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Isolation and characterization of rhizobia from the root nodule of some cultivated legume crops

Som Prasad Paudyal [1], Birendra Kunwar [1], Niroj Paudel [2, 3]*, Bishnu Dev Das [4]

[1] Department of Botany, Trichandra Multiple Campus (Tribhuvan University), Kathmandu, Nepal

[2] National Institute of Horticultural and Herbal Science, Rural Development Administration, Wanju 55365, Republic of Korea

[3] Department of Applied Plant Science, Kangwon National University, Chuncheon 24341, Republic of Korea

[4] Department of Botany, Mahendra Morang Aadarsha Multiple Campus (Tribhuvan University), Biratnagar, Nepal

* Corresponding author: E-mail: nirojjirauna@gmail.com

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ABSTRACT: Biological nitrogen fixation (BNF) as a result of mutual symbiosis between the rhizobia and the cultivated legume have a vital role to balance the nutrient paucity in the soil. Modern researches shows that the BNF can be the important factors regulating and maintaining the defendable agriculture and ensuring food security especially in the developing countries. The use of artificial nitrogen fertilizers to rise crop yield is an internment farming practice, despite its unfavorable effects and hazards to the environment and human population which can be substituted by rhizobial inoculants as a bio-fertilizers. The present study was aimed to isolate and characterize the *Rhizobium* from the nine different selected legumes. The *Rhizobium* bacterium was isolated from the nodules of the nine legume plants on YEMA medium which was found to show white translucent, circular convex colonies and characterized by the non-absorption of Congo red dye. The strains were found to be fast growing except for the rhizobial strains isolated from the Soybean and cowpeas (*Bradyrhizobium*) which were slow growing. The various biochemical tests of the isolated strains like catalase, bromothymol blue, Urea hydrolysis were favorable while Glucose-Peptone Agar (GPA), starch hydrolysis, Citrate utilization were found to be negative. For the ability to utilize the 2% NaCl, the strains TFR showed positive growth, the strains PSR, PhVR, VUR showed the poor tolerance while the rest of the strains showed no tolerance to the 2% NaCl.

Keywords: Biological Nitrogen Fixation; Bio-inoculants; Defendable agriculture; Legumes; *Rhizobium*.

1. INTRODUCTION

Legumes such as beans, peas, nuts, etc. have been used in agriculture since the time of immemorial as food Legumes play several vital roles [1]. Beneficial for crop defense reduced input for the crops rotation for improvement efficiency and incising landscape crises which makes balancing the defect in plant protein formulation in different world for developed population [2-4]. Eighty percent nitrogen produced from the symbiosis is involving leguminous plants and Rhizobiaceae [5].

The unique property of legumes to associate with the nitrogen fixing bacteria to establish symbiosis relation, has helped in increasing the crop production to the mankind. Specificity for nodulation and N_2 fixation varies greatly among the legumes [6]. The multiple interaction and signaling bacteria and host cells which is early stage here flavonoids changes the NodD proteins as activated form and its binding nod box initiates the transcription of inducible nod operons [7]. Most of the *Rhizobium* abundant extra-cellular or exo-polysachharide slime that range from watery consistency to a tenacious gum [8, 9]. According to the growth rate (generation time) on laboratory media, rhizobia, are broadly classified into two groups, slow grower and fast grower. Slower growers (generation time > 6 h) refers to those rhizobia associated soybean and cowpea whereas, fast growers (generation time < 6 h) refers to those rhizobia associated with bean and pea [8, 10]. Fred et al. [11] stated that the host ranges is an important factors in defining the species and alsoin describing the morphological and physiological differences. The Bergey's manual of systematic bacteriology is used for the classification of the rhizobia [8]. On the basis of small sub-unit ribosomal RNA data, the rhizobia are sub-divided into 3 genera; *Rhizobium, Bradirhizobium* and *Azorhizobium* [12]. Young et al. [13] classify, about 38 species in five genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Azorhizobium* are recognized. Weir [14] currently listed out the 98 species of rhizobial under 13 genera. Legume have power of symbiotic relationship with soil bacteria for trapping N_2 from the atmosphere which convert into ammonia. The temperature for artificial nodulation, survival and persistence of rhizobial strain is depending upon the natural habit, temperature various strain [15]. When the flavonoids in the rhizosphere , starts communication between the plant host and rhizobia [16]. Master regulation of symbiosis is determined by the flavonoid binding transcriptional regular Nod [17].

