ISOLATION AND CHARACTERIZATION OF *RHIZOBIUM* STRAINS FROM THE ROOT NODULES OF TWO SELECTED LEGUMES*

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Abstract

Rhizobium is an important microorganism for the environment because of its nitrogen-fixing ability when in symbiotic relationship with plants (mainly legumes). The present study was conducted to isolate and characterize the rhizobia from the root nodules of *Vigna mungo* (L.) Hepper (Black gram) and *Vigna unguiculata* subsp. *unguiculata* (L.) Walp. (Cowpea). The experiment was performed at the Microbiology Laboratory, Department of Botany, University of Yangon in 2022. The two isolated bacterial strains were given named as (PVMR from black gram.) and (PVUR from cowpea). Both isolates on YEMA medium showed white, circular, entire margin, convex elevation, produced mucoid and translucent colonies. The microscopic examination revealed that both isolates are gram negative and rod shaped. In two bacterial strains: PVMR was identified as *Rhizobium* sp. and and PVUR was identified as *Bradyrhizobium* sp. based on the results of authentication test. Biochemical characterization of both strains showed positive reaction with catalase, methyl red test, nitrate reduction, and urea test, while negative reaction was observed in citrate utilization, gelatin hydrolysis, hydrogen sulphide production, indole, Triple Sugar Iron Agar (TSI), and Vogas Proskauer test. In antibiotic resistance test, PVMR strain resistance to only Penicillin and sensitive to Amoxicillin, Chloramphenicol, Tetracycline, whereas, PVUR strain resistance to Amoxicillin, Chloramphenicol, Penicillin but sensitive to Tetracycline. The plant inoculation assay indicated the improvement of plant growth and nodules formation in the treatments inoculated with two rhizobial isolates compared with non-inoculation treatments.

Keywords root nodules, *Rhizobium*, *Bradyrhizobium*, black gram, cowpea

Introduction

Nitrogen is one of the most important elements in atmosphere (approximately 80%), that is used to make various products that plant require for their development. Nitrogen deficiency is a limiting factor to plant growth and has significant ecological and agricultural implications. The extensive use of synthetic nitrogen fertilizers to get high yield is not only expensive but also a threat to the environmental balance and contributes to global warming (Vitousek, 1997). Therefore, eco-friendly and cost-effective agro-technologies to increase crop production are required including the process of biological nitrogen fixation, which can be done by certain soil microbes both symbiotically and as well as non-symbiotically. The ability to fix atmospheric nitrogen comes from the symbiotic relationship between legumes and rhizobia (Howard and Rees, 1996).

Biological nitrogen fixation (BNF) is a natural process where rhizobia and leguminous plants with nodules in their root systems are able to convert the nitrogen gas into a form that is usable for plant life. The symbiotic relationships between leguminous plants and rhizobia have a great importance to agricultural production and reduces the requirement for nitrogenous fertilizer (Dilworth and Parker, 1969; Hunter *et al*., 2007). This association between the host plant (legumes) and rhizobia is mutually beneficial. *Rhizobium* is a well-known group of bacteria that acts as the primary symbiotic fixer of nitrogen. Bacteria of family Rhizobiaceae, including six genera namely *Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium and Bradyrhizobium* are symbiotic and effectively convert atmospheric nitrogen to usable forms which is utilized by the host (Okazaki *et al*., 2004). According to the growth rate

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(generation time) on laboratory media, rhizobia, are broadly classified into two groups, fast grower and slow grower. Fast growers (generation time < 6 hrs) refer to those rhizobia associated with bean and pea whereas, slower growers (generation time > 6 hrs) refer to those rhizobia associated soybean and cowpea (Jordan and Allen., 1974; Paudyal, 2002).

