academicJournals

Vol. 12(22), pp. 1874-1880, 1 June, 2017
DOI: 10.5897/AJAR2017.12321
Article Number: E34C71B64540
ISSN 1991-637X

http://www.academicjournals.org/AJAR

African Journal of Agricultural Research

*Full Length Research Paper*

# Isolation, identification and molecular characterization of *Rhizobium* species from *Sesbania bispinosa* cultivated in Bangladesh

**Nusrat Nahar, Anowara Begum and Humaira Akhter***

Department of Microbiology, University of Dhaka, Dhaka-1000, Bangladesh.

Received 20 March, 2017; Accepted 20 April, 2017

**The investigation was carried out to study the characteristics of rhizobial strains isolated from the leguminous plant, *Sesbania bispinosa* growing in different regions of Bangladesh. Forty-four isolates were studied for biochemical and molecular characteristics. Isolates were able to utilize different carbohydrates. All isolates showed complete resistance to cloxacillin and penicillin G that results in increased survivability of rhizobial populations in antibiotic stressed conditions. Isolates were able to form nodule in the plant infection test. The majority of the strains showed positive results for *nodC* and *nifH* gene amplification which are the typical characteristics of *Rhizobium* species. Genetic relatedness was assessed by comparing the sequences of 16S rRNA. Two distinct clusters were seen in the dendrogram constructed by the Complete Linkage method. The isolates R7, R8, R17, R33 and R3 were distinct from the 20 reference strains. The first cluster was phylogenetically distinct from the reference strains and might have evolved from a distinct lineage. Isolate R4 was placed adjacent to *Rhizobium cnuense*. So, the findings may represent new species of *Rhizobium*. This study helps to identify an ideal strain of *Rhizobium* from *S. bispinosa* that can be function as biofertilizer when released in the soil and contribute to sustainable agricultural practices by improving yields.**

**Key words:** *Rhizobium, Sesbania bispinosa*, nodulation gene, nitrogen fixation gene, sequencing.

## INTRODUCTION

Nitrogen is one of the most abundant nutrient supplement for plant growth and is not directly available to plants because of its strong triple covalent bond. Increasing global population demands large food production which can be achieved by using plant beneficial microorganisms. Rhizobia are plant growth promoting bacteria that plays a key role in enhancing nitrogen fixation ability of leguminous plants by converting nitrogen to ammonia and provide organic nitrogenous compound to the plants. Rhizobia form nodules on root to fix nitrogen where nod factor and flavonoids initiate nodulation (Oldroyd, 2013). Nodulins are *nod* gene protein that are associated with Nod factor assembly and *nod* gene is the determinant of host range (Perret et al., 2000). The *nifH* gene is selected as a nitrogen fixation marker. Rhizobia take up carbon sources derived for

*Corresponding author. E-mail: humaira@du.ac.bd.

plant photosynthesis. Rhizobial isolates harbour plasmid. Mulligan et al. (1985) demonstrated that nodulation genes, the regulatory genes, and the nitrogen fixing genes are located on large symbiotic plasmid. Antibiotics accumulation in soils after excretion from feces and urine was reported by Ji et al. (2012). These antibiotics are lethal to susceptible rhizobial population and decreased rhizobial persistence in the soil and ultimately total nitrogen fixation. Cole and Elkan (1973, 1979) demonstrated that antibiotic resistance is a plasmid-born character. Cole et al. (1973) and Frioni et al. (2001) reported that greatest difference of antibiotics tolerance was found between fast and slow growing rhizobia. The antibiotic-resistant *Rhizobium* occupies large number of nodules in legumes (Belachew, 2010; Gemell et al., 1993). *Sesbania* is an important green manure that fixes large quantities of $N_2$. In the wet season, *Sesbania bispinosa* grew rapidly, exhibiting high level of nitrogen accumulation in soil Ladha et al. (1992) and Ndoye et al. (1988). *S. bispinosa* has not been much investigated regarding the rhizobial infection in Bangladesh. Carbohydrate utilization test is used to characterize rhizobia. The utilization of different carbohydrates serve as a diagnostic tool in the differentiation of *Rhizobium* species (El-Idrissi et al., 1996; Graham et al., 1991). Molecular methods including polymerase chain reaction (PCR) and 16S rRNA sequencing are more reliable for identification of bacterial isolates.

The aim of study was to characterize forty-four *Rhizobium* species isolated from *S. bispinosa* and also to identify the antibiotic resistant *Rhizobium* which has increased survivability and can be used as biofertilizer to increase productivity.

## MATERIALS AND METHODS

### Isolation of *Rhizobium* strains

Pink and healthy root nodules of *S. bispinosa* were washed in water. It was then transferred to 3% $H_2O_2$ solution and soaked for 2-3 min. To remove the traces of $H_2O_2$ solution, nodules were rinsed 5-6 times in sterile distilled water. Nodules were washed in 70% ethanol and then sterile glass rod was used to crush the nodule. Nodule suspension was streaked on yeast-mannitol agar (YMA) plates and incubated at 30°C for 24 h (Agrawal et al., 2012). Well isolated typical single colonies were restreaked on freshly prepared YMA plates in order to obtain pure cultures and colony characteristics were observed.

