VOLUME 10 ISSUE 1 2024

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 10, No. 1, 973-983 (2024)

International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

Editor-in-Chief: Prof. Ajai Kumar Srivastav

Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

Isolation and Characterization of Rhizobial Strains from Some Leguminous Plant of Gorakhpur Region (India) and Their Assessment for PGPR Traits

Gupta Archana, Srivastava Srishti and Singh Pooja*

Bacteriology and Natural Pesticide Lab, Department of Botany, Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur 273009, Uttar Pradesh, India

**Corresponding Author*

Received: 12th March, 2024; **Accepted:** 24th May, 2024; **Published online:** 18th June, 2024

https://doi.org/10.33745/ijzi.2024.v10i01.102

Abstract: Root nodulating bacteria have immense potential as biofertilizers in sustainable agriculture that aid in soil fertility without the use of chemical fertilizers. Current work was carried out to isolate and characterize rhizobial strains from the root nodules of some legume plants. Plant samples were taken from 3different localities of Gorakhpur. A total of 18rhizobial isolates from various leguminous plants were examined for their morphological, biochemical and other attributes. All isolates were found to be rod shaped, Gram negative, fast growing and indole producers. These isolates were further analysed for their PGPR traits.

Keywords: Legumes, Root nodules, Rhizobia, Biochemical characterization, PGPR

Citation: Gupta Archana, Srivastava Srishti and Singh Pooja: Isolation and characterization of rhizobial strains from some leguminous plant of Gorakhpur region (India) and their assessment for PGPR traits. Intern. J. Zool. Invest. 10(1): 973-983, 2024.

https://doi.org/10.33745/ijzi.2024.v10i01.102

Introduction

Nitrogen, is an important component of amino acids, nucleic acids, nucleotides, chlorophyll, enzymes and hormones, and is a crucial nutrient for plants. However, almost all soils are deficient of nitrogen; hence nitrogen is considered a limiting element (Laurette *et al.*, 2015). Although nitrogen occupies about 78% of the atmospheric air, it is not readily accessible to plant unless in the form of soil nitrate (Arora, 2006). Legumes are beneficial both ecologically and agriculturally because they are responsible for a considerable role of the worldwideflux of nitrogen from atmospheric N_2 to fixed forms likeNH_3, NO_3, and organic nitrogen (Zahran *et al.*, 1991). Leguminous plants have a peculiar ability to establish symbiosis with nitrogen fixing bacteria of the family Rhizobiaceae. The group of bacteria that are members of genera *Rhizobium, Bradyrhizobium, Allorhizobium, Rinorhizobium, and Mesorhizobium* are referred to as "rhizobia" (Martinez-Romero, 2003; Willems, 2006). These bacteria can form nodules on their host plants,

where they fix nitrogen (Peoples *et al*., 1995). Many leguminous crops yield considerably enhanced when rhizobium inoculants were used, reducing the need for more costly synthetic fertilizers that degrade soil quality (Laurette *et al*., 2015).

Synthetic fertilizers for improving soil fertility are rarely available to most farmers especially in the rural areas of India. These fertilizers are not only costly and scarce, but are alsolost rapidly due to leaching, runoff and volatilization, causing water and atmospheric pollution. A natural phenomenon referred to as Biological Nitrogen-Fixation(BNF), is used by several bacteria and leguminous plants with nodules to convert nitrogen into a form that plants can use.A well conventional practice for maintaining soil fertility has been the cultivation of leguminous plants which replenish atmospheric nitrogen through symbiosis with rhizobia in rotation with non-leguminous plants (Deka and Azad, 2006).

The physiological state of the host plant and rhizobial strains has a significant impact on the Biological Nitrogen Fixation process. There are different methods by which rhizobial strains respond to various factors. These are considered to be essential criteria for differentiating these bacteria. Current work is an attempt to isolate some rhizobial strains from few selected fabaceous plants of Gorakhpur region and characterise them for their rhizobial and PGPR traits.

Materials and Methods

Sample collections and Isolation of rhizobial bacteria from root nodules:

Fabaceous plants with fresh and healthy pink root nodules were collected from 3 different localities of Gorakhpur region -- Sahjanwa, Pipraich and Railway side fields (Table 1; Fig. 1). Rajendran *et al*. (2008) used Yeast Extract Mannitol Agar (YEMA) to isolate *Rhizobium*. In this study, firm, pink, healthy, and undamaged nodules were chosen for isolation. The collected nodules were washed in running water to remove adhering soil particles and were surface sterilised using 0.1% $HgCl_2$ followed by washing in sterile water 5-6 times. The nodules were placed in 70% ethanol for 2 min and again washed in sterile water. Finally, they were crushed in 1 ml sterile distilled water with the help of flat sterile glass rod and made into uniform suspension. Nodule extract was serially diluted by adding 1 ml of extract in 9 ml of sterile distilled water. Using a sterilised glass spreader, 0.1 ml of each dilution's suspension was aseptically applied to each plate (triplets) on yeast extract mannitol agar with congo red dye (CRYEMA) 0.025 µg/ml (Fig. 2). The morphology of the colonies was assessed after 3-4 days of incubation at 27±1°C. The culture was purified by taking single colony through subsequent streaking. Bacterial colonies that were obtained were round, white, translucent, and shining. A single colony was picked up and taken to YEMA slant for further examination.

