore, the resulting quantitative data were interpreted descriptively. Before inoculation, bacterial cultures were standardized using a McFarland standard to reduce variation in the initial cell density among isolates.

Origin of the Bacterial Isolates

The bacterial isolates were originally obtained from the peanut rhizosphere and maintained in the culture collection of the Agricultural Microbiology Laboratory, Faculty of Agriculture, University of Mataram, Indonesia. In addition, *Escherichia coli* was included as a reference strain. The *E. coli* strain was obtained from the culture collection of the Biology Laboratory, Faculty of Education, University of Mataram. Before the IAA assays, all bacterial isolates were purified and maintained at 4 °C until further analysis to preserve culture viability.

Rejuvenation, Bacterial Culture Preparation, and Qualitative IAA Assay

Bacterial isolates were rejuvenated by streaking onto Nutrient Agar (NA) and incubating overnight at 28 °C to obtain fresh, pure cultures. A single colony of each isolate was subsequently transferred into Nutrient Broth (NB) supplemented with 1 mM L-tryptophan and incubated at 28 °C for 24 h. For comparison, a parallel culture of each isolate was prepared in NB without L-tryptophan. After incubation, the bacterial cultures were centrifuged, and the supernatants were collected for qualitative detection of IAA.

For the qualitative assay, 2 mL of Salkowski reagent containing FeCl_3 and HClO_4 was added to 1 mL of culture supernatant. The mixture was then incubated in the dark at room temperature for 30 min. The development of a pink to reddish-pink color was interpreted as a positive reaction indicating the presence of IAA or other Salkowski-reactive indolic compounds (Gordon & Weber, 1951).

Quantitative Measurement of IAA Production

For quantitative analysis, 0.5 mL of each bacterial suspension was standardized to a 0.5 McFarland standard, corresponding to approximately 1.5×10^8 CFU/mL (Rosmania & Yanti, 2020), and subsequently inoculated into 30 mL of NB supplemented with 1 mM L-tryptophan. The culture sampled immediately after inoculation was designated as day 0. Separate cultures designated for days 1, 2, and 3 were incubated in a shaker incubator at 150 rpm for 24, 48, and 72 h, respectively.

After the designated incubation period, the bacterial cultures were centrifuged at $10,000 \times g$ for 10 min at 4 °C, and the resulting supernatants were transferred to clean test tubes and reacted with Salkowski reagent. IAA-associated color development was quantified by measuring the absorbance at 530 nm using a spectrophotometer. The absorbance values were subsequently compared with a standard curve prepared from pure IAA at concentrations ranging from 0 to 30 ppm to estimate the IAA concentration in each bacterial culture supernatant (Patten & Glick, 1996).

Preparation of the IAA Standard Curve

A standard curve was prepared using a series of pure IAA solutions derived from an IAA stock solution reported as 0.01 g L^{-1} (1000 ppm). The stock solution was diluted to obtain IAA standards ranging from 0 to 30 ppm at 5 ppm intervals. For each standard concentration, 0.5 mL of IAA solution was mixed with 1 mL of Salkowski reagent and incubated in the dark for 30 min until stable color development was achieved. Absorbance was then measured at 530 nm using a UV-Vis spectrophotometer. The resulting absorbance values were used to construct the calibration curve for estimating IAA concentrations in the bacterial culture supernatants.

Data Collection and Processing

Photographic data were collected using a mobile-phone camera to document bacterial colony morphology and color development during the qualitative IAA assay. Quantitative data, including absorbance values and estimated IAA concentrations, were tabulated and graphically presented using Microsoft Excel, while Microsoft Word was used for manuscript preparation and data presentation.

RESULTS AND DISCUSSION

Purification of IAA-Producing Bacteria

Figure 1 shows the results of the purification of previously selected bacterial isolates known to produce ACC deaminase, as reported by Jaya et al. (2024). Bacterial colonies grown in a single Petri dish appeared to have uniform morphology and were free of contamination. The six isolates were coded as I1, I3, I6, I7, I8, and I9.

Purification of IAA-producing bacteria from the culture collection was intended to ensure the purity of the isolates for use in the rejuvenation process on new media. This purification process was repeated several times to obtain a truly pure isolate. This step was necessary before subsequent IAA production screening. This also minimized the potential contamination by other microorganisms during IAA production, allowing different bacterial isolates to be more reliably assessed for their inherent characteristics.