Leguminous plants possess a unique ability to establish symbiosis with nitrogen fixing bacteria of the family Rhizobiaceae. Among the legume crops, black gram and cowpea are economically important pulse in Myanmar. Black gram (*Vigna mungo* L. Hepper) is one of the main leguminous crops that provide chief source of food. It is rich in proteins and contains amino acids higher quantities than any other cereals and pulses. Black gram fulfills major part of nitrogen requirement by symbiotic nitrogen fixation with the help of bacterium called Rhizobia (Pareek et al, 1978). Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the most ancient crops and an important source of food, income and livestock feed as well as forms a major component of tropical farming systems due to its ability for improvement of soil fertility through nitrogen fixation and as a cover crop (Sanginga *et al*., 2003). In addition, Antova *et al*., 2014 stated that cowpea has a unique ability to fix atmospheric nitrogen under stressed conditions. The cultivation of cowpea stimulates the proliferation of rhizobia in a field for their ability to enhance soil rhizobia populations (Mulongoy & Ayanaba, 1986).

Nitrogen fixing leguminous plants not only supports plant growth but also improve soil nitrogen status for associated crops. The rhizobial inoculants are now widely used in various parts of the world because they are inexpensive, environment-friendly, and easy to use without side effects and improve crop production (Tena *et al*., 2016). Therefore, rhizobium inoculation has become a popular agronomic practice to provide adequate amounts of nitrogen to leguminous plants, instead of the use of nitrogenous fertilizer. For this reason, the present research was aimed to isolate rhizobial strains from the root nodules of two different selected legume plants, to conduct the authentication test for rhizobial isolates which is differentiate from other contaminating microbes, to identify the rhizobial strains using some biochemical tests, to determine the antibiotic susceptibility test of isolated rhizobial strains and to evaluate the plant growth parameters of two rhizobial strains by plant inoculation assay.

Materials and Methods

1. Sample collection and isolation of bacteria from root nodules

The root nodules of *Vigna mungo* (L.) Hepper (Black gram) were collected from Hinthada Township, Ayeyarwaddy Region and *Vigna unguiculata* subsp. *unguiculata* (L.) Walp. (Cowpea) was collected from Hmawbi Township, Yangon Region. The collected root nodules were washed in running tap water to remove the adhering soil particles. The healthy and undamaged nodules were detached from the roots. For the surface sterilization, the nodules were washed with distilled water and sterilized with 70% ethanol for 30 seconds, followed by 1% of sodium hypochlorite solution for 2-3 minutes. Then, rinsed thoroughly with sterilized distilled water (3-5 times) to remove the chemicals. The sterilized nodules of different legumes were crushed with sterile glass rod or forceps in the microcentrifuge tube containing 0.5 ml of N- saline (0.85% NaCl). Then, one loopful of the nodule suspension was streaked on petri plates containing yeast extract mannitol agar (YEMA) medium supplemented with 0.0025% (w/v) congo red as an indicator. The plates were incubated for 3-5 days at room temperature. The purified cultures were maintained on YEMA agar slants, stored at 4°C in refrigerator for further study (Singh *et al*., 2008, Vincent, 1970).

2. Morphological and Microscopical characterization

The colony morphology of bacterial isolates including colony color, form, elevation, margin, mucosity, opacity was determined by observing the colonies on YEMA plates with congo red indicator after incubation period at room temperature. The microscopical characterization was conducted by gram staining.

Gram Staining

The pure cultures of bacterial strains were selected for more specific identification of the colonies. For gram staining, 24 hours old culture was evenly smeared on a clean slide and gently heated to fix by passing over a Bunsen burner. First, stained with crystal violet for 1 minute and second, immersed in Gram's iodine for one minute. Then, ethanol was applied for 30 sec and the counter stain safranine was applied as the final step. Followed by a gentle wash with distilled water after finishing each step. The slides were air dried and examined under the microscope. Gram-negative bacteria retain the pink/red colour while Gram-positive bacteria retain the crystal-violet (Somasegaran and Hoben, 1994).

3. Authentication Tests

Five different authentication (confirmatory) tests such as growth on YEMA with Congo red, Bromothymol blue test, Glucose- peptone agar test, Keto-lactose test, and Hoffer's alkaline test were performed to confirm the isolates as rhizobia.

(i) Growth on YEMA with Congo red

The rhizobial isolates was streaked on CR-YEMA medium (congo red yeast extract mannitol agar) and observed for absorption of congo red dye (Somasegaran *et al*., 1994).