### Biochemical testing for identification

All isolates were processed for oxidase test as described by Kovaks (1956); catalase and nitrate reduction test as determined by Graham and Parker (1964). Urea agar slants were used to determine the urease activity that was incubated for 7 days at 30°C (Christensen, 1946). Isolates were also tested for indole production as described by Lowe (1962), gelatin liquefication and motility test as mentioned by Arora (2003), and ONPG (O-nitro phenyl--β-D-galactoside) test as described by Cappuccino (2007). For carbohydrate utilization tests, the basal medium used was that of Bishop et al. (1976) and for carbohydrate utilization test, different carbohydrates were substituted for mannitol, and $KN0_3$ (0.6 g/liter) was used as the nitrogen source.

**Table 1.** Antibiotic used in the susceptibility testing of *Rhizobium* isolates.

| Name of antibiotic | Concentration (µg) |
|---|---|
| Kanamycin | 30 |
| Gentamicin | 10 |
| Cephalexin | 30 |
| Streptomycin | 10 |
| Cloxacillin | 5 |
| Penicillin G | 10 |
| Polymixin B | 300 |
| Erythromycin | 15 |
| Nalidixic acid | 30 |
| Rifampicin | 5 |

### Determination of the antibiotic susceptibility

Bacterial suspension was prepared on nutrient broth and lawn on Muller-Hinton agar plates by using sterile cotton swab. Antibiotic discs were placed aseptically and plates were then incubated at 30°C for 24 h. The diameter was measured in millimeters after incubation. Name of the antibiotic and their concentration are given in Table 1.

### Plant infection test

Plant infection test was performed to confirm whether the isolates were able to form nodule and fix atmospheric nitrogen in modified Jensen's Agar medium. Seed was surface sterilized by covering with 3% hydrogen peroxide solution and left for 2 min. After washing three times in sterile water, surface sterilized seeds of *S. bispinosa* were spread onto surface of water agar plates. Plates were inverted and left in dark until germination occurred for 2 days to 1 week. Germinated seeds were aseptically transferred to big size test tubes containing modified Jensen's agar medium and roots were pointing downward with the contact of the agar. When leaves appeared, 1 ml of rhizobial suspension was added at their base. Plants were grown in light for approximately 8 weeks.

### Detection of *nifH* and *nodC* genes

Amplification was performed in a 12.5µL reaction volume for each specimen containing 2.5 µL (100 ng/µL) of template DNA. The reaction volume was prepared by mixing the following reagents: 7 µL of DEPC treated water, 1 µL of 20 mM $MgCl_2$, 0.2 µL of 10 mM dNTP, 0.625 µL of 10 mM forward and 0.625 µL of 10 mM reverse primer, 0.05 µL of Taq polymerase and 2.5 µL of template DNA. Mastermix was transferred to each PCR tube and then the corresponding DNA samples (2.5 µL) were added to each tube. For the amplification of the *nifH* gene, the PCR contents were subjected to initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 45 s. Annealing, elongation and final extension phase were maintained at 63°C for 45 s, 72°C for 45 s and 72°C for 7 min, respectively. An identical regime was

**Table 2.** Biochemical characteristics of isolates.

| Test | Results |
|---|---|
| Oxidase | Positive |
| Catalase | Positive |
| Urease production | Positive |
| Nitrate reduction | Positive |
| Motility | Positive |
| ONPG (O-nitro phenyl--β-D-galactoside) | Positive |
| Indole production | Negative |
| Gelatin liquefication | Negative |

maintained for *nodC* detection except the fact that the annealing was maintained at 54°C. The nucleotide sequences of the primers were as follows: *nifH* (forward 5′-CGTTTTACGGCAAGGGCGGTATCGGCA-3 and reverse 5′-TCCTCCAGCTCCTCCATGGTGATCGG-3′) (Perret and Broughtn, 1998) and *nodC* (forward 5′- GCCATAGTGGCAACCGTCGT-3′ and reverse 5′- CTCGCCGCTGCAAGT-3′) (Jacob et al., 1985).

#### Phylogenetic analysis

For the amplification of the 16S rRNA gene, the PCR contents were subjected to initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s. Annealing, elongation and final extension phase were maintained at 46°C for 45 s, 72°C for 1.5 min and 72°C for 8 min, respectively. PCR was conducted using 16S rRNA primers (Uni_euF:5′-AGAGTTTGATCCTGGCTCAG-3′ and Uni_euR:5′- TACCTTGTTTTACGACTT-3′) (Singh et al., 2013). The chromatogram sequences were inspected with Chromas 2.3 (Technelysium, Australia), and contigs was prepared using SeqMan II (DNASTAR, Madison, Wis.). The dendrogram was constructed using the online software Phylogeny.fr (http://www.phylogeny.fr/simple_phylogeny.cgi).

