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Screening and Optimization of Indole-3-Acetic Acid Production by Cowpea (*Vigna unguiculata* L. Walp.) Rhizobia Isolates

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Abstract

The production of indole-3-acetic acid (IAA) is a key trait for rhizobacteria to improve plant growth. For this, 14 rhizobia isolated from root nodules of *Vigna unguiculata* were first screened for their ability to produce IAA. Then, purified IAA samples were compared to standard IAA using thin-layer chromatography (TLC). The four best-producing isolates were selected for optimization of IAA production under different cultural conditions. Results showed that all of tested isolates were capable of producing IAA, with the highest concentration of 59.93 µg/ml recorded for VuChEl3 isolate, followed by VuJaEl3, VuJaEl14 and VuPlEl2 isolates recording concentrations of 29.1 µg/ml, 22.3 µg/ml and 12.4 µg/ml respectively. TLC analysis confirmed that IAA matched authentic IAA, showing the same retention factor (R_f) value of 0.9. Mannitol and yeast extract were found to be the best carbon and nitrogen sources for IAA production. An incubation temperature of 30˚C, a pH of 6.5, an incubation time of 72 h, and respective tryptophan (Trp) and NaCl concentrations of 1 mg/ml and 0% were found to be optimal parameters resulting in a maximum IAA yield of 118.43 µg/ml.

Keywords

Indole-3-Acetic Acid, Rhizobia, *Vigna unguiculata*, Optimization, Tryptophan

1. Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is the most important and traditionally cultivated grain legumes, often grown in association with other food crops in tropical regions [1]. Its cultivation also contributes to soil fertility restoration due to its strong potential for biological nitrogen fixation in symbiosis with rhizobia. These

bacteria transform atmospheric nitrogen into ammoniacal and subsequently into organic form of nitrogen [2]. Beyond nitrogen fixation, rhizobia also play a crucial role in maintaining and regenerating soil fertility and improving plant productivity, through their ability to solubilize inorganic phosphorus and synthesize growth-promoting substances such as indole-3-acetic acid (IAA), one of the most physiologically active auxins.

IAA is the most common natural auxin in plants. IAA is synthesized from indole, and there is evidence that plants can produce it directly from tryptophan [3], with root exudates being the main tryptophan resource in the soil [4]. Although rhizobia are known for their ability to fix nitrogen, they are also reported to synthesize IAA under *in vitro* conditions [5]. IAA acts as an important signal molecule in the regulation of plant development [4]. This phytohormone is involved in trophic responses, differentiation, gene regulation [6] and the initiation and elongation of cell division in roots. Rhizobia strains secrete IAA, which plays a significant role in plant growth and nodule formation and development [7]. In legumes such as cowpea, IAA plays a role in root nodule formation and seed germination [8].

However, IAA biosynthesis is affected by several environmental factors. Its production increases under high pH conditions and in the presence of greater quantities of tryptophan [4]. On the other hand, factors such as acidification, osmotic stress and carbon source limitation can negatively affect IAA biosynthesis.

The aim of this study is to improve IAA production. First, indigenous cowpea rhizobia isolate capable of producing IAA were screened. Secondly, IAA was purified, and optimization experiments were conducted to determine physicochemical conditions that maximize IAA production.

2. Material and Methods

2.1. Isolation of Cowpea Rhizobia from Nodules

Nodules were harvested, according to the method of Vincent [9], from the roots of *Vigna unguiculata* in Elogbatindi in the southern region of Cameroon. The nodules were immersed in 95% (v/v) ethanol for 30 seconds, then transferred to a mercury chloride ($HgCl_2$) solution 0.1% for 1 minute. They were rinsed five times using sterile distilled water under aseptic conditions. The nodules were crushed in a drop of sterile distilled water, and the slurry was streaked on surface of culture medium of yeast extract mannitol agar (YEMA) medium supplemented with Congo Red (0.025 g/l) stain. Plates were incubated inverted at 30˚C for 3 - 7 days. The emergence of mucus white colonies on the surface of culture media indicated the presence *Rhizobium* colonies.