Morphological Characterization of Rhizobial Isolates:

The colony appearance (i.e. colour, size, shape, elevation, edge of the rhizobial colony and the growth rate) were ascertained by observing the colonies on YEMA-plates of one night lastedcultures at 27±1°C (Pervin *et al.*, 2017). The isolates were examined under a microscope using the Gram's staining (Fig. 3).

Gram's staining:

HiMedia K001-1 KT Gram's Stains Kit, was used to perform Gram's staining. YEM Broth was used to create an overnight rhizobial culture. Subsequently, smear was made on a glass slide and it was fixed by placing it over a mild flame. The smear was exposed to 1-2 ml of crystal violet for 30 sec, and it was then rinsed with distilled water for 30-40 sec. Slide was exposed to Gram's iodine for 30 sec and was washed again. Then, 1-2 ml of decolourising agent was used for 15-30 sec, and the slide was once again cleaned by using distilled water. Finally, the slide was counter-stained with safranin, washed and dried. The slide was then studied under a light microscope with oil immersion and 100x magnification (Reetha *et al.*,

Table 1: List of Legume plants and isolated Rhizobial strains

Region	Month of survey	Plant	Strain name
Pipraich	November	*Vicia* sp.	V.C.1 V.C.2
Pipraich	November	*Lathyrus* sp.	L.1 L.2
Pipraich	December	*Pisum sativum*	P.S.1 P.S.2
Sahjanwa	January	*Phaseolus vulgaris*	P.V.1 P.V.2
Sahjanwa	December	*Trigonella foenum-graecum*	T.F.1 T.F.2
Sahjanwa	July	*Mucuna pruriens*	M.P.1 M.P.2
Gorakhpur	May	*Crotolaria juncea*	C.J.1 C.J.2
Gorakhpur	October	*Alysicarpus parviflorus*	A.P.1 A.P.2
Gorakhpur	March	*Clover trifolium*	C.T.1 C.T.2

Fig. 1: Root nodules formed on leguminous plant.

2014). Results are given in Table 2.

Biochemical Characterization:

All the isolates were standardized by the method of McFarland and the concentration of the inoculum was adjusted to 1×10^{8}CFU/ml. About 30 µl of the inoculum suspension was streaked onto Petri plates containing YEMA. The plates were incubated at 27 ± 2°C for 4-5 days (Kucuk *et al.*, 2006; György *et al*., 2010). Each test was performed in triplicates. Catalase activity, indole test, citrate utilization test, Gelatin test, Voges Proskauer test (V.P.), and confirmatory tests such as Congo red test (Vincent ,1970), Keto lactose test (Bernaerts and De Ley, 1963) and Bromothymol Blue (BTB) agar test (Somasegaran and Hoben, 1994) were performed to differentiate *Rhizobium* and *Agrobacterium.* Rhizobial tests for PGPR traits performed were HCN test, Siderophore production and Ammonium production test. The growth of rhizobial colonies was recorded as + for slight growth, ++ for abundant growth and - for no growth. The results are given in Table 3.

Indole Test:

Production of indole acetic acid was observed in inoculated tryptophan broth (2 g/l). After 5-6 days of incubation (at 27 ± 2°), Kovac's reagent (1 ml) was added to the broth culture in a test tube. After 10-15 min, red coloured ring formation is positive

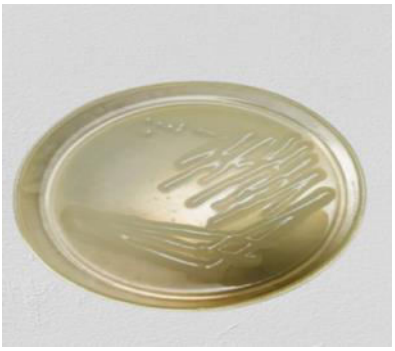

Fig. 2: Bacterial growth on YEMA medium.

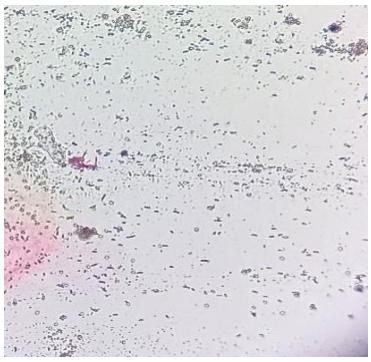

Fig. 3: Grams staining of isolates.

indole test (MacWilliams, 2016).