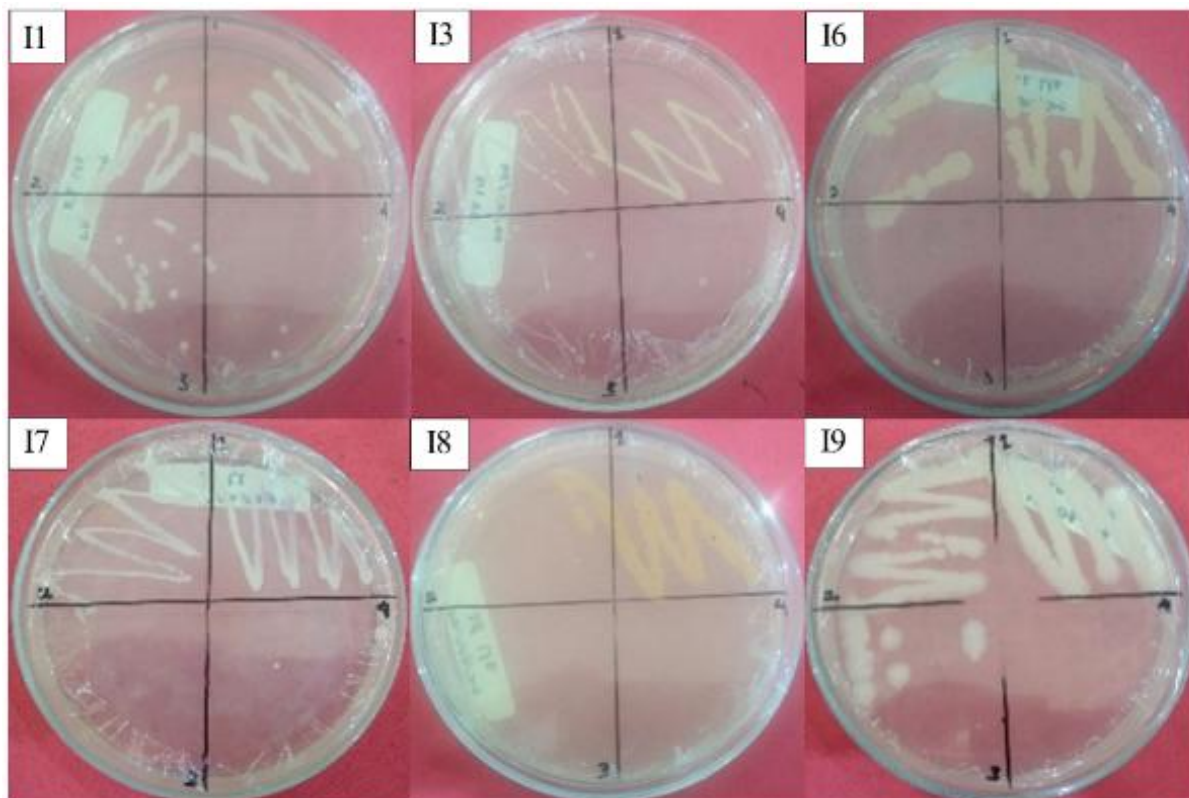


Figure 1. Purified isolates of ACC deaminase-producing bacteria. I1, I3, I6, I7, I8, and I9 are isolate codes.

Qualitative Characteristics of IAA-Producing Bacteria

Qualitative assay for the six rejuvenated bacterial isolates showed a colour change from the typical clear gold colour of NB medium to pink, indicating the presence of IAA produced and released by the bacteria into the medium (Figure 2). Qualitative tests were conducted on bacterial isolates to determine whether they produced IAA. The colour change that occurred in the NB medium indicated a reaction between IAA and the Fe contained in the Salkowski reagent to form the $\text{Fe}_2(\text{OH})_2(\text{Al})_4$ complex. There are two types of reactions: complex and redox (Gang et al., 2019). The intensity of the colour change reflects differences in the amount of IAA or other reactive indolic compounds present in the medium. This qualitative indicator provides preliminary evidence of IAA-producing potential among the isolates and supports their further evaluation on a quantitative assay. However, because the Salkowski reagent is not completely specific to IAA, the colour development should be interpreted as an indication of IAA or IAA-like indolic compounds rather than as definitive confirmation of IAA production.

The addition of L-tryptophan to the growth medium is an important factor for bacterial isolates to produce IAA. IAA synthesis by bacteria in the presence of L-

tryptophan indicates that L-tryptophan can serve as a precursor for IAA synthesis. Tryptophan is used physiologically in IAA synthesis in plants and in microorganisms, including bacteria (Li et al., 2018; Nonhebel, 2015). According to Karnwal (2009), IAA production by rhizobacteria is associated with the availability of the amino acid in plant roots, particularly when the precursors are sufficiently high. This phenomenon naturally occurs when plant roots secrete exudates into the rhizosphere, which are then consumed by rhizobacteria to synthesize IAA (Kravchenko et al., 2004). Rhizobacteria have been widely reported to produce various phytohormones, including IAA. IAA is an auxin naturally synthesized by plant tissue, influencing plant growth rate, cell elongation, and cell differentiation (Advinda et al., 2018; Roopa et al., 2023).