2.2. Screening of Isolates for IAA Production

Fourteen cowpea rhizobia isolates were tested for their ability to produce IAA. IAA levels in each isolate were determined using the colorimetric Salkowski reagent prepared with 50 ml of 35% perchloric acid ($HClO_4$) and 1 ml of 0.5 M

iron (III) chloride ($FeCl_3$) [10]. Isolates were cultured in 100 ml YEM medium supplemented with 0.1 mg/ml of L-tryptophan, at 28˚C ± 2˚C for 3 days at a shaking speed of 150 rpm in an incubator. Afterward, cultures were centrifuged at 10,000 rpm for 10 minutes at 4˚C, and the supernatant was mixed with Salkowski reagent (1:2) and two drops of orthophosphoric acid. After 30 minutes in the dark at 28˚C ± 2˚C, absorbance was measured at 530 nm. IAA concentration was calculated using a standard IAA curve. All experiments were performed in triplicate.

2.3. Extraction and Detection of IAA by Thin-Layer Chromatography (TLC)

2.3.1. Extraction of Crude IAA

The isolate with the highest IAA production was selected. A colony was inoculated into 100 ml of YEM medium supplemented with 1 mg/ml L-tryptophan and incubated at 30˚C for 3 days. Extraction was performed after 72 hours [11]. The culture broth was centrifuged at 7000 rpm for 10 minutes. The supernatant was mixed with ethyl acetate (1:2 ratio), stirred and the organic phase was recovered and concentrated by drying on a rotary evaporator at 45˚C. The dried extract was recovered and dissolved in 0.5 ml methanol.

2.3.2. Detection of IAA by Thin-Layer Chromatography

Thin-layer chromatography is used on silica gel plates (GF254, 0.25 mm thickness). The migration system used was a solvent system: propanol:water (8:2). Both the extract and the standard IAA were spotted on the plate. Chromatogram was developed with the Salkowski's reagent [12].

The retention factor (Rf) of the standard IAA and the extract IAA are calculated according to the formula $Rf = di/ds$, where di represents the distance moved by the compound (measured at the center of the spot) and ds is the solvent front distance.

2.4. Optimization of IAA Production

The production of IAA was optimized for four selected isolates: VuChEl3, VuJaEl3, VuJaEl14 and VuPlEl2.

2.4.1. Effect of Carbon Sources

The effect of carbon sources on IAA production was studied by replacing mannitol in medium supplemented with 0.1 mg/ml of L-tryptophan by 8 carbon sources at 1% concentration. The carbon sources used are glucose, fructose, sucrose, maltose, starch, cellulose, glycerol, sorbitol and mannitol as a control. The cultures were incubated at 30˚C in a shaker at 150 rpm for 3 days. IAA production was then measured [10].

2.4.2. Effect of Nitrogen Sources

Five nitrogen sources (glycine, asparagine, casein, ammonium chloride, potassium nitrate) were tested by replacing yeast extract at 0.1% concentration. After 72 h

incubation, IAA production was quantified.

2.4.3. Effect of Different NaCl Concentrations

Rhizobia isolates were tested for their ability to produce IAA at NaCl concentrations ranging from 0 to 10%. Each flask containing 100 ml of YEM-Tryptophan medium and increasing concentrations of NaCl was inoculated with 1 ml of a bacterial culture of each isolate corresponding to a McFarland scale concentration of 10^8 CFU/ml, and the cultures were incubated at 30˚C in a shaking incubator. After 72 h incubation, IAA production is quantified using Salkowski's reagent.

2.4.4. Effect of pH

IAA production was assessed and at pH levels: 6.5, 5.5, 5, 4.5, 4, and 3.5 in YEM medium supplemented with L-tryptophan. Each flask containing 100 ml of YEM-Tryptophan medium adjusted to different pH levels was inoculated with 1 ml of a bacterial culture of each isolate corresponding to a McFarland scale concentration of 10^8 CFU/ml, and the cultures were incubated at 30˚C in a shaking incubator. After 72 hours of incubation, IAA production was quantified using Salkowski's reagent.

2.4.5. Effect of Incubation Time

Erlenmeyer containing 100 ml of YEM-Tryptophan medium was inoculated with 1 ml of a bacterial culture of each isolate (10^8 CFU/ml). Cultures were incubated at 30˚C in a shaking incubator. The concentration of IAA produced was measured at 24 h, 48 h, 72 h, 96 h, 120 h and 144 h of incubation.