Methyl Red and Voges proskauer (*MR-VP*) *test*:

MR-VP broth composition is: Peptone-7 g/l, Dextrose- 5 g/l, Potassium phosphate – 5 g/l and distilled water 1000 ml. Methyl Red solution: Methyl Red – 100 mg, 95% Ethanol - 300 ml and distilled water 200 ml. Voges-Proskauer Reagent–I: ɑ- Napthol (5 g), Ethanol 100 ml. Voges-Proskauer Reagent-II: KOH- 40 g/100 ml water.

48 h old YEM broth culture with rhizobial strains was prepared. Rhizobial culture was incubated into MR-VP broth media and incubated for 48 h at 28°C. 3-5 drops of methyl red solution were added. For MR test, 9-10 drops of VP-I reagent were added in culture tubes while for VP test, 2-3 drops of VP-II reagent were added in the culture tubes. It did not turn to red colour showing negative test for MR-VP and a positive response for the presence of *Rhizobia*. Formation of red colour indicates positive result for MR and VP test (Ijutov, 1963; Clark and Lubs, 2015).

Citrate Utilization:

Simmon Citrate Agar 24.28 g/l (HiMedia) was the media for citrate utilization test. Each culture tube was filled with 10 ml of medium, and slants were prepared. They were streaked using bacterial inoculums and left overnight, maintaining a temperature of 27 ± 2° for 48 h. If isolates do not change from green to blue colour, they are citrate negative (Abdulkadir and Waliyu, 2012).

Gelatin Hydrolysis:

The test was performed to determine capability of the nodulating bacteria to produce gelatinase enzyme. Gelatin medium was prepared using 5 g/l peptone, 3 g/l beef extract and 12 g/l gelatin. Actively grown cultures were inoculated on nutrient gelatin medium for 48 h (Aneja, 2022). If the media remained in solid form and did not liquefy, it confirmed negative gelatin hydrolysis and absence of gelatinase enzyme.

Catalase Activity:

48 h-old isolates were submerged with H_2O_2 and

Table 2: Morphological characterizations of Rhizobial strains

Isolates	Form	Surface	Colony color	Opacity	Texture	Arrangement	YEMA growth	Mucus	Gram's stn.
A.P.1	Circular	Granular	Whitish	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-Ve
A.P.2	Circular	Smooth	Transluscent	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-Ve
C.J.1	Circular	Smooth	Transluscent	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-Ve
C.J.2	Circular	Smooth	Transluscent	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-Ve
C.T.1.	Circular	Smooth	Transluscent	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-Ve
C.T.2.	Circular	Smooth	Transluscent	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-Ve
L.A.1	Circular	Granular	Whitish	Opaque	Creamy	Cluster/ Single	Small mucoid	Low	-Ve
L.A.2	Circular	Granular	Whitish	Opaque	Creamy	Cluster/ Single	Small mucoid	Low	-Ve
M.P.1	Circular	Smooth	Transluscent	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-Ve
M.P.2	Circular	Granular	Whitish	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-Ve
P.S.1.	Circular	Granular	Whitish	Opaque	Creamy	Cluster/ Single	Small mucoid	Low	-Ve
P.S.2.	Circular	Granular	Whitish	Opaque	Creamy	Cluster/ Single	Small mucoid	Low	-Ve
P.V.1	Circular	Smooth	Transluscent	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-Ve
P.V.2	Circular	Smooth	Transluscent	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-Ve
T.F.1.	Circular	Smooth	Transluscent	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-VE
T.F.2.	Circular	Smooth	Transluscent	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-VE
V.F.1	Circular	Smooth	Transluscent	Opaque	Slimy	Cluster/ Single	Large mucoid	High	-VE
V.F.2	Circular	Smooth	Transluscent	Opaque	Creamy	Cluster/ Single	Large mucoid	High	-VE

the rhizobial colonies were observed for release of bubbles in the form of O_2 (Graham and Parker, 1964).

Confirmatory tests for the rhizobial strains:

After purification, growth of culture on glucose peptoneagar (GPA) medium, Congo red medium and 3 Keto lactose tests were performed as confirmatory testsfor strains of *Rhizobium.* The results are given in Table 4.

Growth on Glucose Peptone Agar (GPA) medium:

The peptone-glucose agar medium was prepared by dissolving peptone (10 g), glucose (5 g), agar (15 g) and 10 ml of Bromothymol blue in 1,000 ml of distilled water and the pH was adjusted to 6.8. Rhizobial isolates that were at least three days old, cultured in YEMB were streaked onto glucose Peptone agar (GPA) medium. The Petri plates were incubated at 27 ± 2°C for 5 days to observe the growth and change in the colour. Rhizobial colonies showed no growth or very poor growth on glucose peptone agar medium and caused very little change in pH when incubated at 27 ± 2°. Excessive growth is a sign of contamination (Vincent, 1970).