Nod factors modify different molecules spcially consist of oligosaccharides consisting of four or five beta-1,4-linked N-acetyl glucosamine residues with a fatty acid residue replacing the N-acetyl group at the non-reducing end [16]. The genes *nodABC* conserved in all rhizobia except strains *BTAi1* and *ORS278* [18]. The root nodules of *Cicer arietinum* plants harbor the nitrogen-fixing bacterium *Rhizobium* from the isolate and identify *Rhizobium* from *Cicer arietinum* root nodules by using CRYEMA medium [19].

Isolation and characterization of *Rhizobia* are given by Shahzad et al. [20]. Prajapati et al. [21] isolated *R. meliloti* on CRYEMA from the root nodules and rhizosphere soil of fenugreek, out of 9 isolates 4 absorbed congo red and 5 did not. Cultural characteristic for colony color and shape except for colony size are similar from the isolates.

The *Rhizobium* is isolated from the root nodule of the *Vicia faba* [22]. Morphologically, physiologically, biochemically and on the basis of molecular biology were tested [23]. The aim of the present research is isolation of *Rhizobium* strain from root nodule of the selected legume plants with characterization of the *Rhizobium* bacteria. And, also to find the authentication of the bacteria by nodulation method to the specific host and check the viability of the bacterium for trans-inoculation.

2. MATERIALS AND METHODS

2.1. Collection of root nodules

Nodules from nine different cultivated varieties of the legumes were collected from the Sanfebagar municipality-08, Kantain, Achham, Nepal. The nodules from the following plants were collected (*Vigna mungo*, *Glycine max, Cicer arientinum*, *Pisum sativum*, *Vicia faba, Trigonella foenugraecum, Vigna unguiculata*, *Lablab purpureus, Phaseolus vulgaris*).

2.2. Isolation of bacteria from root nodules

Collected root nodule were washed in tap water to remove the adhering soil particles. They were dipped in 0.1% mercuric chloride ($HgCl_2$) and sodium hypochlorite solution for about 20 seconds in sterile Petriplates. The nodules were then washed several times with sterile distilled water to remove the traces of $HgCl_2$. The surface sterilized nodules were then crushed in sterile water kept in a test tube. The resulting suspension was streaked on the surface of Yeast Extract Mannitol Agar (YEMA). All the chemicals were accurately weighed and mixed thoroughly in distilled water in the calibrated conical flask. The flask containing the medium was plugged with the non-absorbent cotton. It was then autoclaved at 15 lbs. pressure at 121°C for about 30 minutes. The medium was poured in the sterile petridishes (approx. 20-ml in each) aseptically and allowed to solidify. The plates were then incubated at 30±1°C for 24 hrs. so as to avoid the contaminated plates. These were then streaked with crushed suspension of nodules in triplicates. The plates were incubated at 28±1°C for 12 hrs. The colonies developed were picked up by the help of platinum loop and transferred to the YEMA slants for further study. Composition of YEMA media (Mannitol, 10 g; K_2HPO_4, 0.5 g; $MgSO_4$•7 H_2O, 0.2 g; NaCl, 0.1 g; Yeast extract, 1 g; Agar extract, 15 g; Distilled water; 1 L and pH 7).

All the chemicals were accurately weighed and mixed thoroughly in distilled water in the calibrated conical flask. The flask containing the medium was plugged with the non-absorbent cotton. It was then autoclaved at 15 lbs. pressure at 121°C for about 30 minutes. The medium was poured in the sterile petridishes (approx. 20 ml in each) aseptically and allowed to solidify. The plates were then incubated at 30±1°C for 24 hrs. So as to avoid the contaminated plates. These were then streaked with crushed suspension of nodules in triplicates. The plates were incubated at 28±1°C for 12 hrs. The colonies developed were picked up by the help of platinum loop and transferred to the YEMA slants for further study.

2.3. Morphological, biochemical and molecular characterization of the isolated strains

Nine strains with five replicates each from the selected nine different legumes specimens were isolated and the strains were characterized by the following tests.

2.3.1. Gram staining

Gram staining [24] of the cultured isolates were done to provide information as presumptive tests of *Rhizobium.*A drop of 12 h old culture was evenly smeared on a clean slide and heated gently to fix. Crystal violet was applied with a dropper and was allowed to react for exactly 1 minute, followed by a gentle wash in a tap water. Iodine was then applied and allowed to react for 1 minute. Then, iodinated alcohol was applied and left for 5 minutes. Finally, the counter stain Safranine was applied to react for 5 minutes. It was washed with tap water very gently. The slides were air dried and examined under oil immersion in a light microscope.

2.3.2. Growth on YEMA medium

The isolated strains were streaked o the YEMA plates to see the colony size, morphology and other characteristics.

2.3.3. Growth on Congo Red+ YEMA

YEMA incorporated with 0.0025% (W/V) congo red so as to differentiate the contamination along with the rhizobial colony in the medium. Rhizobial colonies remain white, translucent, elevated and excessively mucilaginous. Results of plates were recorded after 24 hrs.

