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Cultural and Biochemical Characteristics of Rhizobium Present in Nodule of *Pongamia Pinnata* (l.) Pierre

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***Abstract:** The present investigation is an attempt to study the impact of rocky terrain on Rhizobium bacteria of root nodules isolated from legume trees. To study the morphological, cultural, and biochemical characteristics of bacterial strain the bacteria obtained from nodules of selected legume i.e. Pongamia pinnata. Rhizobia inhabited in root nodules of plant, grown in Kota university campus, rhizobia were isolated and inoculated on Yeast Extract Mannitol Agar (YEMA) medium and it's morphological, cultural and biochemical characteristics were studied. It was observed that colonies were circular or irregular; light creamish, glistering, gelatinous, convex with entire margins. The bacteria were gram negative, rod shaped, aerobic, non-spore forming and slow-moving bacteria arranged single, in pairs and in clusters. It showed negative chemical reaction for indole, while showed positive reaction for citrate utilization, catalase, urease. By the help of bio chemical characteristics it was confirmed that isolated bacterial culture may be of Rhizobium pongamie and rocky terrain with high temperature of environment does not have any negative effect on the characters of Rhizobium, our findings was supported by many earlier investigations.*

***Keywords:** Rhizobium, Pongamia Pinnata, Yeast Extract Mannitol Agar*

I. INTRODUCTION

Biological nitrogen fixation is a component of sustainable agriculture and Rhizobia inoculants have been applied frequently as bio-fertilizers [8] and [26]. Each major legume group is nodulated by different species of Rhizobium [5]. *Pongamia pinnata* a common legume plant of hadoti region is selected for the study purpose. *Pongamia pinnata (L)* is recommended as oil and biodiesel producing tree of Fabaceae family [17]. It shows significant resistance to abiotic stress such as water deficit and salinity. It is found in various geoclimatic regions and wide range of environment as humid, subtropical to tropical, semiarid (i.e. with various temp range 15-50 ^{0}C and pH <7 sodic and alkaline soils), [15]. It is also able to tolerate a broad range of climatic and edaphic conditions so it can be cultivated in marginal land [16] and [12].

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It can be cultivated on rocky terrain like Kota city and recommended in environmental management due to its stress resistant and insecticidal properties. *Pongamia* is identified as bio resource in agro forestry and urban landscaping to amelioration of problem soil especially in case of degraded, eroded and polluted soils. *Pongamia* is also satisfying all the sustainability criteria expected from a revenue tree.

Pongamia can be grown on the soil of Kota university campus because of its better adaptation towards the local geoclimatic conditions and also of its nodulation properties. The previous researches show that *Pongamia* show good nitrogen fixing properties and symbiotic association with different strain of Rhizobium. [11] and [12].

II. MATERIALS AND METHODS

The saplings of Pongamia pinnata of 3-4 month were uprooted from university campus during April 2024. Distinct nodules were excised from 6-8 inches deep roots.

Nodules were surface sterilization with 70 %(v/v) alcohol for 1 min, then treated with 10% (w/v) sodium hypochlorite for 15 min. and washed with sterilized water. A sterile blade was used to split a 2 mm sterile nodule in two halves, and a blunt needle was used to scoop out the central part of nodules. The excised nodules were macerated and diluted in 500 µL of saline water. Approximately 100 µL of the inoculums was spread on yeast extract- mannitol agar plate and incubated at 28oC for 3 days. After incubation for 4 days transparent to white single colonies were transferred to YEMA slants. Isolated single colonies were repeatedly streaked on congo red-YEM agar to confirm the culture's purity. Purified isolates were maintained in YEM broth containing 20 % (v/v) glycerol at -80oC for future use [25] [27], [6] and [1].

A. Characterization of Isolates

The cultural, morphological and bio-chemical characteristics of the isolates were studied by following procedure

B. Cultural Characteristics

The shape, color, opacity, margin and elevation of the colonies of theisolates grown on standard YEMA plates were observed.

C. Morphological Characteristics

The shape, oxygen demand, motility, gram stain reaction of bacteriawas observed under microscope using standard procedure.

D. Biochemical Characteristics

Biochemical characteristics of the Rhizobium isolates were studied using different tests like Indole, Citrate utilization, Urease test, Catalase test by following procedure given below:

E. Indole Test

To find out how well an organism can break down the amino acid tryptophan. The tube containing SIM broth containing 30 grams of beef /yeast extract, peptone 3gm, Ferrous ammonium sulfate-0.2gm, Sodium thiosulphate-0.025, Refined water-1000ml, pH-7.3 were prepared and then autoclaved (121°C; 15 psi; 15 min). Rhizobium was inoculated in broth and incubated at 30±2°C for two days. Uninoculated broth served as the control. After incubation, 1 mL of Kovac's reagent (isoamyl alcohol, para-dimethyl-aminobenzaldehyde and concentrated hydrochloric acid) was added to each test tube, including the control. Tubes were shaken gently every 10-15 min and allowed to stand until the reagent surfaced.The formation of a red ring indicates a positive resultand yellow ring indicated a negative result.