Congo red absorption test:

About 10 ml of stock solution of Congo red (0.25g of Congo Red in 100 ml distilled water) was added to 100 ml of YEMA medium and

Table 3: Biochemical Characterization of bacterial isolates

Isolates	Indole Test	Methyl Red Test	Citrate test	Gelatinase Test	Voges proskauer	Catalase Test
A.P.1	-ve (Yellow)	Yellow/ Fast	No color change	-ve	Yellow	Yes
A.P.2	-ve (Yellow)	Yellow/ Fast	No Color change	-ve	Yellow	Yes
C.J.1	-ve (Yellow)	Yellow/ Fast	No Color change	-ve	Yellow	Yes
C.J.2	-ve (Yellow)	Yellow/ Fast	No Color change	-ve	Yellow	Yes
C.T.	-ve (Yellow)	Yellow/ Fast	No Color change	-ve	Yellow	Yes
C.T.2.	-ve (Yellow)	Yellow/ Fast	No Color change	_ve	Yellow	Yes
L.A.1	-ve (Yellow)	Yellow/ Fast	No Color change	-ve	Yellow	Yes
L.A.2	-ve (Yellow)	Yellow/ Fast	No Color change	-ve	Yellow	Yes
M.P.1	-ve (Yellow)	Yellow/ Fast	Color change	-ve	Yellow	Yes
M.P.2	-ve (Yellow)	Yellow/ Fast	No Color change	-ve	Yellow	Yes
P.S.1.	-ve (Yellow)	Yellow/ Fast	No Color change	-ve	Yellow	Yes
P.S.2.	-ve (Yellow)	Yellow/ Fast	No Color change	-ve	Yellow	Yes
P.V.1	-ve (Yellow)	Yellow/ Fast	Color change	-ve	Yellow	Yes
P.V.2	-ve (Yellow)	Yellow/ Fast	Color not change	-ve	Yellow	Yes
T.F.1.	-ve (Yellow)	Yellow/ Fast	Color not change	-ve	Yellow	Yes
T.F.2.	-ve (Yellow)	Yellow/ Fast	Color not change	-ve	Yellow	Yes
V.F.1	-ve (Yellow)	Yellow/ Fast	Color change	-ve	Yellow	Yes
V.F.2	-ve (Yellow)	Yellow/ Fast	No Color change	-ve	Yellow	Yes

Table 4: Conformity tests

Isolates	GROWTH ON GPA	CONGO RED ABSORPTION	KETO-LACTOSE TEST	YEMA+BTB
A.P.1	Negative	non- absorbing	Negative	Yellow
A.P.2	Negative	non- absorbing	Negative	Yellow
C.J.1	Negative	non- absorbing	Negative	Yellow
C.J.2	Negative	non- absorbing	Negative	Yellow
C.T.	Negative	non- absorbing	Negative	Yellow
C.T.2.	Negative	non- absorbing	Negative	Yellow
L.A.1	Negative	non- absorbing	Negative	Yellow
L.A.2	Negative	non- absorbing	Negative	Yellow
M.P.1	Negative	non- absorbing	Negative	Yellow
M.P.2	Negative	non- absorbing	Negative	Yellow
P.S.1.	Negative	non- absorbing	Negative	Yellow
P.S.2.	Negative	non- absorbing	Negative	Yellow
P.V.1	Negative	non- absorbing	Negative	Yellow
P.V.2	Negative	non- absorbing	Negative	Yellow
T.F.1	Negative	non- absorbing	Negative	Yellow
T.F.2.	Negative	non- absorbing	Negative	Yellow
V.F.1	Negative	non- absorbing	Negative	Yellow
V.F.2	Negative	non- absorbing	Negative	Yellow

autoclaved. The three-day old culture grown in YEMA was streaked onto Petri plant containing Congo red supplemented YEMA medium. The Petri plates were sealed with parafilm and incubated at 27 ± 2°C for 5 days. The colonies of *Rhizobium* isolates obtained did not show absorption of Congo red.

Keto-lactose test:

To distinguish rhizobial strains from other contaminating bacteria, this test is frequently used. Keto-lactose agar medium was prepared and isolates were streaked on plates and then incubated at 27 ± 2° for 48 h. After 1 h incubation period at 27 ± 2°C the plates being flooded with Benedict's Reagent, results were observed. Absence of development of yellow colour indicate that lactose cannot be utilised by *the* rhizobial strains (Bernaerts and De Ley, 1963).