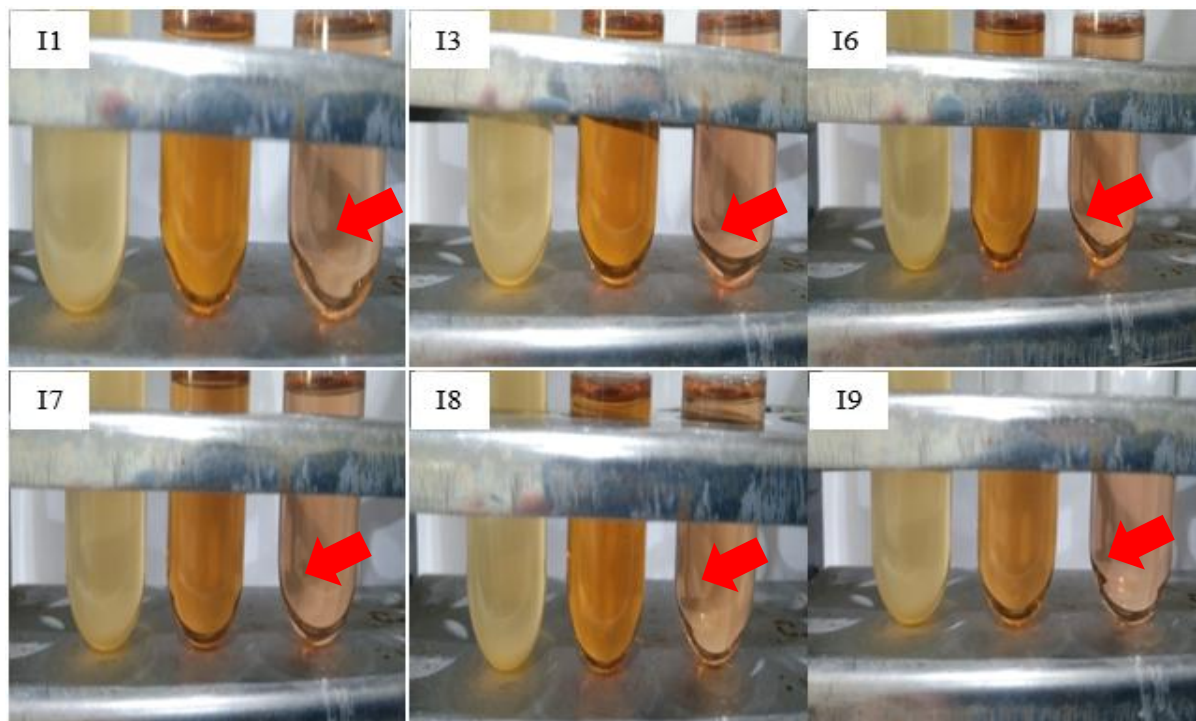


Figure 2. Qualitative test results of IAA-producing bacterial isolates on NB media. Supernatant without Salkowski (left), supernatant without the addition of L-tryptophan with Salkowski (centre), and supernatant with the addition of L-tryptophan and Salkowski (pink to reddish pink indicating IAA activity).

Quantitative Characteristics of IAA-Producing Bacteria

The population density of bacterial isolates was measured and standardized using the McFarland method (McFarland, 1907) before assessing their ability to produce IAA. The absorbance of bacterial cultures in NB medium was measured and compared with the McFarland standard to obtain a bacterial population of 1.5×10^8 CFU/mL. The absorbance values of the six bacterial isolates were relatively similar, ranging from 0.89 to 1.08, as shown in Table 1.

