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Identification and Biochemical characterization of Plant growth promoting *Rhizobium* and evaluating its potential as Bioinoculant isolated from root nodule of Cowpea (*Vigna unguiculata*)

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Abstract

Cowpea is a leguminous crop which is used as a food for humans, feed for livestocks and serves as the revenue generating agricultural crop for farmers. *Rhizobium* is a legume specific organism, which has the potential to fix atmospheric nitrogen. Rhizobia can be characterized by different methods. In this study, *Rhizobium* is isolated from the root nodule of cowpea plant. The isolated *Rhizobium* strain was biochemically characterized by different methods and screened for plant growth hormone producing traits. It was observed that the *Rhizobium* isolate significantly produced IAA, Ammonia, Siderophore and HCN. The maximum Indole Acetic Acid and Ammonia production was recorded as 101.5 µg/ml and 79 µg/ml respectively. The isolate also tested positive for siderophore and HCN production. From this study, we conclude that the *Rhizobium* strain can be used for improving plant growth promotion.

Key words: Cowpea, *Rhizobium*, IAA, Ammonia, Siderophore and HCN production.

Cowpea (*Vigna unguiculata*) is a multipurpose legume crop that serves as a useful and consistent source of income. It also supplies sustenance for human and livestock. Cowpea is a crucial part of cropping systems because it can fix atmospheric nitrogen through biological nitrogen fixation, a symbiotic relationship between the legume host and soil-dwelling bacteria known as rhizobia[14]. Cowpea can fix about 240 kg / ha of atmospheric nitrogen with effective fertilizer management, making roughly 60-70 kg/ ha nitrogen accessible for succeeding crops cultivated in rotation with it (Kebede *et al.,* 2021). The

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Cereal crop's nitrogen utilization efficiency can rise from 20 per cent in continuous cereal monoculture to 28 per cent when cowpea is used to fix up to 88 kg of nitrogen per hectare[3]. Cowpea can contribute up to 150 kg / ha of net nitrogen to soil, particularly when paired with N2 fixing rhizobia Degefu *et al.*[8]. Further, using cowpea in crop production plants could result in up to 337 kg N / ha of nitrogen fixation. This possibility of a strong symbiotic relationship between *Rhizobium* and nitrogen fixation could be a source of nitrogen supply for crop production systems, which is economically alluring and eco - friendly to the environment and soil system[22].

As the Nitrogen fixation by *Rhizobium* bacteria plays significant role in providing an adequate amount of nitrogen to the legumes. Symbiotic relationship between rhizobia and legumes is a major source of stabilized nitrogen (ammonia) in the biosphere, and this process increases agricultural productivity which reduces dependence on nitrogenous fertilizers[24]. The importance of studying *Rhizobium* bacteria and their infection to cowpea plants under arid and semi - arid conditions. Several of them can be used as efficient inoculant in inducing infestation and nitrogen fixation on the host plant. The strains of *Rhizobium* were capable to produce nodules when induced *Rhizobium* as inoculants and resulted in significant changes in cowpea plant characteristics[1,4].

Isolation of Rhizobium - a root nodule bacteria :

Rhizobium was isolated from root nodules of healthy and actively growing plants. The plant at 40 days after emergence was uprooted carefully to obtain root nodules. The root nodules were detached and washed under running tap water until adhering soil particles were fully removed. The washed root nodules were dipped in 0.1 per cent mercuric chloride solution for 30 seconds followed by 75 per cent ethanol for another 30 seconds and rinsed with sterile distilled water for 10 times. Surface - disinfected nodules were transferred to test tubes containing 5 mL of sterilized distilled water, where they were crushed with a sterilized glass rod to obtain a milky suspension. A 0.3 mL aliquot of this suspension was placed on yeast extract mannitol agar (YEMA) medium plates containing Congo red following the pour plate technique. YEMA medium contains 10.0 g/L mannitol, 1 g/L yeast, 0.5 g/ L K_2HPO_4, g/L $MgSO_4.7H_2O$, 0.1 g/L NaCl, 0.025 g/L Congo red and 20.0 g/L agar powder. The pH of culture medium was adjusted to 6.8±0.2 at 25ºC, then autoclaved. Sterilized YEMA medium was poured into Petri dishes with plated *Rhizobium* suspension. After solidification of medium, Petri dishes were sealed by parafilm to avoid contamination and incubated at 28±1ºC for 24 - 48 hours. After 24- 48 hours of incubation translucent, raised and mucilaginous *Rhizobium* colonies were formed and typical single colonies were restreaked 3-4 times on fresh YEMA plates to obtain pure cultures.

Morphological characterization of isolated Rhizobium isolate :

Gram staining :

Gram-stained *Rhizobium* colonies were observed under Microscope with a raised smooth edge and a musky smell. It was

determined that rod shaped were Gram-negative[12].