2.3.4. Growth on Glucose Peptone Agar (GPA)

Rhizobia cannot or poorly utilize peptone whereas *Agrobacterium* can utilize and grow very fast on this medium. Isolates were streaked upon the Glucose Peptone Agar (GPA) medium and the plates were incubated for 7 days at 30°C. The composition of the medium is as followed the Composition of Growth on Glucose Peptone Agar (GPA; Glucose, 10 g; Peptone, 20 g; NaCl, 5.0 g; Agar-agar, 15 g; Distilled water, 1L; pH 7).

2.3.5. Utilization of citrate

YEM agar containing sodium citrate in place of mannitol is supplemented with 1 ml of bromothymol (BTB) blue solution and incubated at 30°C. After 7 days of incubation changes in color and growth was recorded.

2.3.6. Ability to produce 3-ketolactase

Mannitol in YEM agar is replaced with lactose. The plates were streaked and incubated for 48 h at 30°C. After incubation the plates were flooded by pouring Benedicts reagent and further incubated for 1 h. Appearance of yellow ring around the colonies indicates the production of 3-ketolactase.

2.3.7. Growth on 2% NaCl

Tolerance to sodium chloride stress was determined on YEMA plates containing 2% NaCl. The plates were incubated with bacterial streak for 7 days and observed for the growth.

2.3.8. Acid production in YEM agar

YEM agar amended with 0.001% bromo-thymol blue indicator dye and then inoculated with the exponentially growing culture, which was incubated at 28°C. After 72 h, the change in color of the media along the region of bacterial growth was observed.

2.3.9. Catalase activity

The cultures were streaked upon YEM agar plates and indicated for 72 h at 30°C. After incubation the cultures were flooded over by hydrogen peroxide (H_2O_2).

2.3.10. Hydrolysis of urea

Exponential phase culture inoculated with in the tubes containing YEM medium repair with 2% (W/V) urea and 0.012% phenol red. It was incubated for 7 days at 30°C.

2.3.11. Hydrolysis of starch

Mannitol, in Yem agar is replaced with the modified medium is then streaked upon with the cultures of isolated strains and incubated for 48 h for 30°C. After incubation the plates are flooded over by iodine solution.

2.3.12. Utilization of various carbon sources

For Carbohydrates utilization basal medium was used. Different carbohydrates were substituted for mannitol in YEM agar medium in which yeast extract is reduced. The plates were streaked with the log phase culture and incubated for 72 h at 28°C. The results were noted for bacterial growth comparing with that of

control. Carbohydrates used were mannitol, fructose, glucose, sucrose, lactose, maltose, sorbitol, dextrose, rhamnose, trehalose, raffinose, arabinose and starch.

2.3.13. Plant infection test and cross inoculation studies

All the isolates were checked for the nodulation on their original hosts as well as other legumes. This study was carried out in the earthenware pots. The seeds were collected from the local market. The garden soil was autoclaved so as to disinfect from the micro-organisms and pre-existing rhizobia and mixed with the sterilized coco pit. The mixed soil containing sterilized coco pit and the garden soil in the ratio 2:1 was filled in the pots. Surface sterilized seeds were soaked in water overnight and were inoculated by exponentially growing log phase bacteria. The seeds were then sown on the pots. Plants were watered and uprooted after 30 days to observe the nodulation.

3. RESULTS

3.1. Morphological characteristics

All the strains isolated on YEM agar medium each from the nine different plant species were cultured and showed the best positive growth at 28±2°C in YEM agar medium and morphologically tested. The strains were found to be fast growing except for the rhizobial strains isolated from the soybean and cowpeas (*Bradyrhizobium*) which were slow growing. All the strains were motile, gram negative, rod shaped. The colonies on YEM agar were circular, non-spreading, translucent, convex, smooth, entire and odorless with 2-4 mm in diameter after 48 h of incubation at 28±1°C. The excessive secretion of the mucilaginous exopolysaccharide was found. The isolates were characterized by the non-absorption of Congo red dye up to 24 h of growth but afterwards the colonies started to absorb it. Gram's staining of the isolates was confirmed by microscopic observations and all the rhizobial isolates were found to be gram negative and rod shaped (Table 1).

Table 1. Morphological characteristic of rhizobial strain.