The quantitative IAA assay showed that *E. coli*, the reference strain, also produced IAA, but with a different pattern and at a lower concentration than the other bacterial isolates. IAA concentrations were determined from absorbance measurements of the supernatant and compared with the pure IAA levels in the standard curve (Figure 3). The IAA concentrations in Table 1 show that all seven isolates produced IAA at 24 hours of age, varying from day 0, when the culture reached 10^8 CFU/mL. The IAA concentration in independent cultures was measured at 0, 24, 48, and 72 hours after incubation to support the continued proliferation of the bacterial

cultures under controlled conditions. However, *E. coli*, as a reference strain, also produced IAA, with the lowest value of 2.62 ppm and the highest on day 2 at 25.82 ppm, then decreasing on day 3 (Guo et al., 2019). Although IAA biosynthesis in bacteria is widely distributed across the strains, including *E. coli*, the complexity of biosynthetic pathways varies among taxa, particularly in rhizobacteria. They appear to have a stronger capability for IAA production than bacteria from non-rhizosphere environments. (Zhang et al., 2019). The other six bacterial isolates had the highest IAA concentration, namely, isolate I3 at 26.77 ppm, and the lowest, isolate I7, at 20.58 ppm. On the first day, the highest value was observed for isolate I9 at 30.02 ppm, and the lowest for isolate I6 at 24.06 ppm. On day 2, the highest value was obtained in isolate I9 at 30.00 ppm and the lowest in isolate I6 at 21.05 ppm. Meanwhile, on day 3, the highest value was obtained in isolate I9 at 34.75 ppm and the lowest in isolate I3 at 25.41 ppm.

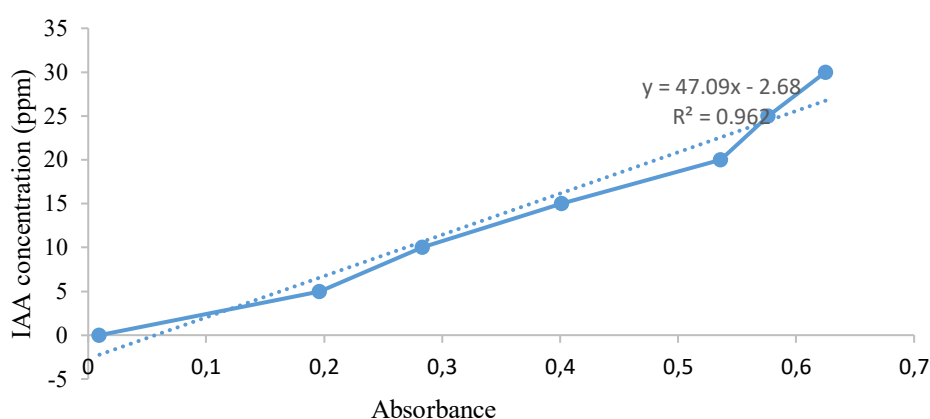


Figure 3. Standard curve of pure IAA

Table 1. Absorbance, population, and IAA concentration of the bacterial isolates

Isolate	Absorbance ($\lambda=600$ nm)	Population (CFU/ml)	IAA concentration (ppm) on day							
			0		1		2		3	
			Abs.	Conc.	Abs.	Conc.	Abs.	Conc.	Abs.	Conc.
I1	1.00	1.5×10^8	0.56	23.62	0.66	28.38	0.62	26.49	0.62	26.62
I3	0.95	1.5×10^8	0.64	26.77	0.64	27.46	0.54	22.85	0.60	25.41
I6	1.04	1.5×10^8	0.52	21.68	0.57	24.06	0.50	21.05	0.62	26.74
I7	1.08	1.5×10^8	0.49	20.58	0.60	25.76	0.58	24.46	0.72	31.35
I8	0.95	1.5×10^8	0.54	22.61	0.61	25.96	0.66	28.19	0.60	25.70
I9	1.05	1.5×10^8	0.55	23.43	0.69	30.02	0.69	30.00	0.79	34.75
<i>E. coli</i>	1.00	1.5×10^8	0.11	2.62	0.61	13.31	0.61	25.82	0.42	17.06

Abs: absorbance; Conc: Concentration

In general, IAA production from the six isolates other than *E. coli* was already visible on day 0, ranging from 20.58 to 26.77 ppm, and then increased gradually on day 1 to 24.06-30.02 ppm. In addition, on day 2, IAA production decreased slightly but not significantly, ranging from 21.05 to 30.00 ppm, followed by a further increase on day 3 to 25.41-34.75 ppm. Based on the increase in IAA levels over 72 hours of incubation, isolate I9 shows the greatest potential for synthesising the phytohormone IAA (Figure 4).

IAA production varied across incubation period. I9 consistently showed relatively high IAA production. I9 reached the highest recorded value after three days of incubation. This pattern may be associated with strain-specific differences in IAA biosynthetic activity and bacterial growth dynamics (Melo et al., 2016). The peak production at day 3 may signify efficient conversion of tryptophan into IAA during the

late exponential or early stationary phase. This is consistent with research by Wahyudi et al. (2011) and Zahroya et al. (2020), which reported that secondary metabolites such as IAA can be produced at their highest levels during the stationary phase. Similar variation in the period of maximum production has been reported among isolates (Lebrazi et al., 2020). In contrast, the distinctive pattern observed in *E. coli* may reflect differences in its metabolic response to the culture conditions compared with indigenous plant-associated isolates, indicating that the bacterium is not a rhizobacterial type commonly involved in plant growth promotion.