Biochemical characterization of Rhizobium isolates

Motility test :

Motility test were done according to Elbeltagy *et al.,*[10]. A well-isolated colony was picked by using sterile needle, then stab into a semi-solid nutrient agar (0.2% agar) medium to within 1 cm of the tube's bottom. Incubate the colony for eighteen hours at 35ºC. Positive result is indicated by a red turbid area exter away from the line of inoculation. A negative test is indicated by growth along the inoculation line.

Oxidase test :

The oxidase test was used to determine *Rhizobium* oxidase activity. The 1% N, N, N, N-tetramethyl-p-phenylene diamine was dissolved in warm water to create the oxidase reagent, which was then kept in a dark bottle at a temperature of 25 to 30°C. Using the wet filter-paper approach, 24 hold *Rhizobium* colonies were transferred to a strip of filter paper after being dipped in this reagent. Colonies that were oxidase - positive quickly changed from lavender to dark purple to black.

Catalase test :

Catalase activity of *Rhizobium* was determined by the CAT test by placing 24 hours colonies on a glass slide and adding one drop of 3 % H_2O_2. The gas bubbles was appeared on glass slide it indicate the presence of CAT.

Citrate test :

Bromothymol blue and sodium citrate in an equivalent proportion to mannitol in YEM agar were used to test the ability to utilize citrate. Fresh cultures were incubated for 48 hours on modified conditions[16]. A positive outcome was seen after incubation when green changed to blue.

Methyl red test :

In sterile test tubes containing methyl red-Voges Proskauer broth, 5 mL of *Rhizobium* root nodule suspension was added after each test tube had been cultured at 30°C for two days[20]. A yellow soup denotes a negative outcome whereas red broth denotes a positive one.

Strach hydrolysis test :

A medium made of starch and agar was prepared[11]. In order for the medium to harden, it was placed into sterile Petri dishes. For four days, *Rhizobium* was incubated at 30 ± 2°C after being injected into Petri dishes. Following incubation, 5 mL of the iodine solution (0.340 g iodine and 0.660 g potassium iodide in 100 mL distilled water) was added. The ability to hydrolyze starch was demonstrated by the formation of a clear zone around colonies.

Voges - Proskauer *test :*

The individual *Rhizobium* isolates were inoculated into 5 mL sterile test tube

which contains methyl red-Voges Proskauer broth and it was incubated at 30 ± 2°C for two days. Barrit's reagents A and B (5 mL each) were added following the incubation. The development of a red colour indicated a negative result[21].

Gelatinase test :

To test the gelatinase activity, log phase cultures from YEM broth were swabbed onto YEM agar plates that contained 0.4% (w/v) gelatine. Plates were incubated at 28 ± 1°C for 7 days. An effective outcome is shown by the creation of a clear zone surrounding the culture.

Indole test :

The indole test was conducted according to the steps laid out by MacFaddin[19]; Hemraj *et al.*[11]. 10 mL test tubes (4 mL/test tube) were filled with tryptone broth medium which was then autoclaved (121°C; 15 pressure; 15 min). Inoculated *Rhizobium* was placed in broth and allowed to grow for two days at 30 ± 2°C. The control was soup that had not been infected. Each test tube, including the control, received 1 mL of Kovac's reagent (isoamyl alcohol, para-dimethyl amino benzaldehyde, and concentrated hydrochloric acid) following incubation. Every 10-15 minutes, tubes were gently shaken and let to stand until the reagent bubbled to the surface. A red ring formed when a result was positive, while a yellow ring when the result was negative.

Urease test :

Christensen's medium was prepared and sterilized at 121ºC for 15 minutes at 15 lb/inch2. The medium was dispensed into test tubes as slants. The test cultures were inoculated heavily over the entire slope surface. The cultures were then incubated at 37ºC for 24 - 48 hours. After incubation, the slant was observed for change in color.

Evaluation for Different PGP traits :

Siderophore Production :

The bacterial culture was spotted on a Chrome Azurol S (CAS) agar medium plate for the siderophore production qualitative assay. The CAS agar plate was then incubated for six days at 28ºC. A clear orange-yellow halo zone was formed around the spotted culture after incubation, which indicates a positive result for siderophore production.

HCN Production :

HCN was evaluated by using the protocols mentioned by Kremer and Souissi[17]. The bacterial isolate was streaked into a King's B agar plate supplemented with 4.4 g of glycine per liter. After that, streaked plate was placed with a Whatman No. 1 filter paper dipped in the picric acid solution (0.05% solution in 2% sodium carbonate). The streaked plate was covered by Parafilm and incubated at 28ºC for 5 days. The positive result for HCN production was showed by change in the color of the lid from yellow to orange- brown[2].