Acid-Base production Test (*YEMA +BTB*):

Isolated strains were streaked on YEMA containing bromothymol blue, and incubated. The colour observed was yellow which signified acid production; as a positive test. While blue colour (alkali production) was a negative result (Jordan, 1984).

Characterization of bacterial isolates for PGPR traits:

HCN Production test:

All of the isolates were checked for the ability to produce hydrogen cyanide by slightly altering the procedure. King's B agar medium was added with 4.4 g glycine/l and the media was prepared. The prepared media was streaked with the bacterial isolates. At the top of the plate, Whatman filter paper No. 1 soaked in a solution of 0.5% picric acid and 2% sodium carbonate was kept. Plates were sealed with parafilm and incubated at 27±2 °C for 4 days. HCN production was indicated by a hue changing from orange to red (Reetha *et al*., 2014).

Siderophore production:

Siderophore production was estimated qualitatively. Chrome Azurol S (CAS) Agar medium was used for the detection of siderophores. Each isolate was grown in synthetic medium, containing 0.5 µM of iron and incubated for 24 h on a rotary shaker at room temperature. The culture supernatant was examined using the CAS plates to determine whether siderophores were present. Culture supernatant was streaked on to the CAS agar plates and incubated at room temperature for 48 h. Formation of yellow to orange coloured zone around the rhizobial colonies indicated siderophore production (Schwyn and Neilands, 1987).

Ammonium Production:

The isolates were tested for ammonia production

by inoculating the isolates into 10 ml of pre-sterilized peptone water in the test tubes. The tubes were incubated for 48-72 h at 27±2 °C. Nessler's reagent (0.5 ml) was added in each tube. Change in colour of the medium from brown to yellow colour was taken as positive test for ammonia production (Bhattacharyya *et al*., 2020).

Results and Discussion

Nitrogen fixing (Rhizobia) bacteria of the soil usually called *Rhizobium* have a considerable importance in agriculture because of their capacity to fix the atmospheric nitrogen in symbiosis with the legumes.

A total of 18 Rhizobacterial strain were successfully isolated from root nodule of different leguminous plant at different localities. These strains were named as C.T. (*Clover trifolium*), T.F. (*Trigonella foenum-graecum*), P.S. (*Pisum sativum*), P.V. (*Phaseolus vulgaris*), L.A. (*Lathyrus aphaca*), V.F. (*Vicia faba*), M.P. (*Mucuna pruriens*), C.J. (*Crotalaria juncea*), A.P. (*Alysicarpus spp.*) (Table 1).

The majority of the isolates generated circular, smooth/ granular surface, whitish/translusent, opaque, slimy colonies indicating that they were *Rhizobium* spp. It was found that each isolate was rod-shaped and Gram negative. Motile and