SN	Strain characteristic	Rhizobial strain
1	Shape	Circular
2	Size	2-2.5 mm
3	Color	White
4	Opacity	Translucent
5	Bacterium shape	Rod shaped
6	Gram staining	Gram negative
7	Motility	Mobile

3.2. Cultural and biochemical tests

Rhizobial isolates were studied for their biochemical characteristics. Best growth of the rhizobial isolates were observed on YEMA medium (Figure 1). Very poor or no growth was observed on Glucose Peptone Agar (GPA). All the strains were unable to produce 3-keto lactase when the strains were cultured at 30°C in the YEM agar medium, Mannitol replaced with lactose. Catalase activity was found to be positive for all the strains isolated from 10 different plant species. For the ability to utilize the 2% NaCl, the strains TFR showed positive growth, the strains PSR, PhVR, VUR showed the poor tolerance (out of 5 strains 3 showed

positive) while the rest of the strains showed no tolerance to the 2% NaCl. All the strains showed the positive urea hydrolysis test which was conferred by change in color of the petriplates into yellow/orange. Study showed that the isolates acidified the YEM agar bromothymol blue after 24 to 48 h determined by change in color except the GMR and VUR strains which showed the negative test for the acid production. Citrate utilization test and hydrolysis of starch was found to be negative (Table 2).

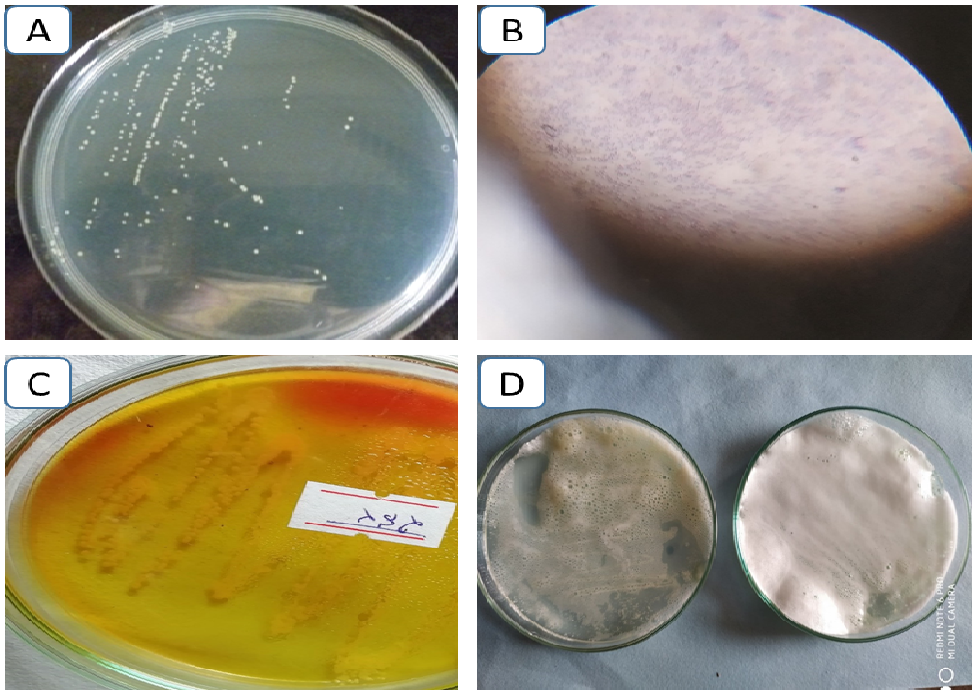

Figure 1. A – Rhizobial colonies isolated on YEM agar medium; B – Morphology of the isolates under after Gram staining; C – Colonies of rhizobia isolates on yeast mannitol agar medium containing bromothymol blue (yellowish, smooth margins, medium sized, round colonies), D – Catalase activity test.

Table 2. Biochemical characterization of the strains from the nine different legumes ("G(-)" – Gram-negative, "-" – No growth observed, "+" – Positive growth was observed).

Test	Strains of rhizobia								
	VMR	**GMR**	**CAR**	**PSR**	**VFR**	**TFR**	**VUR**	**LPR**	**PhVR**
Gram Stain	G(-)	G(-)	G(-)	G(-)	G(-)	G(-)	G(-)	G(-)	G(-)
Acid production	+	+	+	+	+	+	+	+	+
Growth on GPA	-	-	-	-	-	-	-	-	-
Citrate Utilization Test	-	-	-	-	-	-	-	-	-
Ability to Utilize 3-keto-lactase	-	-	-	-	-	-	-	-	-
Growth on 2% Nacl	-	-	-	poor	-	+	poor	-	poor
Catalase Activity	+	+	+	+	+	+	+	+	+
Urea Hydrolysis	+	+	+	+	+	+	+	+	+
Starch Hydrolysis	-	-	-	-	-	-	-	-	-

VMR – *Vigna mungo* (L.) Hepper strains, GMR – *Glycine max* (L.) Merr. strains, CAR – *Cicer arientinum* L. strains, PSR – *Pisum sativum* L. strains, VFR – *Vicia faba* L. strains, TFR – *Trigonella foenugraecum* L. strains, VUR – *Vigna unguiculata* (L.) Walp strains, LPR – *Lablab purpureus* (L.) Sweet strains, PVR – *Phaseolus vulgaris* L. strains.