2.4.6. Effect of Temperatures

The optimum temperature to produce IAA by cowpea rhizobia isolates was determined by incubating cultures at different temperatures. Erlenmeyer containing 100 ml of YEM-Tryptophan medium is inoculated with 1 ml of a bacterial culture of each isolate (10^8 CFU/ml). Cultures were incubated at 4˚C, 25˚C, 30˚C, 35˚C, 40˚C, 45˚C and 50˚C in a shaking incubator. After 72 hours of incubation, IAA production was determined using Salkowski's reagent.

2.4.7. Effect of L-Tryptophan Concentration

The effect of L-tryptophan concentrations on IAA production was studied using YEM medium supplemented with L-tryptophan at concentrations of 0 mg/ml, 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml, 2 mg/ml, 2.5 mg/ml. Cultures were incubated at 30˚C in a shaker at 150 rpm for 3 days. After 72 hours of incubation, IAA production is determined using Salkowski's reagent.

2.5. Statistical Analysis

Statistical analysis of the data was carried out using GraphPad Prism 5.0 software. Analysis of variance (ANOVA) was performed using the parametric ANOVA procedure to test for differences. The Duncan test was used to assess significant differences between means at the 5% probability threshold.

3. Results

3.1. Isolation of Rhizobia

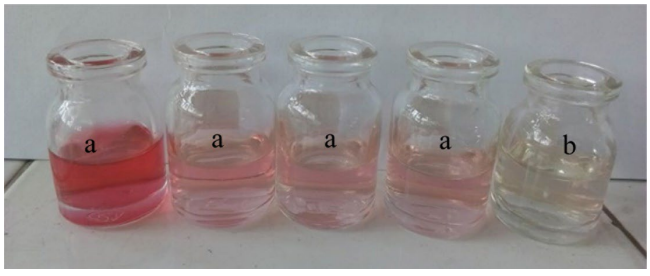
In this study, a total of 14 rhizobia isolates were isolated. All isolates showed light pink on their growth on YEMA Congo red medium. This result indicates that they could not absorb the red color totally. A great diversity in the rhizobial population colonizing the root of *Vigna unguiculata* was also noticed (Table 1).

Table 1. Morphological and biochemical characterization of cowpea rhizobia isolates.

Isolates codes	Growth	Shape	Color	Texture	Mucus	Gram	Bromothymol blue test	Catalase	Oxidase	Exopolysaccharides production
VuChEl3	Fast	Circular	White	Rounded	Present	Negative	Yellow	Positive	Positive	Positive
VuChEl9	Fast	Circular	Creamy	Smooth	Absent	Negative	Yellow	Positive	Positive	Positive
VuPlEl1	Fast	Circular	Beige	Smooth	Absent	Negative	Yellow	Positive	Positive	Positive
VuPlEl2	Fast	Circular	White	Rounded	Present	Negative	Yellow	Positive	Positive	Positive
VuPlEl3	Fast	Circular	White	Rounded	Present	Negative	Yellow	Positive	Positive	Positive
VuPlEl6	Fast	Circular	Creamy	Rounded	Present	Negative	Yellow	Positive	Positive	Positive
VuJaEl1	Fast	Circular	White	Rounded	Present	Negative	Yellow	Positive	Positive	Positive
VuJaEl2	Fast	Circular	Beige	Smooth	Present	Negative	Yellow	Positive	Positive	Positive
VuJaEl3	Fast	Circular	Creamy	Smooth	Present	Negative	Yellow	Positive	Positive	Positive
VuJaEl4	Fast	Circular	White	Smooth	Absent	Negative	Yellow	Positive	Positive	Positive
VuJaEl5	Fast	Circular	Creamy	Rounded	Present	Negative	Yellow	Positive	Positive	Positive
VuJaEl7	Fast	Circular	White	Rounded	Present	Negative	Yellow	Positive	Positive	Positive
VuJaEl13	Fast	Circular	White	Rounded	Present	Negative	Yellow	Positive	Positive	Positive
VuJaEl14	Fast	Circular	White	Rounded	Present	Negative	Yellow	Positive	Positive	Positive