(ii) Bromothymol blue Test

The bromothymol blue test was performed to differentiate between fast and slow growers of *Rhizobium* species (Vincent,1970).

(iii) Growth on Glucose Peptone Agar (GPA)

GPA test was performed to determine the capability of the *Rhizobium* strains to utilize glucose as the sole carbon for its growth medium (Singh *et al*., 2008).

(iv) Keto-lactose Test

The isolates were streaked on the medium and incubated for 2-3 days. Then, Benedict's reagent was added on the plates and kept at room temperature for 1-2 hours (Bernaertz and Daley, 1963).

(v) Hoffer's alkaline Test

The test was conducted to determine the difference between *Agrobacterium* which grows at higher pH level and *Rhizobium* which unable to do so. The isolates were inoculated in Hoffer's alkaline broth and observed the bacterial growth after 24 - 48 hours (Hofer, 1935).

4. Biochemical characterization

Different Biochemical characterization test such as catalase test, citrate utilization test, gelatin hydrolysis test, hydrogen sulphide production test, indole test, methyl red (MR) test, nitrate reduction test, Triple Sugar Iron Agar (TSI) test, urea test, and vogas proskauer (VP) test was done for the identification of *Rhizobium* strains (MacFaddin 2000, Koser, 1923, Sadowsky *et al*., 1983, Vincent,1970)

5. Determination the Antibiotic Susceptibility

The two isolated bacterial strains were test for antibiotic sensitivity by disc diffusion on YEMA agar medium. As described in Lupwayi and Haque (1994), the stock solution of each antibiotic was prepared by dissolving 2g of each antibiotic in 100 ml of water. 200 µl of actively grown cultures of each *Rhizobium* was spread on the YEMA medium using a sterilized cotton swab. Antibiotic discs with the different concentration (30µg/ml for Chloramphenicol and Tetracycline) (10µg/ml for Amoxicillin and Penicillin) were placed equidistantly at the center of plates and incubated overnight at room temperature. The sensitivity or the resistances of *Rhizobium* isolates to antibiotics were determined by observation of absence or presence of growth around the discs. The isolates which showed growth around a particular antibiotic are resistant to that corresponding antibiotic, whereas the isolates whose growth is inhibited by a particular antibiotic seem to be sensitive to that antibiotic.

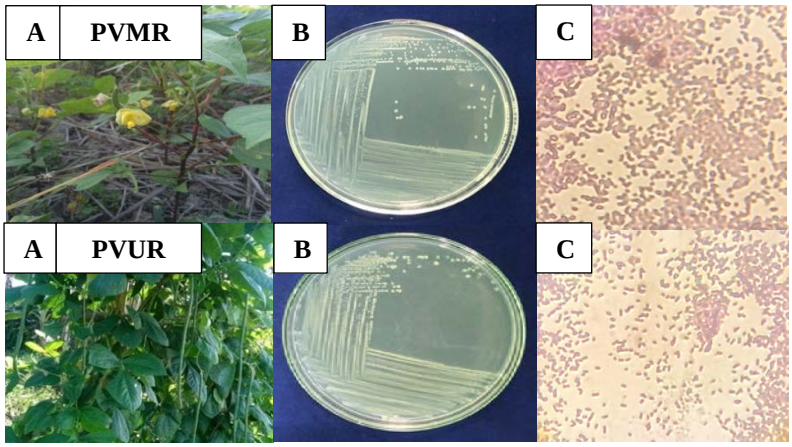
6. Evaluation the effect of two *Rhizobium* strains on plant growth