F. Citrate Utilization

To distinguish the capacity to mature citrate as sole carbon source. The cylinder containing (Simmons Citrate Agar) Ammonium di hydrogen phosphate-1.0gm, Di potassium phosphate-1.0gm, Sodium chloride-5.0gm, Sodium citrate-2.0gm, Magnesium sulfate-0.2gm, Agar-15gm, Bromothymol Blue-0.08gm, Refined water-1000ml, pH-6.9 were vaccinated by *Rhizobium* culture, following 24-48 hours of hatching change in the shade of the media was noticed and result showed change in shade of medium. By replacing mannitol from YEMA agar with an equal amount of sodium citrate and bromothymol blue, the ability to utilize citrates was determined. After inoculating the plates with the altered media, they were left to incubate for 48 hours.

G. Urease Production

To determine the enzyme's capacity to break down urea. The container containing (Urea Broth), 0.1 g of yeast extract, and potassium di hydrogen phosphate-9.1gm, Di potassium hydrogen phosphate- 9.5gm,Phenol red-0.01gm, Refined water1000ml,pH-6.8(*after sterilization with a 20 percent urea solution (previously sterilized through filtration) inoculated with a culture of isolated bacteria, such as Rhizobium. Following 24-48 hours of during incubation, as well as a change in the media's color and the media's color was observed.

H. Catalase Activity

To determine whether some bacteria can break down hydrogen peroxide by producing catalase, an enzyme. Slide with two to three drops of (Trypticase soya stock) Trypticase-15gm, sodium chloride-5.0gm, Refined water-1000ml, pH-7.3 were immunized by 24-48 hours incubatedmicroorganism's culture. Upon adding 3% hydrogen peroxide, observe after a few seconds. the change on the slide Bubbles were showed up on the slide. Different Hydrogen peroxide was sprayed all over 48-hour-old isolates and noticed for freedom of fizz of oxygen around the bacterial colonies.

III. RESULT AND DISCUSSION

Pongamia pinnata (L.) Pierre (Pongamia) is a tree which traditionally, has been used in Hadoti region of Rajasthan as a source of medicines, manure, wood, fodder, fuel ,biopesticide [3] and [9] Because the seeds of this plants contain around 40% oil, attracted attention for its potential as a biofuel source. It may be grown on marginal lands since it can withstand a wide range of climatic and edaphic conditions. [12]. Pongamia is a member of the Papilionoidea sub-family of Fabaceae (*Leguminosae*) family which is characterized by the ability to fix nitrogen symbiotically. Its ability to fix nitrogen symbiotically poses a significant advantage as all other first-generation biofuel sources are non-legumes **[**8**]** [10] and [20] [32]. According to [18], the use of crops with high bioenergy potential for the phytoremediation of contaminated soils can potentially provide both socio-economic and ecological benefits. It can also be part of climate change mitigation strategies as it has a high carbon sequestration potential ([19].

The community structure of *Pongamia* root-nodule bacteria has been addressed by a few studies that assessed nodulation ability by endogenous rhizobia their stimulatory effect on nodule number and plant growth [28] [29] [30] [31]. In this present study, we extend the work on *Pongamia* root-modulating bacteria to isolate and characterize the*Rhizobium* species from the root nodules of biodiesel crop *P. pinnata* from Kota Rajasthan [2] and [14]

The Rhizobium isolates grow well on YEMA medium within 3-5 days of incubation. The colonies in YEMA medium were circular, mucoid, glistening, elevated, white and translucent. Bacteria were reported Gram-negative and rod-shaped. The morphological characters of Rhizobium isolates were similar to those reported by [13]. Biochemical characteristics of Rhizobium isolates were analyzed by catalase production, citrate utilization, urease & indole. The results of morphological and biochemical characters are summarized in Table 2. It was found that it gave positive results for the catalase, urease, citrate utilization but negative results for indole. Shahzad [22**]** also used the same tests to confirm that similar isolated bacterial strains were Rhizobium spp. Positive sugar tests during the isolation and characterization of *Rhizobium meliloti* were noted by [4], [22] and [24].

Table-1: Morphological and Cultural Characters

1	Shape	Circular
2	Colour	White Creamish
3	Opacity	Opaque
4	Margine	Regular
5	Elevation	Convex
6	Bacteria shape	Rod
7	Oxygen demand	Aerobic
8	Motility	Motile
9	Gram staining	Gram -ve

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Table-2: Biochemical Characters

S. no	Test	Result
1	Production of Indole from tryptophane	-ve
2	Citrate utilization	+ve
3	Production of ammonia from urea	+ve
4	Catalase	+ve

A gram-negative, nonmotile, fast-growing, rod-shaped, bacterial strain is isolated from root nodules of *Pongamia* that grew optimal at 28°C, pH 7.0 in presence of 2% NaCl.(Graham and Parker 1964,Kaseri*et al* 2013) [7].