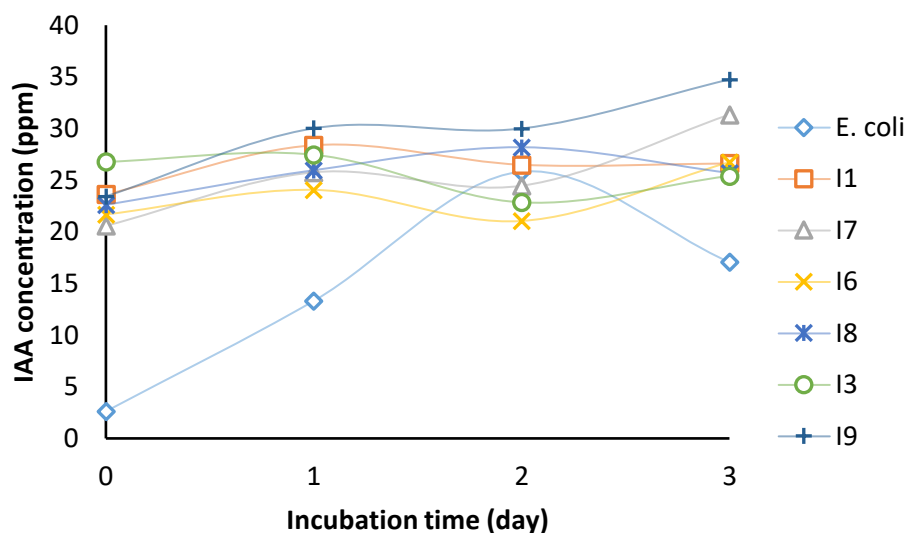


Figure 4. IAA production trend of bacterial isolates and comparison with each other

IAA-producing bacteria play a crucial role in supporting plant growth by stimulating cell division and root and stem elongation, thereby potentially developing root surface area for nutrient and water absorption. In this study, although I9 showed the highest production on day 3, elevated bacterial IAA may also induce ACC and ethylene signalling in plants when auxin levels are excessive. In these situations, rhizobacteria possessing ACC deaminase activity promote plant development by degrading ACC, thereby reducing ethylene accumulation. By this mechanism, IAA and ACC deaminase-producing rhizobacteria could help maintain root growth and enhance plant tolerance to abiotic stresses.

Although plants can produce IAA endogenously, exogenous IAA production by rhizobacteria may promote plant growth. The concentration of IAA produced by bacterial isolates varies with species and several growth factors, including tryptophan availability in the medium, pH, and the isolate's optimum growth conditions (Rini et al., 2020). Based on the research results of Dewi et al. (2016), bacterial isolates that produce IAA with I9 or higher are considered the most promising for this purpose. The application of bacterial isolates to produce IAA as a biofertilizer, or their use to improve soil quality and fertility, is very important for supporting sustainable agricultural practices.

This study has a few limitations. As the quantitative IAA screening was conducted without replicates, the IAA assay values should be interpreted as preliminary results rather than statistically validated determinations. Incorporating independent replications to examine experimental variability and robustness of the values should be addressed in future studies. In addition, the calibration range of the

standard curve for pure IAA is limited, necessitating an extended range for future studies; the resulting IAA levels should be interpreted with caution.

CONCLUSION

All six rhizobacterial isolates obtained from the peanut rhizosphere showed the ability to produce IAA, indicating their potential as plant growth-promoting rhizobacteria (PGPR). Although IAA production varied among the isolates, isolate I9 exhibited the highest IAA-producing capacity and showed an increasing production trend over the incubation period. These results suggest that isolate I9 is a promising candidate for further evaluation as a PGPR.

The combined occurrence of IAA production and previously identified ACC deaminase activity may contribute to plant growth promotion and improved tolerance to environmental stress. However, because these effects were not directly evaluated in the present study, further research is required to validate the plant growth-promoting performance of the isolates under controlled conditions, including glasshouse pot experiments.

RECOMMENDATION

Based on the results of this study, further evaluation is required in the glasshouse and in field experiments to ensure the effectiveness of isolate I9. The need for molecular identification of the isolate is also important, as it is a potential candidate for biofertilizer formulation, interaction and hypersensitivity tests with plants under abiotic stress, and evaluation of the impact of isolate application on soil health and hypersensitivity.

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