The plant inoculation experiment, including all the process (inoculum and seed preparation, inoculation test and experimental design for plant assay) were performed. The soil was sterilized by autoclaving at 121°C for 15 minutes. The two *rhizobium* isolates were grown in 50 mL falcon tube containing 20 mL of YEMA broth and incubated in a shaking condition at 150 rpm for 3 days at room temperature (Somasegaran and Hoben 1985). The similar sized seeds of cowpea and black gram were surface sterilized with distilled water and placed on the sterilized paper for 2-3 days. The good germinated seedlings were selected and transferred to the plastic cup (top) containing autoclaved soil and each cup containing 3 seeds were inoculated with 1 ml of bacterial solution with different concentration. The nitrogen-free nutrient solution B & D (Broughton and Dilworth, 1970) were supplied into the plastic cup (bottom) for all treatments. The three inoculation treatments were performed: 1. no inoculation (control); 2. 10^{-1} cfu/ml, and 3. 10^{-5} cfu/ml respectively. These cups were kept in a place full of sun light for proper growth. Plant weight, plant height, and nodule number of both plants were evaluated after 15 days of post-inoculation (Vincent, 1970).

Results

1. Morphological and Microscopical characterization of isolated bacteria

The two bacterial strains (one from each legume) were isolated from the root nodules of two selected legumes plant. The two strains were designated name as PVMR for *Vigna mungo* L. (Black gram) and PVUR for *Vigna unguiculata* subsp. *unguiculata* L. (Cowpea). Colonies of both isolates showed similar morphology and produced white, circular, entire margin, convex elevation, and mucoid colonies when grown on YEMA plates (Figure. 1B). Gram's staining of the isolates was confirmed by microscopic observations and both isolates were found to be gram negative, and rod shaped (Figure. 1C).



PVMR= *Vigna mungo* L.; PVUR= *Vigna unguiculata* subsp. *unguiculata* L.

Figure 1 (A) Habit of plants; (B) Rhizobial colonies on YEMA medium; (C) Gram staining of isolates under the microscope

2. Authentication tests

Table 1 Authentication tests of two *Rhizobium* strains

Confirmatory Tests	**Results**	
	PVMR	**PVUR**
Growth on Congo red medium	White	White
Bromothymol Blue test	Yellow/ Fast grower	Blue/ Slow grower
Growth glucose peptone agar (GPA)	No growth	No growth
Production of ketolactose test	Negative	Negative
Hoffer's alkaline test	No growth	No growth

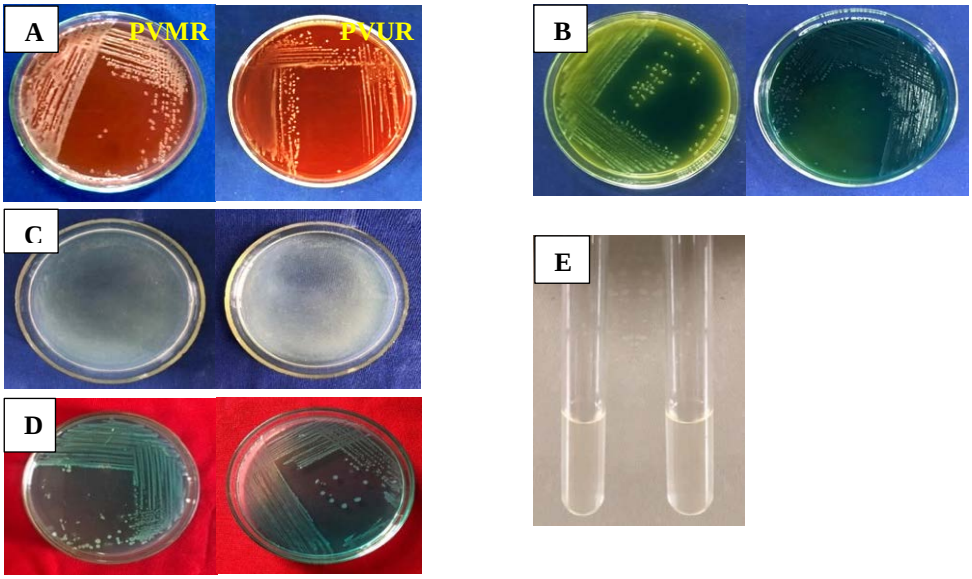


Figure 2 (A) *Rhizobium* colonies on CR-YEMA; (B) Bromothymol Blue test; (C) Glucose Peptone Agar (GPA) test; (D) Keto-lactose test; (E) Hoffer's alkaline Test