3.3. Utilization of various carbon sources

Test for the utilization of various carbon sources for the five replicate strains each from nine isolated rhizobia, showed that there was a complete inability of the strains to utilize the starch but in other carbon sources the growth was found to be good. Fructose, glucose, sucrose, lactose, maltose, dextrose anhydrous, arabinose, trehalose and starch were used as the sources of the carbon and all the strains showed good growth on all the carbon sources except the starch (Table 3).

Table 3. Utilization of carbon sources ("+" – Growth occurred, "-" – No growth occurred).

Source of carbon	Strains of rhizobia								
	VMR	GMR	CAR	PSR	VFR	TFR	VUR	LPR	PhVR
Carbohydrate	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+
Dextrose Anhydrous	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	-	-	-

3.4. Authentication of nodulation

The nodulation is present and absent which is authenticate of the different strain (Table 4). The strain shows the present the nodules (Figure 2) whether the nodulation test under control environment shows absence of root nodules (Figure 3).

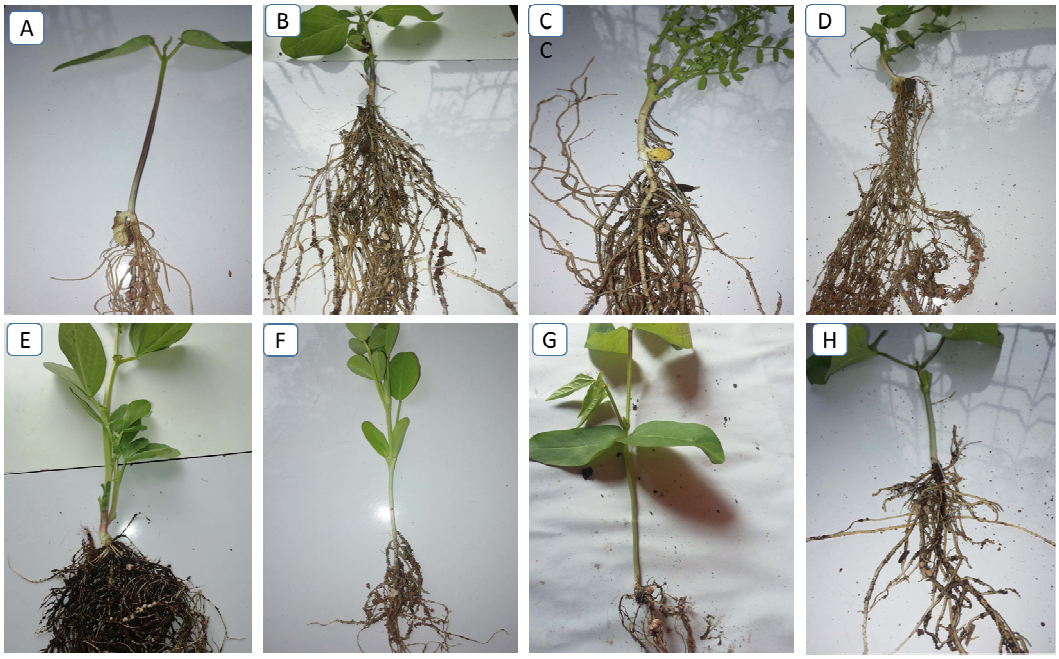

Figure 2. Nodulation test showing the root nodules in A – *Vigna mungo*, B – *Glycine max*, C – *Cicer arientunum*, D – *Pisum sativum*; E – *Vicia faba*, F – *Trigonella fonugraecum*, G – *Vigna unguiculata*, H – *Phaseolus vulgaris*.