3.2. Screening of Isolates for IAA Production

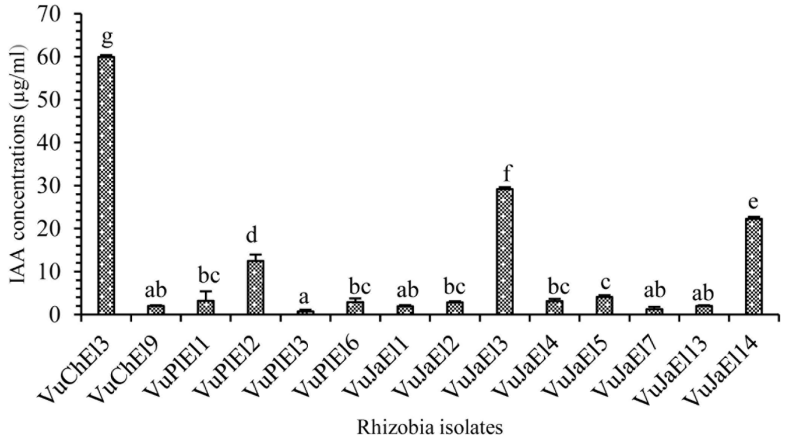
The colorimetric assay in YEM medium supplemented with L-tryptophan showed that all the 14 tested isolates were able to produce IAA at varying concentrations. Pink color development was initially visible at the highest IAA concentration within minutes and continued to intensify over 30 minutes (Figure 1).



Note: a: Tasks that produced IAA; b: Control.

Figure 1. Colorimetric detection of IAA production by cowpea rhizobia isolates using Salkowski's reagent.

Figure 2 shows the concentrations of IAA produced by cowpea rhizobia isolates. IAA production is significantly higher in the VuChEl3 isolate (59.93 µg/ml) and significantly lower in the VuPlEl3 isolate (0.68 µg/ml). The VuJaEl3, VuJaEl14, and VuPlEl2 isolates proved to be good IAA producers, with concentrations of 29.1 µg/ml, 22.3 µg/ml and 12.4 µg/ml, respectively.



Note: Bars with the same letter are not significantly different at $P < 0.05$.

Figure 2. IAA concentrations produced by cowpea rhizobia isolates.

3.3. Detection by Thin Layer Chromatography

IAA produced by isolate VuChEl3 was compared with a standard IAA sample by TLC. A pink spot appeared on the chromatogram (Figure 3) with an Rf value

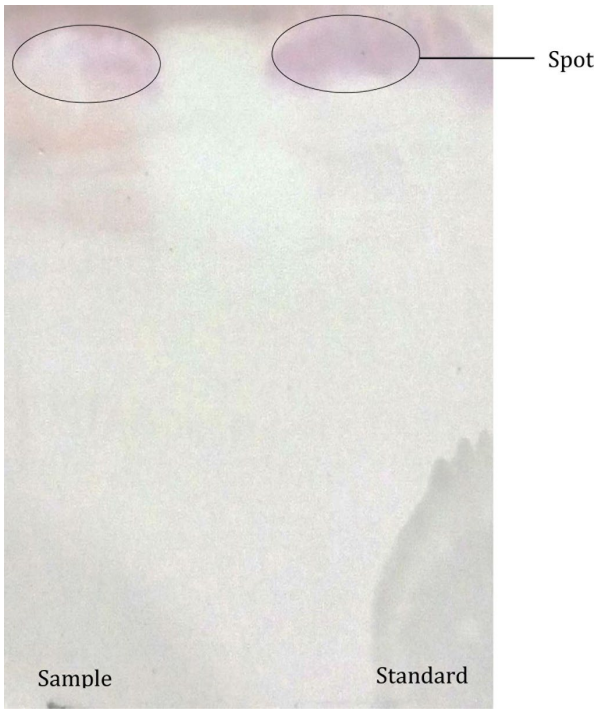


Figure 3. Chromatogram of cowpea rhizobium isolates detecting IAA using Salkowiski's reagent compared with standard.