3. Biochemical Characterization

Table 2 Biochemical Characterization of two *Rhizobium* strains

Biochemical Tests	Results	
	PVMR	PVUR
Catalase test	+	+
Citrate utilization test	-	-
Gelatin hydrolysis test	-	-
Hydrogen sulphide production test	-	-
Indole test	-	-
Methyl red (MR) test	+	+
Nitrate reduction test	+	+
Triple Sugar Iron (TSI) test	-	-
Urea Test	+	+
Vogas Proskauer (VP) test	-	-

+ (positive test), - (negative test)

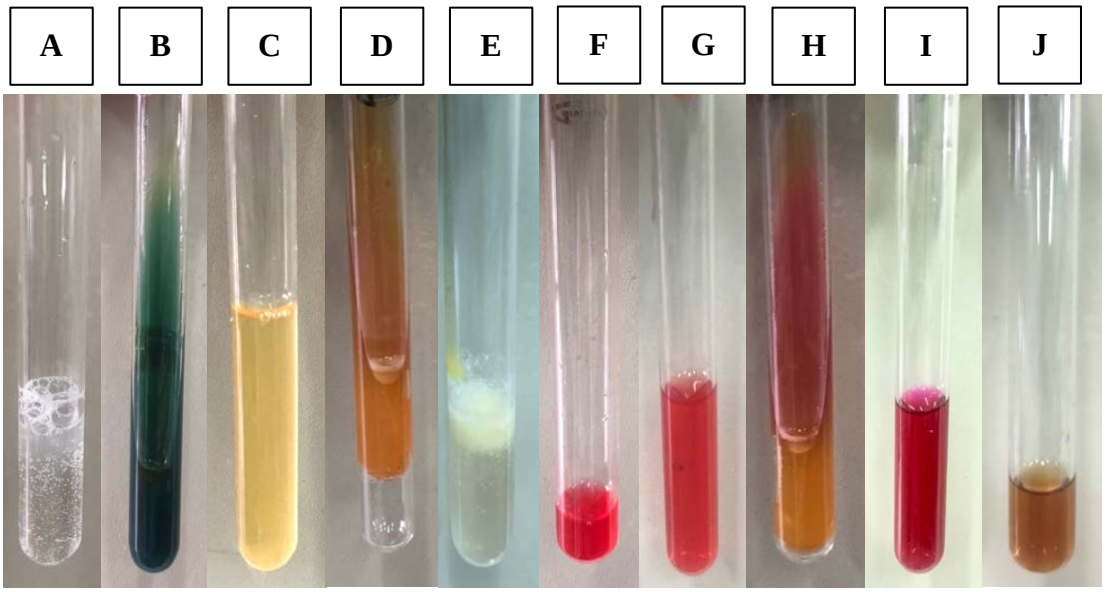
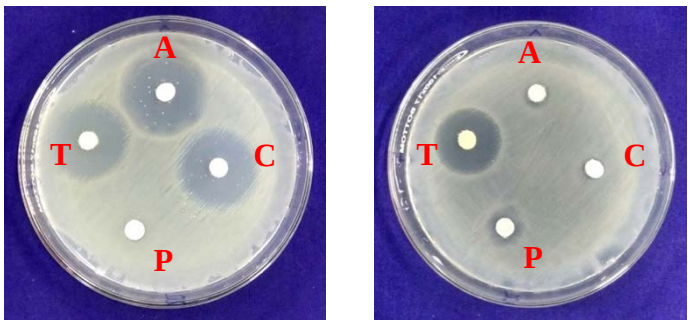


Figure 3 Biochemical tests of two *Rhizobium* strains (A) Catalase test; (B) Citrate utilization test; (C) Gelatinase hydrolysis test; (D) Hydrogen sulphide test; (E) Indole test; (F) Methyl red (MR) test; (G) Nitrate reduction test; (H) Triple sugar iron (TSI) test; (I) Urease test; (J) Voges Proskauer (VP) test

4. Determination on the Antibiotic Susceptibility



PVMR **PVUR**

Figure 4 Antibiotic Susceptibility test of two *Rhizobium* strains (PVMR and PVUR) by disc diffusion test. Amoxicillin (A), Chloramphenicol (C), Penicillin (P), and Tetracycline (T)

5. Evaluation the effect of two *Rhizobium* strains on plant growth

The effect of two rhizobial isolates on the plant growth of cowpea and black gram was evaluated at 15 days post inoculation. The two treatments (10^{-1} cfu/ml and 10^{-5} cfu/ml) inoculated with two rhizobial isolates indicated the highest plant height and plant weight compared with the uninoculated treatment (control) in both selected plants (Figure. 5, 6).