PLATE-1

Fig. 1: Root Nodules of *Pongamia Pinneta*

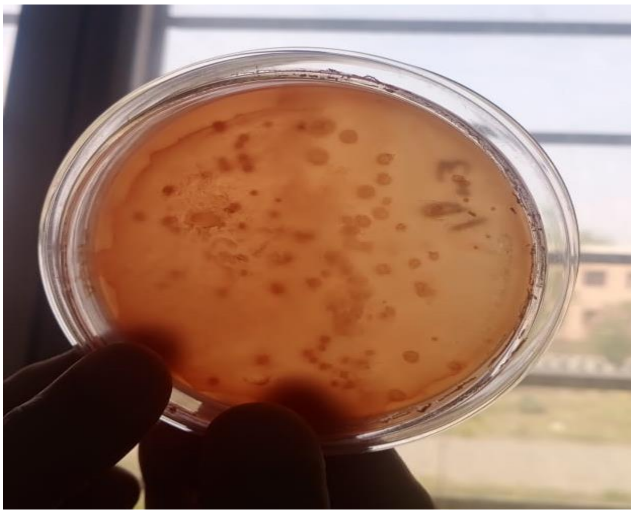

Fig. 2: Morphological and Cultural Characters; Gram Staining

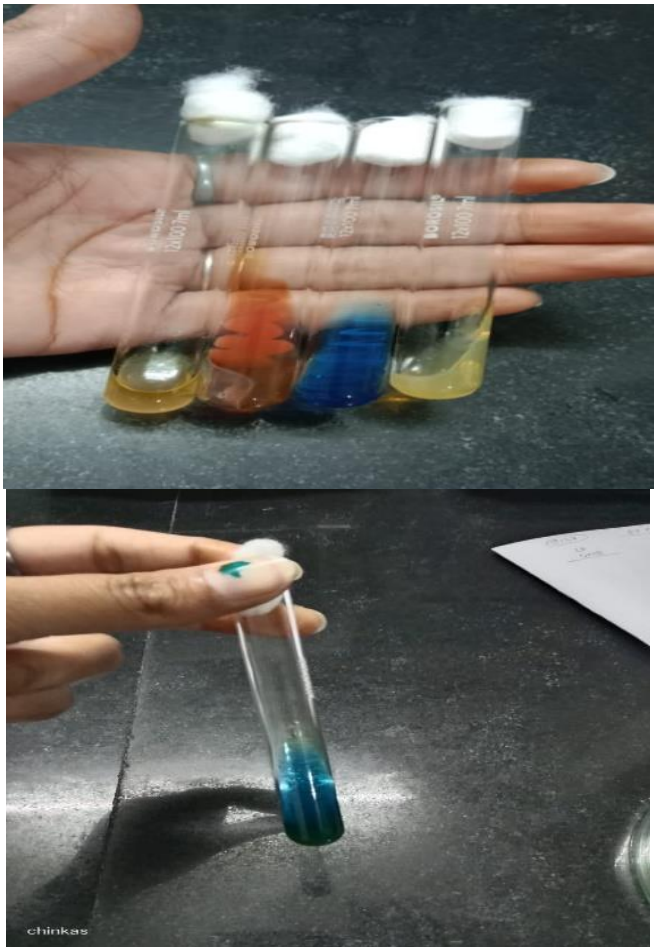

Fig. 3: Biochemical Characterization of Rhizobium

IV. CONCLUSION

The ability of rhizobium to grown in diverse environmental conditions will be important for the establishment and success of *pongamia* plantation on the less or unfertile rocky terrain. *Pongamia pinnata* has an added advantage of N_2-fixing ability and tolerance to stress conditions as compared with other biodiesel crops. (Shen Tian et al., 2023) [23].

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It harbours "rhizobia" as an endophytic bacterial community on its root nodules. The genus Rhizobium was founded by Frank (1890) based on its characters to form nodules on roots of legume plants. This property is the only valid test in the identification of the organism. Apart from it, some diagnostic features of Rhizobium could be conveniently not only determined and identify the organism but also delineate different species. (Shahrajabian& Cheng 2021) [21].

The importance of characterizing indigenous rhizobia of *Pongamia* cannot be overemphasized. Still there has not been any detailed study of phenotypic characteristics and symbiotic effectiveness of rhizobia isolates which naturally nodulated *Pongamia* considering its potential value in sustainable agriculture and role in agroforestry. Therefore, in this work, we attempt to isolate the nitrogen-fixing rhizobial symbiont strain from nodules of *P. pinnata*.

It is concluded that there is a urgent need to create awareness to cultivate legume tree species in this area that will not only improve soil fertility but will also be helpful in improving socio- economic status.

DECLARATION STATEMENT

After aggregating input from all authors, I must verify the accuracy of the following information as the article's author.

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