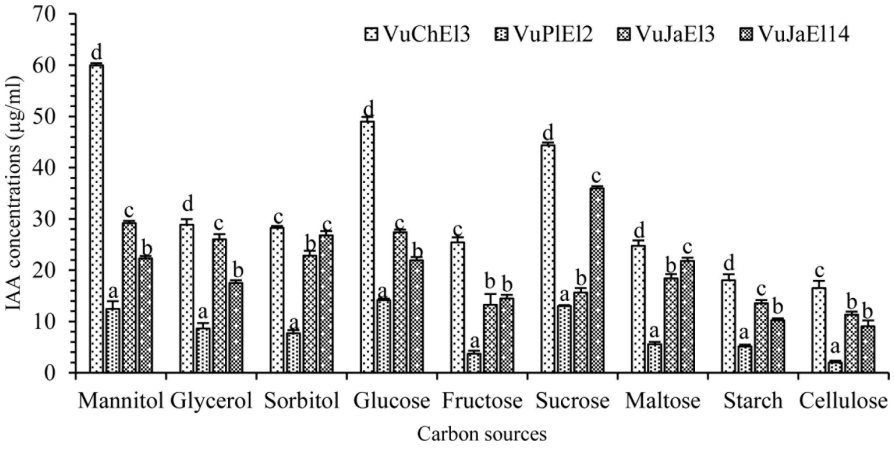
matching that of standard IAA (0.9), confirming IAA production by cowpea-nodulating isolates.

3.4. Effect of Different Carbon Sources on IAA Production

Isolates VuChEl3 and VuJaEl3 produce the highest amount of IAA in the presence of mannitol, at 59.93 µg/ml and 29.18 µg/ml, respectively. Isolate VuPlEl2 produces maximum IAA in the presence of glucose (14.18 µg/ml) and isolate VuJaEl14 in the presence of sucrose (35.93 µg/ml). Of all the 9 carbon sources used, maximum quantity of IAA was produced in the presence of mannitol, followed by glucose and sucrose. In contrast, IAA production remained low in the presence of cellulose (Figure 4).

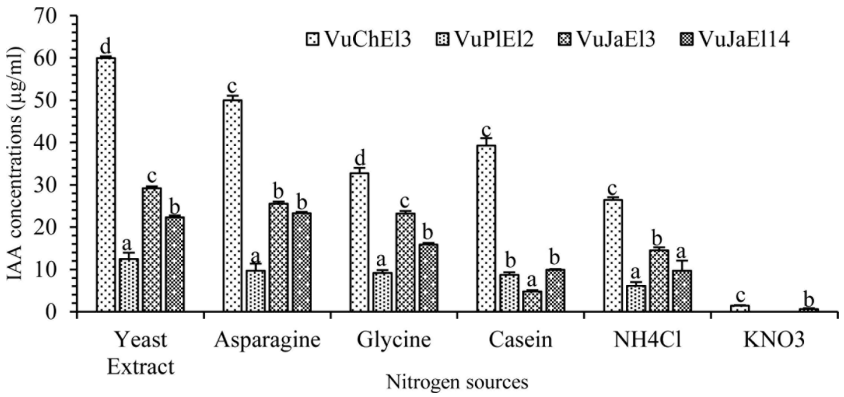
3.5. Effect of Different Nitrogen Sources on IAA Production

Yeast extract proved to be the best source of organic nitrogen for IAA production in three isolates: VuChEl3, VuPlEl2 and VuJaEl3, which produced 59.93 µg/ml, 12.43 µg/ml and 29.18 µg/ml of IAA respectively (Figure 5). VuJaEl4 isolate produced



Note: Bars with the same letter are not significantly different at the $P < 0.05$.

Figure 4. Effect of carbon sources on IAA production by cowpea rhizobia isolates.



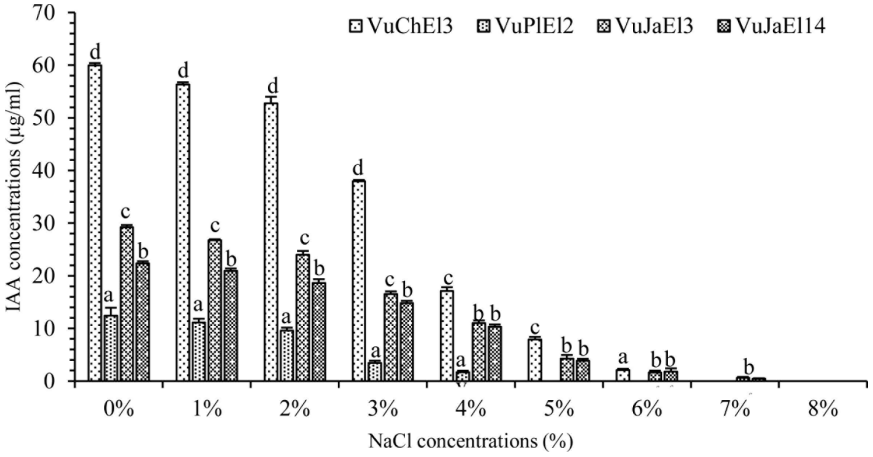
Note: Bars with the same letter are not significantly different at the $P < 0.05$.