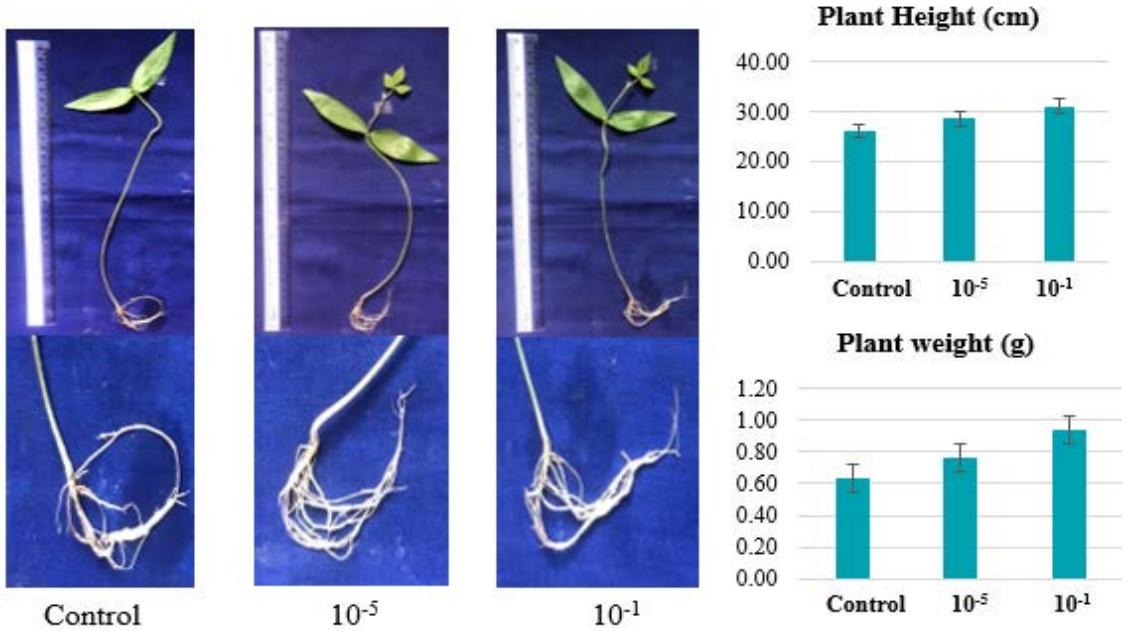


Figure 5 Comparison of plant growth parameters (plant height and plant weight) of black gram, inoculated with no-inoculation (control), 10^{-5} cfu/ml and 10^{-1} cfu/ml of rhizobial isolate PVMR strain at 15 days after sowing. The histograms at each treatment are significantly different at 15 days, $P > 0.05$ (t-test).