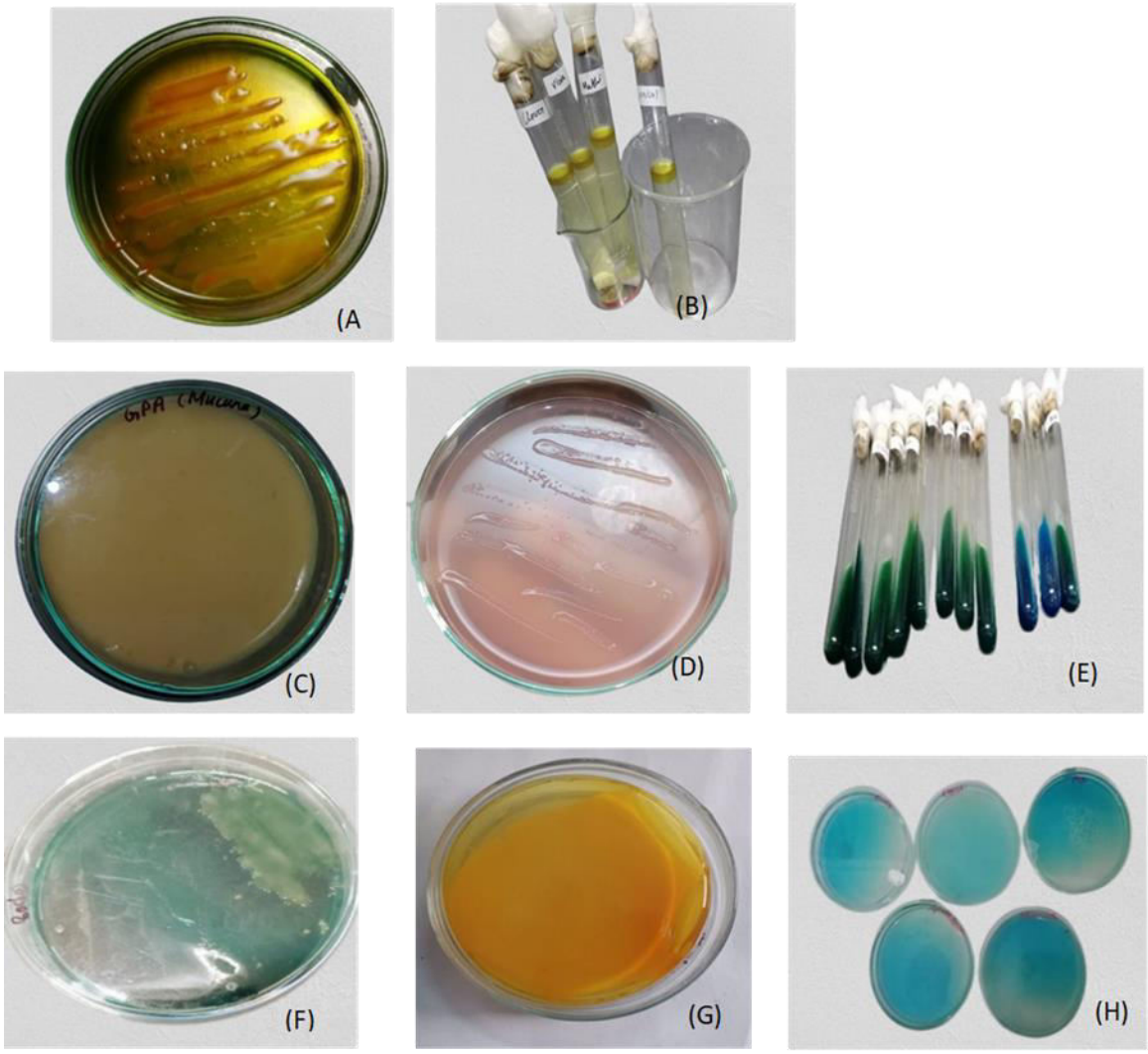

Fig. 4: (A)Bromothymol blue fast-growing rhizobial strain; (B) Voges Proskauer; (C) No growth on GPA test; (D) Growth on CREMA medium; (E) Citrate test ; (F) Siderophore test; (G) HCN test; (H) 3- Keto lactose test.

rod-shaped cells were observed under light microscope with 100X magnification (Fig. 4). The results showing the colony morphology, Gram's reaction and cell shape of selected rhizobial isolates are presented in Table 2. Similar observations have also been reported by other researchers (Deora and Singhal, 2010; Deshawal and Chaubey, 2014). Based on the aforementioned data, it may be clearly inferred that all of the bacterial isolates were rhizobial species.

All isolates were tested by selective biochemical tests which are presented in Table 3. All strains showed negative result for methyl red, indole test, VP (Voges Proskauer) and gelatin test. For citrate utilization test, all the strains except M.P.1, P.V.1 and V.F.1 showing negative citrate test (no change in colour). For catalase test, all isolates showed positive test. Similar observations have been reported by Mujahidy (2013) and Rajpoot and Panwar (2013). Pervin *et al.* (2017) also observed catalase positive and citrate negative activities in Rhizobial isolates from nodules.

In Congo red test, all the isolates showed poor absorption of dye Congo red. This fact gave further evidence for purity of Rhizobial isolates.

Table 5: Characterization of rhizobial isolates for PGPR traits

ISOLATES	HCN	SIDEROPHORE	AMMONIUM PRODUCTION
A.P.1	+++	++	+
A.P.2	+++	++	+++
C.J.1	++	+	+
C.J.2	+	+	+
C.T.1.	+	+	+
C.T.2.	+	+	+
L.A.1	+	+	+
L.A.2	+	+	+
M.P.1	++	+	+
M.P.2	++	+	+
P.S.1.	+++	+	+
P.S.2.	+++	+	+
P.V.1	+++	+++	++
P.V.2	+++	+++	++
T.F.1.	++	+	++
T.F.2.	++	+	++
V.F.1	++	++	+
V.F.2	++	++	+

Purified isolates could be classified as fast (turn medium yellow) and slow growing (turn medium Blue) rhizobiaon YEMA supplemented with Bromo thymol blue. Blue coloured colonies indicated alkali production while yellow colonies indicated acid production. All the isolates studied were fast growing Rhizobia. Similar result is also reported by Sharma *et al. (*2009), Gauri *et al*. (2011), and Gachande and Khansole (2011).

Further conformity of rhizobial strains performed on 3-keto lactose Agar (Table 4) showed that all 18 isolates are negative for the production of keto lactose and no yellow zone on copper oxide (Cu_2O) around the colony were observed (Fig. 4). This is a specific observation for rhizobial strains differentiating it from *Agrobacterium*. Further confirmation of the rhizobial strains was done by GPA test. All 18 isolates showed either poor or no growth on the glucose peptone media after one day incubation at 29 ºC. This again helped in distinguishing the rhizobial strains against *Agrobacterium* (Fig. 4).