Figure 5. Effect of nitrogen sources on IAA production by cowpea rhizobia isolates.

a maximum quantity of IAA in the presence of asparagine: 23.31 µg/ml. Potassium nitrate (KNO_3), inhibited IAA production in VuPlEl2 and VuJaEl3, and resulted in significantly low levels in VuChEl3 and VuJaEl14 (1.43 µg/ml and 0.62 µg/ml respectively). Of the six nitrogen sources, yeast extract led to the highest IAA production, followed by asparagine and glycine.

3.6. Effect of Different Concentrations of NaCl on IAA Production

IAA production decreased as NaCl concentration increased (Figure 6). At 0% NaCl, IAA production is maximal and significantly elevated in all isolates. At 1% and 2% of NaCl, IAA production maintains appreciable amounts. At 3% NaCl and above, IAA production drops considerably in all isolates. At 8% NaCl, no isolate production was detected.



Note: Bars with the same letter are not significantly different at the $P < 0.05$.

Figure 6. Effect of different concentrations of NaCl on IAA production by cowpea rhizobia isolates.

3.7. Effect of pH on IAA Production

The amount of IAA produced by each isolate decreased as the pH of the medium became more acidic (Figure 7). All isolates produced high levels of IAA at pH 6.5. From pH 5.5 onwards, IAA production dropped considerably in all isolates. At pH 4 and 3.5, no isolate production was detected.

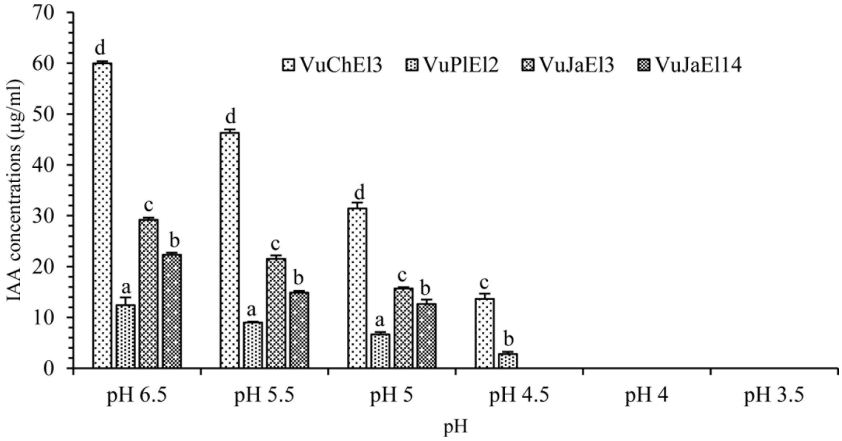
3.8. Effect of Incubation Time on IAA Production

IAA production by cowpea rhizobia isolates was significantly low, ranging from 2 µg/ml to 14.62 µg/ml after 24 hours of incubation, and reached its optimum after 72 hours of incubation (Figure 8). From 96 hours of incubation onwards, IAA production by cowpea-nodulating rhizobia isolates decreased, ranging from 9.12 µg/ml to 38.81 µg/ml.

3.9. Effect of Temperatures on IAA Production

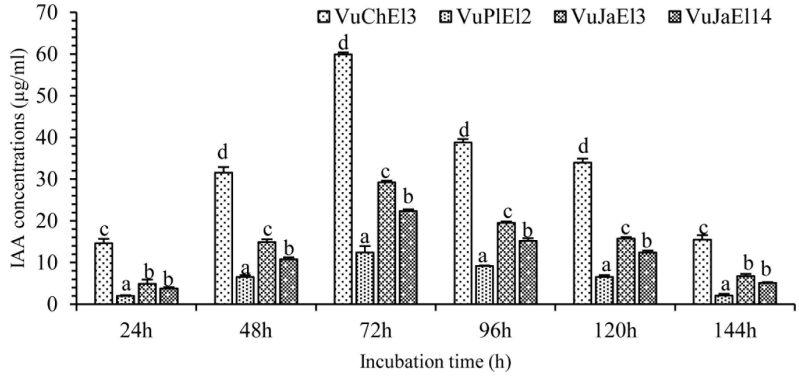
For all the isolates studied, temperature of 30˚C is optimal for IAA production,

with concentrations ranging from 12.43 µg/ml to 59.93 µg/ml (Figure 9). Significantly lower quantities of IAA are produced at 40˚C and 45˚C. At temperatures of 4˚C and 50˚C, no isolates production was detected.