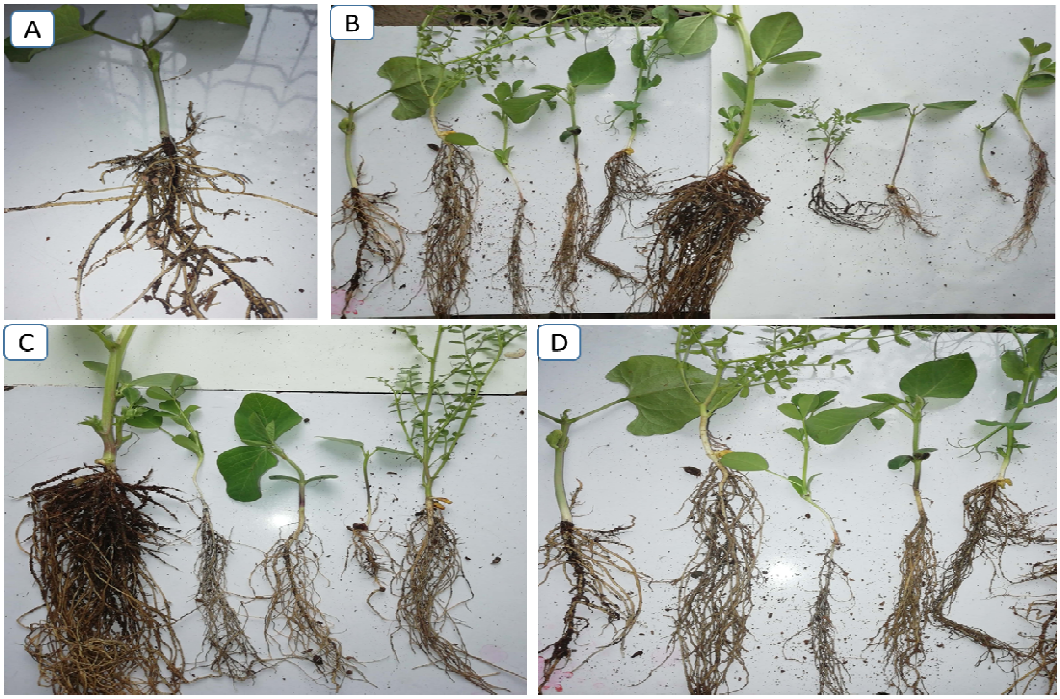

Figure 3. A – Nodulation test showing the root nodules in *Lablab purpureus*. B, C, D – Nodulation test under control environment shows absence of root nodules in *Vigna mungo*, *Glycine max*, *Cicer arientunum*, *Pisum sativum*, *Vicia faba*, *Trigonella foenugraecum*, *Vigna unguiculata*, *Lablab purpues*, *Phaseoulus vulgaris*.

Table 4. Authentication by nodulation and cross inoculation test ("-" – No nodulation occurred, "+" – Nodulation occurred).

Host Plants	Strains of rhizobia								
	VMR	GMR	CAR	PSR	VFR	TFR	VUR	LPR	PhVR
Vigna mungo	+	-	-	-	-	-	-	-	-
Glycine max	-	+	-	-	-	-	-	-	-
Cicer arieunum	-	-	+	-	-	-	-	-	-
Pisum sativum	-	-	-	+	-	-	-	-	-
Vicia faba	-	-	-	+	+	-	+	-	+
Trigonella foenugraecum	-	-	+	-	+	+	-	-	-
Vigna unguiculata	-	+	-	-	-	-	+	-	-
Lablab purpureus	-	-	-	-	-	-	-	+	-
Phaseolus vulgaris	-	-	-	-	-	-	-	-	+

4. DISCUSSION

Allorhizobium, *Bradyrhizobium*, *Mesorhizobium Rhizobium* and *Sinorhizobium* are the genera of bacteria [25, 26] which are collectively referred to as rhizobia, are strictly aerobic, rod-shaped cells, 0.5-0.9 µm x 1.2-3.0 µm in size, non-spore forming, cells stain Gram-negative, fast growing (*Bradyrhizobium*; slow growing), contains the enzyme nitrogenase, motile single polar flagellum or two to six peritrichous flagella, chemoorganotrophic, colonies are white translucent, circular, convex, and raised.

In this research, the strains of root-nodulating bacteria; *Rhizobium*, were isolated from the root nodules of ten different legumes cultivated in different area of the Sanfebar Municipality as an important cereal crop.

The legumes under study included *Vigna mungo*, *Glycine max*, *Cajanus cajan*, *Cicer arientinum*, *Pisum sativum*, *Vicia faba*, *Trigonella foenugraecum*, *Vigna unguiculata*, *Lablab purpureus* and *Phaseolus vulgaris* which has great role in agriculture subsistence. All the strains isolated on YEM agar medium each from the nine different plant species were cultured and showed the best positive growth at 28±2°C in YEM agar medium [11, 19, 27]. The strains were found to be fast growing for the rhizobial strains isolated from *Vigna mungo* [28], *Cajanus cajan* [29], *Cicer arientinum* [19], *Vicia faba* [22], *Trigonella foenugraecum* [30], *Lablab purpureus*) [27] and *Phaseolus vulgaris* [29] while the strains isolated from the *Glycine max* [28, 29] and *Vigna unguiculata* [23, 31] were slow growing (i.e. *Bradyrhizobium*) [32]. All the strains were motile, gram negative, rod shaped and the colonies on YEM agar were circular, translucent, convex, smooth, non-spreading, entire and odorless with 2-4 mm in diameter after 48 h of incubation at 28±1°C. The excessive secretion of the mucilaginous exopolysaccharide was found. These morphological characteristics resembles with the study carried out by different researchers [19, 32] and are also seems to approach close to the characteristics of *Rhizobium* type as described [10]. These morphological characteristics approaches close to *Rhizobium* type, *Mesorhizobium* type for *Cicer, Bradyrhizobium* type for *Glycine max* and *Vigna unguiculata* [33]. The isolates were characterized by the non-absorption of Congo red dye up to 24 h of growth but afterwards the colonies started to absorb it further purity of rhizobial isolates [34].