HCN production by the bacterial isolates has been reported to play a role in suppression of many plant diseases. A positive correlation between production of HCN and suppression of root rot by bacterial isolate has been reported (Defago *et al*., 1990). In present study 18 isolates were tested for HCN production on nutrient Agar plate supplemented with 14% glycine. P.S.1 P.S.2, P.V.1 P.V.2, A.P.1 and A.P.2 showed maximum HCN production (Table 5) and T.F.1, T.F.2, V.F.1, V.F.2, M.P.1, M.P.2 and C.J.1, showed moderate amount of HCN production but C.T.1, C.T.2, L.A.1, L.A.2 and C.J.2 showed poor HCN production (Table 5).Although the role of microbial HCN in disease suppression is not considered to be firmly established. HCN may be involved in the induction of plant resistance towards diseases (Schippers, 1988).

Siderophore production has been reported to be beneficial in growth and promotion of disease suppression in the plants. Among 18 isolates tested, P.V.1 and P.V.2 produced more siderophore while other 16-isolate were moderately positive for Siderophore production. The orange zone surrounding the colony indicated secretion of Siderophore and the diameter of the zone depicted the amount of Siderophore produced by rhizobial isolates. 16 isolates which were found positive for Siderophore production were further subjected for screening of Ammonia production in peptone broth by using Nesslers nutrient broth. Strains P.V.1, P.V.2, A.P.2, T.F.1, T.F.2, showed a clear color change from yellow to brown indicating positive test for ammonia production.

Thus, the most promising strains found with respect to PGPR traits were A.P.2, P.V.1 and P.V.2. These possess significant PGPR characteristics that will meet plant system requirements. They have all soil-fertility enhancing capabilities and can be used industrially as an important biofertilizer. The strains A.P.2., P.V.1 and P.V.2 can further be utilised for various studies related to their compatibilities with synthetic pesticides and natural bio-control agents. This eventually can prove up to what extent synthetic pesticides harm the rhizobial species in soil. In addition, the replacement of these synthetic pesticides with the natural biocontrol agents can also be examined.

Conclusion

We have isolated strains of root nodulating bacteria which fix nitrogen. Therefore, such strains could be used to effectively nodulate legume plants in order to fix nitrogen biologically. These results open novel opportunities for further investigation. Most of the bacterial strains isolated from the leguminous plants were closely related to *Rhizobium* species based on their morphological and biochemical characteristics. *Rhizobium* is a symbiotic microbe that is beneficial for soil fertility because it can fix nitrogen in legume plants. Also, the PGPR activities of the rhizobial species have been tested and confirmed demonstrating their significance as a natural, sustainable bio- fertiliser for plants growth.

Acknowledgements

The authors are thankful to Head, Department of Botany, Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur, U.P., India for providing necessary facilities to conduct the research work

References

Abdulkadir M and Waliyu S. (2012) Screening and isolation of soil bacteria for ability to produce antibiotics. European J Appl Sci. 4(5): 211-215.

Aneja KR. (2022) Experiments in microbiology plant pathology and tissues culture and microbial biotechnogy, 6th edn., New Age International Publishers, New Delhi, India.

Arora DR. (2006) The Text Book of Microbiology, CBS Publisher, New Delhi.

Bernaerts MJ and De Ley J. (1963) A biochemical test for crown gall bacteria. Nature (London) 197: 406-407.

Bhattacharyya C, Banerjee S, Acharya U, Mitra A, Mallick I, Halder A, Halder S, Ghosh A and Ghosh A. (2020) Evaluation of plant growth promotion properties and induction of antioxidative defense mechanism by tea rhizobacteria of Darjeeling, India. Sci Rep. 10(1): 15536.

Clark WM and Lubs HA. (2015) The differentiation of bacteria of the Colon-Arogenes family by the use of indicators. J Infect Dis. 17: 160-173.

Defago G, Berling CH, Burger U, Haas D, Kahr G, Keel C, Voisard C, Wirthner PJ and Wüethrich B. (1990) Suppression of black root rot of tobacco and other root diseases by strains of *Pseudomonas fluorescens*: Potential applications and mechanisms. In: Biological control of soil-borne plant pathogens, Oxon: CAB International, pp.93-108.

Deka AK and Azad P. (2006) Isoaltion of rhizobial strains cultural and biochemical characteristics. Legume Res. 29(3): 209-212.

Deora GS and Singhal K. (2010) Isolation, biochemical characterization and preparation of biofertilizers using Rhizobium strains for commercial use. Biosci Biotech Res Comm. 3(2): 132-136.