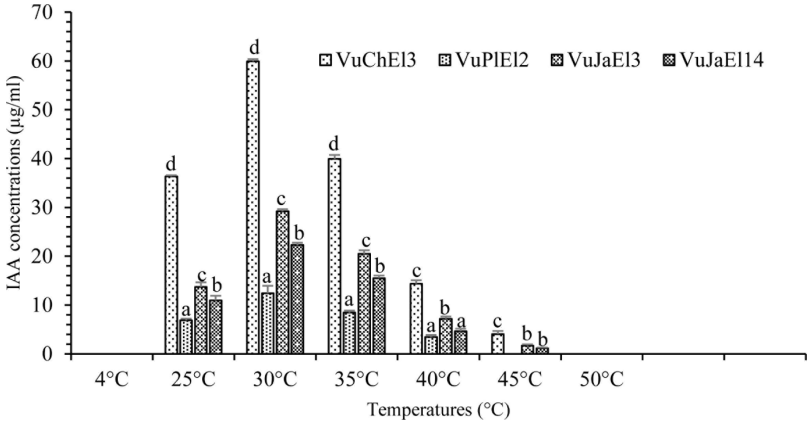
Note: Bars with the same letter are not significantly different at the $P < 0.05$.

Figure 7. Concentrations of IAA produced by cowpea rhizobia isolates under different pH.



Note: Bars with the same letter are not significantly different at the $P < 0.05$.

Figure 8. Concentrations of IAA produced by cowpea rhizobia isolates at different incubation times.

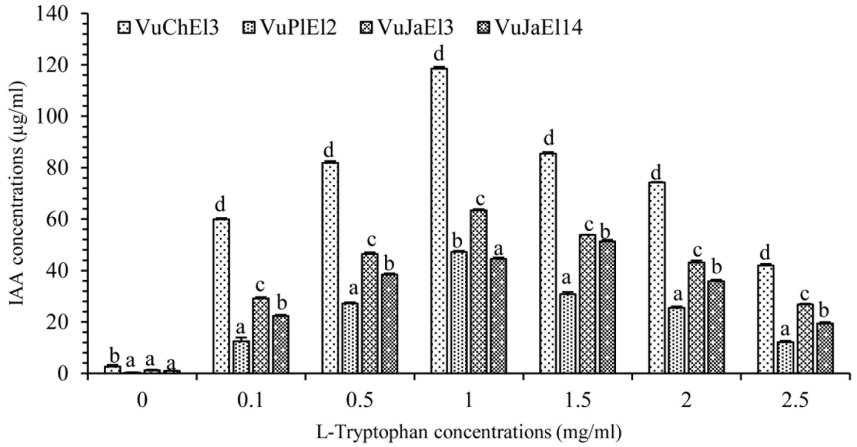


Note: Bars with the same letter are not significantly different at the $P < 0.05$.

Figure 9. Effect of temperature on IAA production by cowpea rhizobia isolates.

3.10. Effect of Tryptophan Concentrations on IAA Production

The effect of L-tryptophan concentration reveals that IAA production varied with the concentration of L-tryptophan in the medium (**Figure 10**). In the absence of L-tryptophan, all isolates produced significantly low quantities of IAA, ranging from 0.31 µg/ml to 2.62 µg/ml. This quantity increased with L-Tryptophan concentration, reaching its optimum at 1 mg/ml L-Tryptophan in the VuChEl3, VuPlEl2 and VuJaEl3 isolates, which produced significantly high quantities of IAA: 118.43 µg/ml, 47.18 µg/ml and 63.43 µg/ml respectively. Above 1 mg/ml L-tryptophan, the amount of IAA decreased in all isolates except VuJaEl14, which produced a maximum amount of IAA in the presence of 1.5 mg/ml L-tryptophan.



Note: Bars with the same letter are not significantly different at the $p < 0.05$.