Biochemical test and incubated growth rate 28±2°C is the confirmation of rhizobia whether is or isn't. Suspending one loopful of organism in a drop of H_2O_2 on a petri-plates were is used for the presence of enzyme catalase in the rhizobial isolates [35]. The bubbles present confirm that positive results. Citrate utilization by the isolates were observed by the growth on petri-plates as directed [35]. 100% of the strains showed the positive test for the catalase activity and negative test for citrate utilization which resembled with result found [8] for the *Rhizobium* isolated from root nodules of *Trigonella foenumgraecum* and *Mucuna pruriens*. Similar result was found [27] on isolates of rhizobia from rhizosphere and root nodules of cowpea, elephant and lablab plants; [19] on isolates of rhizobia from chickpea; Yashita [36] on isolates of rhizobia from lablab beans. All the strains showed no growth on glucose peptone agar (GPA) and were unable to produce 3-keto lactase when the strains were cultured in YEM agar medium, Mannitol replaced with lactose which showed resemblance with the results found [8] on the isolates from root nodules of *Trigonella foenumgraecum* and *Mucuna pruriens*; Amel et al. [22] on the isolates from the root nodules of *Vicia faba.* Paudel et al. [37] focused on soil microbes increase the beneficial of organic and soil nitrogen is correlated for growth and yield in *Vigna unguiculata*.

For the ability to utilize the 2% NaCl, the strains TFR i.e. rhizobial strains from fenugreek showed positive growth which showed similar results with [8] the strains PSR, PhVR, VUR showed the poor tolerance i.e. out of replicates only 3 showed positive results while the rest of the strains showed no tolerance to the 2% NaCl. The result contradicted with the result found [8, 22] on the isolates from root nodules *Vicia faba*. The salt concentration test on the rhizobial strains (*Rhizobium trifolii*, *Rhizobium phaseoli*, *Rhizobium leguminosarum* and *Bradyrhizobium japonicum*) where they found good tolerance and good growth of the rhizobial strains on 2% NaCl. While our result showed resemblance with the result found [27] for the test on the rhizobial strains from Cowpea, elephant and lablab plants and results [36] on strains from lablab plant [19] also found decreased growth of the rhizobial strains in YEM agar medium containing greater than 1% of NaCl.

Determination of growth and survival of rhizobia has been reported which is legume salinity varies from the soil properties, climatic condition and the growth stage of plant. Osmotic stress affect the legume–

Rhizobium interacton for the nodule formation which is from the rhizobia. The rhizobia can tolerate salinity from 4.5 to 5.2 dsm^{-1} [38]. For example, *Rhizobium leguminosarum* for *Phaseolus vulgaris* can tolerate up to 350 mM NaCl concentration in broth culture while those for *Vigna unguiculata* can tolerate up to 450 mM NaCl concentration. However, some *Rhizobium* species are favorable for the moderate salinity soils for fix the nitrogen [23].

All the strains showed the positive urea hydrolysis test. YEM broth was altered with 2% w/v urea in company with 0.012% phenol red and inoculated with log phase culture. After 48 hours of incubation period, it was observed that color of the broth changes from red to orange that is the indication of urea hydrolysis [39].

It means that experimenting microbes are able to use urea as a source of carbon and energy for growth. Similar results were found [19, 36]. In the present study, all isolates were oxidase negative as experiment showed no color change in the region of the colonies after addition of p-aminodimethylaniline oxalate on the surface of isolates [40]. On the media containing mannitol amended with bromthymol blue dye, the strains were found to be producing which was conferred by the change in the color around the colonies [8, 41]. The rhizobial species associated with legumes like pigeon pea, lablab beans, cowpeas, chickpeas, peas, fenugreek, fava beans, common beans are also found to be acid producing by different researchers [19, 27, 28, 36].

Bacterial growth is directly influenced by the change in the temperature as it controls the rate of all cellular activities. In this research, the optimum temperature for growth recorded was at 28±2°C. As incubation temperature is changed, the decreased growth was observed.