Deshwal VK and Chaubey A. (2014) Isolation and characterization of *Rhizobium leguminosarum* from root nodules of *Pisum sativum* L. J Acad Indus Res

Comm. 2(8): 464-467.

Gachande BD and Khansole GS. (2011) Morphological, cultural and biochemical characteristics of *Rhizobium japonicum* syn and *Bradyrhizobium japonicum* of soybean. Biosci Discov 2(1): 1-4.

Gauri, Singh AK, Bhatt RP, Pant S, Bedi MK and Naglot A. (2011) Characterization of Rhizobium isolated from root nodules of *Trifolium alexandrinum.* J Adv Agric Technol. 7(6): 1705-1723.

Graham PH and Parker CA. (1964) Diagnostic features in the characterization of root nodule bacteria of legumes. Plant Soil 20: 383-396.

György E, Mara G, Máthé I, Laslo E, Márialigeti K, Albert B, Oancea F and Lányi S. (2010) Characterization and diversity of the nitrogen fixing microbiota from a specific grassland habitat in the Ciuc Mountains. Romanian Biotechnol Letts. 15(4): 5474-5481.

Ijutov V. (1963) Technique of Voges-Proskauer test. Acta Path Microbiol Scand. 58: 325-335.

Jordan D. (1984) Rhizobaceae. In: Bergey's Manual of Systematic Bacteriology, (eds.) Hendricks D.P., Sneath H.A. and Halt J.H., Orient Longman, New York.

Kleczkowska J, Nutman PS, Skinner FA and Vincent JM. (1968) The identification and classification of Rhizobium. Academic Press, London.

Kucuk CM, Kivanç M and Kinaci E. (2006) Characterization of Rhizobium sp. isolated from Bean. Turk J Biol. 30: 127-132.

Laurette NN, Maxémilienne NB, Henri F, Souleymanou A, Kamdem K, Albert N, Dieudonné N and François-Xavier E. (2015) Isolation and screening of indigenous Bambara groundnut (*Vigna subterranea*) nodulating bacteria for their tolerance to some environmental stresses. Am J Microbiol Res. 3(2): 65-75.

MacWilliams MP. (2016) Indole Test Protocol. American Society for Microbiology. https://asm.org/getattachment/200d3f34-c75e-4072-a7e6-df912c792f62/indole-test-protocol-3202.pdf

Martínez-Romero E. (2003) Diversity of rhizobium-*Phaseolus vulgaris* symbiosis, overview and perspectives. Plant Soil 252: 11-23.

Mujahidy J, Hassan M, Rahman MM and Rashid ANMM. (2013) Isolation and characterization of *Rhizobium* spp. and determination of their potency for growth factor production. Int Res J Biotechnol. 4(7): 117-123.

Peoples MB, Herridge DF and Ladha JK. (1995) Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production. Plant Soil 174: 3-28.

Pervin S, Jannat B, Sanjee S and Farzana T. (2017) Characterization of rhizobia from root nodule and rhizosphere of *Lablab purpureus* and *Vigna sinensis* in Bangladesh. Turkish J Agricult Food Sci Technol. 5(1): 14-17.

Rajendran G, Sing F, Desai AJ and Archana G. (2008) Enhanced growth and nodulation of pigeon pea by coinoculation of *Bacillus* strains along with *Rhizobium sp.* Bioresour Technol. 99(11): 4544–4550.

Rajpoot P and Panwar KS. (2013) Isolation and characterization of rhizobia and their effect on *Vigna radiata* plant. Octa J Biosci. 1(1): 69-76.

Reetha AK, Pavani SL and Mohan S. (2014) Hydrogen cyanide production ability by bacterial antagonist and their antibiotics inhibition potential on *Macrophomina phaseolina* (Tassi.) Goid. Int J Curr Microbiol App Sci. 3(5): 172-178.

Schippers B. (1988) Biological control of pathogens with rhizobacteria. Phil Trans R Soc London B 318: 283-293.

Schwyn B and Neilands JB. (1987) Universal chemical assay for the detection and determination of siderophores. Analytical Biochem. 160(1): 47-56.

Sharma MP, Srivastava K and Sharma SK. (2009) Biochemical characterization and metabolic diversity of soybean rhizobia isolated from Malwa region of Central India. Plant Soil Environ. 56: 375-383.

Somasegaran P and Hoben HJ. (1994) Handbook for Rhizobia: Methods in legume-Rhizobium technology. Springer, New York.

Vincent JM. (1970) Manual for the Practical Study of Root Nodule Bacteria. Blackwell Scientific Publication, Oxford.

Willems A. (2006) The taxonomy of rhizobia an overview. Plant Soil 287: 3-14.

Zahran HH. (1991) Conditions for successful *Rhizobium*-legume symbiosis in saline environments. Biol Fertility Soils 12: .73-80.