Ammonia production :

This method involved inoculating a 72-hour-old bacterial culture in 10 milliliters of

peptone water and incubating it for three days at 28ºC. Nessler's reagent (1 ml) was then added to each inoculation tube. The positive outcome of ammonia production is indicated by the appearance of a yellow-brown color[5]. A spectrophotometer was used to measure the amount of ammonia production at 450 nm by using 0.1–10 µ mol ammonium sulfate standard curve.

IAA production :

By using the colorimetric assay, IAA production of the rhizobial isolate was identified. Then the YEM broth supplemented with 100–500 µg/ml of L-tryptophan was inoculated with the isolate and incubated at 28! for two to seven days. For 12 minutes, the culture was centrifuged at 10,000 rpm. After adding Salkowski reagent to the supernatant, the IAA concentration was identified. The appearance of a pale pink hue signifies an effective result for the production of IAA. Using the standard curve of known IAA concentration (10 - 200 µg/ml), the total amount of IAA was estimated. By combining 2 ml of Salkowski reagent with 2 ml of sterile broth, a negative control was obtained.

Table-1. Morphological and biochemical characterization of *Rhizobium* isolate

Biochemical tests	*Rhizobium* isolate
Gram test	-
Morphology	Rods
Motility test	+
Oxidase test	+
Catalase test	+
Citrate test	-
Methyl red test	+
Starch hydrolysis	+
Urease test	+
Gelatinase test	-
Indole test	+
Voges- Proskauer test	+

(+) Positive result, (-) Negative result

Table-2. Assessment of PGP traits of *Rhizobium* isolate

PGP traits	Result
IAA production	101.5 µg/ml
Ammonia production	79 µg/ml
Siderophore production	+
HCN production	+

Isolation of efficient Rhizobium isolate :

The *Rhizobium* strain was isolated from the root nodules of cowpea.

Morphological characteristics of Rhizobium isolate :

The results of morphological and biochemical characteristics of the isolate was summarized in Table-1. The isolate grew well on YEMA medium. The isolate grew quickly within 3 - 5 days of incubation and failed to absorb Congo red in this medium. Colonies of the isolate in YEMA medium were circular, mucoid, white and translucent. The isolate were Gram-negative and rod-shaped. In congo red medium, *Rhizobium* colonies were white, translucent, glistening, elevated and comparatively smaller than stained colonies of other non-*Rhizobium* isolate.

Biochemical characteristics of Rhizobium isolate :

It was found that the isolate gave

positive results for the oxidase, catalase, indole, urease, motility, voges-Proskauer and starch hydrolysis test and negative results for citrate utilization, Gelatinase test. The same tests were also used to confirm that similar isolated bacterial strains were *Rhizobium* spp. [25].

IAA production :

The screening of PGP traits of the isolate is shown in Table-2. This isolate's production of indolic phytochoromal compounds is an important characteristic. These phyto-chormones promote large-scale root growth and length, which aids the host plant in absorbing more nutrients from the soil[7]. The maximum IAA production was recorded by this isolate is 101.5 µg/ ml (Table-2). Lebraziet *et al.*[18] reported that the *Rhizobium* species produced 116 and 105 µg/ ml IAA. Chaudhary *et al.*[6] reported that the *Rhizobium pusense* isolate produced 110.5 µg/ ml IAA.

Ammonia production :

Ammonia production of the *Rhizobium* isolate was studied and the result showed that *Rhizobium* isolate produced 79 µg/ ml ammonia (Table-2). Karthika *et al.,*[13] pointed out that the high production of ammonia in alkaline soil inhibits the growth of a variety of pathogenic fungi. Chaudhary *et al.,*[6] showed that 81 µg/ ml of ammonia were produced by *Rhizobium pusense* isolate.

Determination of HCN and siderophore production :

The capacity of HCN production by this isolate was demonstrated on a King's B agar medium plate. The plate lid's orange-brown color indicates the positive result for the production of HCN. Mowafy *et al.,*[23] reported that *Rhizobium* MAP7 produced 601.2 mg/l HCN.

A CAS agar plate assay was used to verify the capacity of the isolate to produce siderophores. On the CAS agar plate media, an orange halo zone developed around the inoculated isolate. Mowafy *et al.,*[23] reported that *Rhizobium* MAP7 produced 71.6 per cent siderophore. Dhole *et al.,*[9] reported when *Rhizobium* sp. mixed with both *Bacillus* spp the siderophore production was increased considerably.

Rhizobium was isolated from the root nodules of cowpea plant. The isolate was characterized for different plant growth promoting traits. The isolate was able to produce higher amount of IAA, Ammonia, Siderophore and HCN. This plant growth promoting traits of the *Rhizobium* will eventually increase the plant growth promotion and can be suitably used for the enhancement of crop development.

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