Test for the utilization of various carbon sources showed that there was a complete inability of the strains to utilize the starch but in other carbon sources the growth was found to be good. Fructose, glucose, sucrose, lactose, maltose, dextrose anhydrous, arabinose, trehalose and starch were used as the sources of the carbon and all the strains showed good growth on all the carbon sources except the starch. Similar results were found [8, 41]. For Nepal, use of inoculation practice is far better in terms of nature conservation and the economic perspectives [42]. Furthermore, Paudyal et al. [43] focused on storage carrier on plant productivity showed better plant biomass accumulation and nodulation in case of charcoal, sawdust and garden soil.

Plant infection test was carried out by soaking corresponding sterilized seeds into the rhizobial isolates broth. The soaked seeds were planted into the pots containing sterilized garden soil mixed with coco pit in the ratio 2:1. The plants after uprooting showed that all the ten different legumes showed nodulation when inoculated with the *Rhizobium* isolated from the corresponding plant.

Cross inoculation test showed that *Vicia faba* has been nodulated by rhizobia isolated from *Pisum sativum, Vigna unguiculata,* and *Phaseolus vulgaris, Vigna unguiculata* was nodulated with the rhizobia isolated from *Glycine max. Trigonella foenugraecun* also showed positive cross inoculation test for the *Rhizobium* isolated from *Cicer aerientunum* and *Vicia faba* most of the legumes of the samples while most other legumes did not nodulate with the strains isolated from other hosts.

For rhizobial symbionts legume species greatly varies which is highly specific between the two partners. Host specificity refers to the ability of particular rhizobia species to form nodules on specific legumes. Due to the use of ineffective and non-competitive rhizobia strains as inoculants, the approach of using effective or superior exotic rhizobia strains as inoculants has failed in various environmental condition. For the perfect match, the host specificity play the role between the legume and rhizobia resulting into effective nodules (deep red inside) formation. If cross inoculation with no perfect match has occurred,

ineffective nodules (green or white inside) or no nodules may be formed and nitrogen fixation does not occur [23].

Legumes uniquely contribute many different functions and ecosystem services that are of great value to the agriculture, society and the ecosystem overall. The members of family leguminose (Fabaceae) not only have its importance in production of the proteinaceous grains, but also have the unique ability to associate with the symbiotic bacteria *Rhizobium* which helps to fix the atmospheric free nitrogen into the soil in the forms which can be directly utilized by the plants, which indeed show their importance in maintaining the fertility of the soil, increasing productivity, and indirectly controlling the use of nitrogen synthetic chemical-fertilizers and thereby contribute in mitigating the climate crisis. The symbiotic nitrogen fixation by the legume food, forage, and tree crops can be enhanced by the artificial amendment of rhizobia with the inoculation of effective rhizobial strains. Inoculant carriers can also be prepared on commercial basis as the nitrogen bio-fertilizers.

Cross inoculation test in present study showed that *Vicia faba* has been nodulated by rhizobia isolated from *Pisum sativum, Vigna unguiculata,* and *Phaseolus vulgaris, Vigna unguiculata* was nodulated with the *Rhizobia* isolated from *Glycine max. Trigonella foenugraecun* also showed positive cross inoculation test for the *Rhizobium* isolated from *Cicer aerientunum* and *Vicia faba* most of the legumes of the samples while most other legumes did not nodulate with the strains isolated from other hosts. Ampomah et al. [44] in their study reported that Cowpea has been nodulated by rhizobia isolated from soybean, ground nuts and Bambara ground nuts and these legumes cannot be nodulated by rhizobia isolated from cowpea in their cross inoculation test.

5. CONCLUSION

Preparation of the bio-inoculants of *Rhizobia* and inoculants carriers for the development bio-fertilizers is seem to be essential for substitutes the synthetic chemical fertilizers. The bacterial strains isolated from the root nodules of the legume crops on YEM agar and characterized using different test are rhizobial strains which is confirmed by the authentication by nodulation. The symbiotic nitrogen fixation from the legume food, forage, and tree crops can be enhanced by the artificial amendment of rhizobia with the inoculation of effective rhizobial strains. Inoculant carriers can also be prepared on commercial basis as the nitrogen bio-fertilizers. Established the fact that legumes uniquely associate with the symbiotic bacterium *Rhizobia,* which play the vital role in fixation of the atmospheric free nitrogen in the forms which can be directly utilized by the plants.

Authors' Contributions: SPP deigned and supervised the experiment; BK, conduct and interpreted the experiment; NP, writing original manuscript and interpreted the results; BDD, edit and reviewed the manuscript. All authors have equal contribution. All authors read and approved the final manuscript.

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