Figure 10. Concentrations of IAA produced by cowpea rhizobia isolates with different concentrations of L-tryptophan.

4. Discussion

All rhizobia isolates obtained in this study did not absorb Congo Red, which is consistent with the results of Girija *et al.* [13], who demonstrated that cowpea-nodulating rhizobia in India also failed to absorb this dye.

All tested rhizobia isolates produced IAA but at variable quantities. This result is consistent with the work of Girija *et al.* [13], who showed that cowpea rhizobia isolated in India can produce IAA in variable quantities. Some authors [14] [15] reported that rhizobia strains produce IAA by metabolizing l-tryptophan.

The IAA produced was confirmed by thin-layer chromatography, where the frontal ratio obtained matched with the Rf of the authentic IAA (0.9). Several authors [11] [15] in their work confirmed the IAA produced by rhizobacteria by thin layer chromatography with respective Rf of 0.9 and 0.57.

The effect of carbon and nitrogen sources revealed that the quantities of IAA produced by the 4 rhizobia isolates varied according to the carbon and nitrogen sources used. The greatest quantities of IAA were obtained in the presence of mannitol and yeast extract. These results concur with those of Shouky *et al.* [16], who showed that mannitol and yeast extract are the best carbon and nitrogen sources

for IAA production by *Rhizobium leguminosarum* strains.

The effect of increasing NaCl concentrations on IAA production by rhizobia isolates reveals that IAA production decreases as the NaCl concentration in the culture medium increases. This result is consistent with the work of Yousef [17], who found that IAA production by Bacillus subtilis isolates decreased with increasing NaCl concentration in the culture medium. High concentrations of NaCl cause osmotic stress, disrupting water balance of bacterial cells and resulting in reduced growth and viability. This reduction of growth is directly correlated with decreased IAA production [18].

IAA production by all Rhizobia isolates in this study reached its optimum at pH 6.5. This result agrees with the work of Lebrazi *et al.* [18], who showed that incubation at pH 6.5 was one of the conditions for optimum IAA production by *Rhizobium* sp. Rhizobia primarily synthesize IAA by the tryptophan-dependent indole-3-pyruvic acid (IPyA) and indole-3-acetaldehyde (IAM) pathways. An acidic pH can disrupt these pathways by affecting enzymatic activity and tryptophan availability, thereby reducing IAA production [19].

For the rhizobia isolates in this study, IAA production begins at 24 h and reaches its optimum at 72 hours. After this optimum, IAA production declines in all rhizobia isolates in this study. After this optimum, IAA production declines in all rhizobia isolates in this study. Several authors [14] [16] showed that IAA production in rhizobia strains is maximal after 72 h of incubation. The decrease in IAA production after 72 hours may be due to IAA degrading enzymes such as IAA oxidase and IAA peroxidase [16].

Incubation temperature also appears to be a factor affecting the ability of isolates to produce IAA. For the rhizobia isolates studied, optimal production is obtained at 30˚C. Shokri *et al.* [20] revealed that maximum IAA production is achieved by *Rhizobium sp.* after three days' incubation at 30˚C.

In the absence of L-tryptophan, low quantity of IAA was produced. This result concurs with that of Nalini [21], who reported that bacteria can produce IAA in the absence of L-tryptophan. Isolates VuChEl3, VuPlEl2, and VuJaEl3 produce a maximum quantity of IAA with a concentration of 1 mg/ml L-tryptophan. Ravi Kumar [14] reported that in the presence of 1 mg/ml L-tryptophan concentration, rhizobia isolates produce a maximum of IAA after 72 hours of incubation.

5. Conclusion

In this study, cowpea rhizobia isolates were screened to determine their ability to produce indole-3-acetic acid (IAA), and optimization was carried out with physicochemical parameters such as carbon and nitrogen sources, NaCl concentration, pH, temperature, incubation time and varying concentrations of tryptophan. All 14 cowpea rhizobia isolates obtained produce IAA in varying amounts. The optimal conditions for maximum IAA production (118.43 µg/ml) were achieved using a culture medium containing mannitol and yeast extract supplemented with NaCl concentrations ranging from 0 to 1%, and adjusted to pH 6.5. Maximum IAA pro-

duction was reached after 72 hours at 30˚C in the presence of 1 mg/ml L-tryptophan. The findings of this study provide a basis for potential use of these isolates in the development of biofertilizers.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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