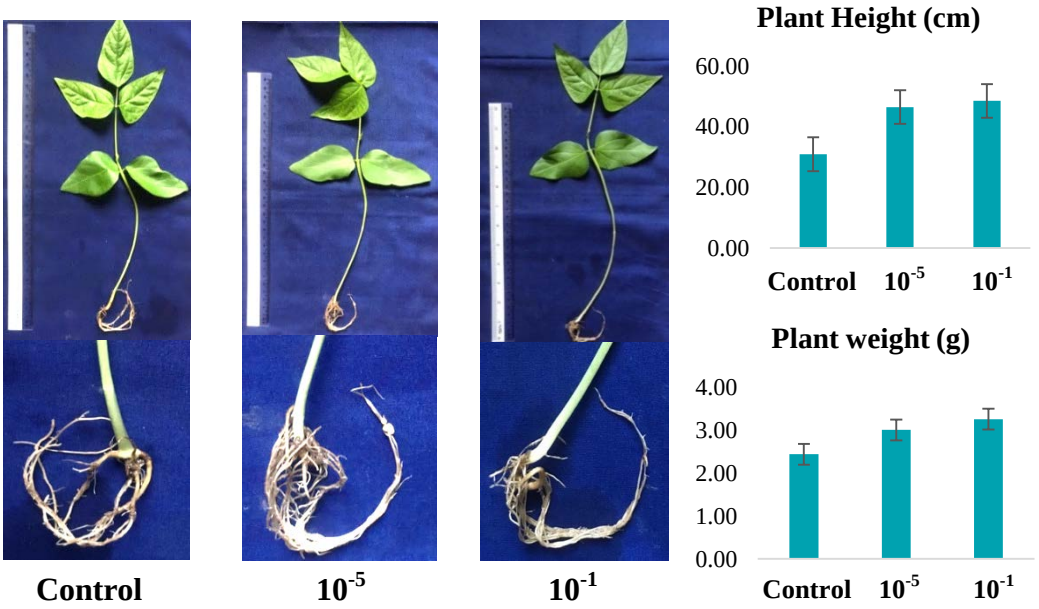


Figure 6 Comparison of plant growth parameters (plant height and plant weight) of cowpea, inoculated with no-inoculation (control), 10^{-5} cfu/ml and 10^{-1} cfu/ml of rhizobial isolate PVUR strain at 15 days after sowing. The histograms at each treatment are significantly different at 15 days, $P > 0.05$ (t-test).

Discussion

In the present study, the two *Rhizobium* strains ('PVMR' from black gram and 'PVUR' from cowpea) were isolated. The colonies of both isolated strains showed white, circular, entire margin, convex elevation, and mucoid colonies production on YEMA medium and microscopic examination revealed that both isolates were rod shaped and gram negative (Figure 1). The morphological and microscopical characteristics resembles with the study carried out by different researchers (Jordan and Allen 1974 and Rasool *et al*., 2015, Keyser *et al*., 1982 and Gauri *et a*l., 2011). The authentication test to confirm the isolates as rhizobia and to differentiate them from other contaminating microbes were conducted and the results of five confirmatory tests was shown in Table 1 and Figure 2.

The results indicated that the colonies of both strains did not absorb the Congo red color on CR-YEMA media (congo red yeast extract mannitol agar) and which is differentiate *Rhizobium* from *Agrobacterium* and other bacterial contaminants (Trinick, 1982). In bromothymol blue (BTB) agar plates, PVMR strain from black gram showed the yellow color indicating the acid production and strain PVUR from cowpea showed the blue color which is indicting the alkali production. Datta *et al*., (2015) showed the fast- growing rhizobial strains isolated from *Vigna mungo* (black gram). Similarly, Shahzad *et a*l., (2012) and Andrews (2017) stated slow-growing strains isolated from *Glycine max* and *Vigna unguiculata* L. From these observations, the present results suggested that PVMR strain might be *Rhizobium* sp. and PVUR strain might be *Bradyrhizobium* sp.

In the GPA test, both isolates showed either poor or no growth on GPA medium after one day indicating the features of rhizobia. The similar observation was reported by Vincent *et al*., (1970). In Keto-lactose test, there is no yellow zone around the colonies after the addition of Benedict's reagent which is the characteristic of *rhizobium*. The negative results on Hofer's

alkaline medium were obtained in the current study which is indicating the *Rhizobium* species normally cannot grow in this medium. These results of both strains similar with the findings of Deshwal and Chaubey (2014) and Deka and Azad (2006).

Moreover, the different biochemical characterization test of two rhizobial isolates (PVMR and PVUR) was performed and results shown in Table 2 and Figure 3. In catalase test, the appearance of bubbles showed the positive result and this agreed with (MacFaddin, 2000). The result of citrate utilization showed the negative result and agreed with (Lupwayi and Hague,1994). Paudyal, 2002 stated that the positive test for the catalase activity and negative test for citrate utilization revealed that is 100% of *Rhizobium* strain and these findings were agreed with the current study. The negative gelatinase activity of *Rhizobium* was observed for both strains and similar with (Hunter *et al*., 2007). There is no hydrogen sulphide production was observed in the current study which is close agreed with (Kumari *et al*. 2010).

The indole test indicated the negative result for both strains which remaining yellow or be slightly cloudy within seconds of adding the reagent and similar result was observed in Shahzad *et al*., (2012). The positive results of methyl red (MR) test agreed with the studies of Raju *et al*., (2017). For nitrate reduction test, a red color indicates a positive and the similar result was agreed with Kumari *et al*. (2010). In TSI test, alkaline (red) slant and acid (yellow) butt showed that only glucose fermentation has taken place and similar results were reported by Kucuk *et al*., (2006). In the current study, both isolated strains showed positive test for urease and similar finding was reported by Gauri *et al*., (2011). The result of Voges-proskauer (VP) showed the negative which has the similar observations with Elsheikh and Wood (1986).

In antibiotic susceptibility test, PVUR (Brady*rhizobium* sp.) and PVMR (*Rhizobium* sp.) showed the different resistance and sensitive results on four antibiotics. PVMR strain indicated that sensitive to Amoxicillin, Chloramphenicol, Tetracycline and which resistance to Penicillin only. In contrast, strain PVUR showed resistance to Amoxicillin, Chloramphenicol, Penicillin but sensitive to Tetracycline (Figure 4). Similarly, Hungaria *et al.,* (2000) stated that the *Rhizobium* isolates were sensitive to tetracycline, kanamycin and streptomycin. Prasuna (2014) found a strain that was resistant to many antibiotics (chloramphenicol, erythromycin, kanamycin, neomycin and penicillin G) and these results were in close agreement with the present findings for both strains.

The plant growth parameters including plant height, plant weight and nodulation efficiency of two rhizobial isolates indicated the positive effects on the two selected plants (black gram and cowpea) at 15 days post inoculation. The improvement of plant growth, leaves number and root structure were observed in the treatment inoculated with two rhizobial isolates compared with the non-inoculation treatment (Figure. 5 and 6). Interestingly, the small nodules formation was recorded in the treatment inoculated with 10^{-1} cfu/ml (PVUR strain) in cowpea while no nodulation was observed in the PVMR strain (black gram). Similarly, a significant increase of nodule number and improved plant height in cowpea was observed when inoculated with *Bradyrhizobium* and close agreement with current study Egamberdiyeva *et al*., 2004, Ndungu, 2017 and Stephen Kyei Boahen *et al*., 2017). According to the report of Maurya *et al*., (1993), and Salam *et al*., (2004), the application of *Rhizobium* significantly increases the plant height, branches/plant and dry matter production in black gram and similar findings found in current study.

Conclusion

The current study indicated that the two isolated strains were the true *Rhizobium* species according to the results of morphological and microscopical characterization, authentication and biochemical test. The rhizobial strain (PVMR) isolated from black gram considered as belonging to the *Rhizobium* group due to fast growing whereas the strain (PVUR) isolated from the cowpea might be *Bradyrhizobium* group which showed slow growing. Based on the results of antibiotic susceptibility test, it can be suggested that these antibiotics resistant strain (PVUR) can survive antibiotic stressed conditions and help to increase rhizobial survivability in the soil and prevent susceptible rhizobial population due to lethal antibiotic compared with less resistance strain (PVMR). In addition, the enhancement of plant height and plant weight were observed from the plants inoculated with both strains after 15 days. Remarkably, the nodulation performance was recorded from the PVUR strain. Therefore, it can be concluded that these findings may give a prospect to do extensive research by using highly performed isolates and also play an important role in sustainable agriculture system. However, it is still necessary to conduct further screening the different plant growth promoting traits and nitrogen fixing ability of both strains as well as to explore the application as potential biofertilizer instead of synthetic fertilizer under laboratory and field conditions

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