## RESULTS AND DISCUSSION

Isolates were initially studied for morphological and cultural characteristics. The colonies were circular, convex, gummy with entire margin and no pigmentation on YMA agar plates after 24 h incubation. The isolates were positive for catalase, oxidase, motility, ONPG (O-nitro phenyl--β-D-galactoside) tests and negative for indole production and gelatin liquefication tests (Table 2). These findings were in close agreement with Naz et al. (2009). Isolates also showed positive result for nitrate reductase and urease test. Same test results were found by Sadowsky et al. (1983). They were helpful in presumptive identification of the genus of the isolates. It was also found that isolates were able to grow before 24 h incubation. So, it is concluded that our representative isolates were fast grower.

Carbohydrate utilization capacity deduced that isolates were able to utilize different carbohydrates. Isolates were unable to utilize sucrose, succinate and lactose (Table 3). Same tests results were also observed by Sadowsky et al. (1983). Glenn and Dilworth (1981) reported that

**Table 3.** Sugar fermentation tests of the isolates.

| Carbohydrate | Results |
|---|---|
| Arabinose | Positive |
| Sucrose | Negative |
| Fructose | Positive |
| Glucose | Positive |
| Mannitol | Positive |
| Mannose | Positive |
| Rhamnose | Positive |
| Succinate | Negative |
| Xylose | Positive |
| Lactose | Negative |

catabolic enzymes and disaccharides uptake systems were absent in slow growing rhizobia. That is why fast grower rhizobia utilize a wide variety of carbohydrate unlike slow grower rhizobia (Fred et al., 1932; Graham and Parker, 1964). These results also confirmed that majority of our isolates were fast grower.

Antimicrobial resistance has emerged over last few decades due to abuse of these drugs. *Rhizobium* was examined for their genetic basis for resistance to antibiotics (Figure 1). All isolates were resistant to penicillin G and cloxacillin and 84.1% isolates were resistant to cephalexin. The resistant isolates might have beta-lactamase enzyme that conferred the resistance property to these isolates. *Rhizobium laguerreae* resistance to ampicillin, penicillin antibiotics was reported by Saidi et al. (2014). Twenty-five (56.8%) isolates were resistant to kanamycin, 36.4% isolates were resistant to gentamicin and 47.72% isolates were resistant to streptomycin. Streptomycin, kanamycin and gentamicin are aminoglycoside antibiotics and conferred resistance by interrupting protein synthesis. Nineteen (43.2%) isolates were resistant to erythromycin. Since erythromycin molecules are large, they are unable to pass through the outer cell membrane. Prasuna et al. (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 our findings. About 38.6, 25 and 4.5% of isolates were resistant to rifampicin, nalidixic acid and polymixin B, respectively (Figure 2). Mihaylova et al. (2014) reported that *Rhizobium* strains were sensitive to gentamicin and polymyxin B. So, these isolates showed higher antibiotic resistance towards beta-lactamase antibiotics. Antibiotic resistant *Rhizobium* increases rhizobial survivability in the soil (Naamala et al., 2016). So that, these antibiotics resistant *Rhizobium* can survive antibiotic stressed conditions and help to increase soil productivity. Presence of *nod* gene was confirmed by plant infection test. Twenty strains were studied for plant infection test and positive results were obtained for

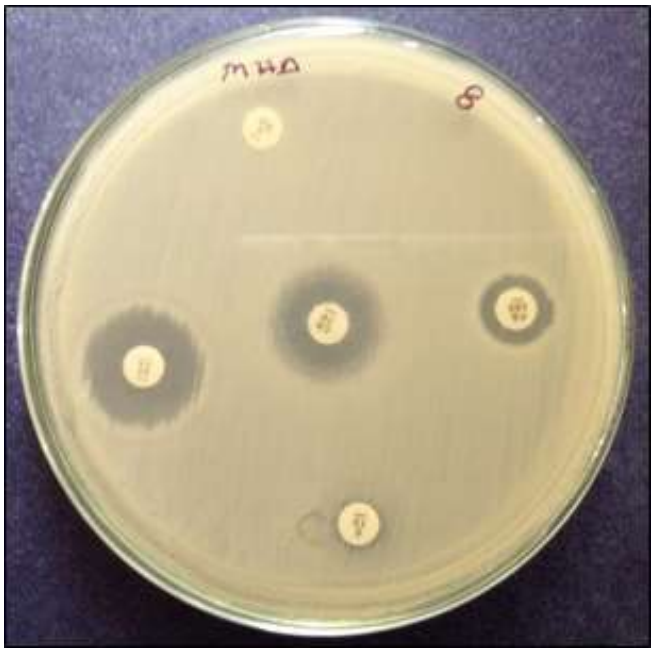

**Figure 1.** Antibiotic susceptibility test of *Rhizobium* species by disc diffusion test.

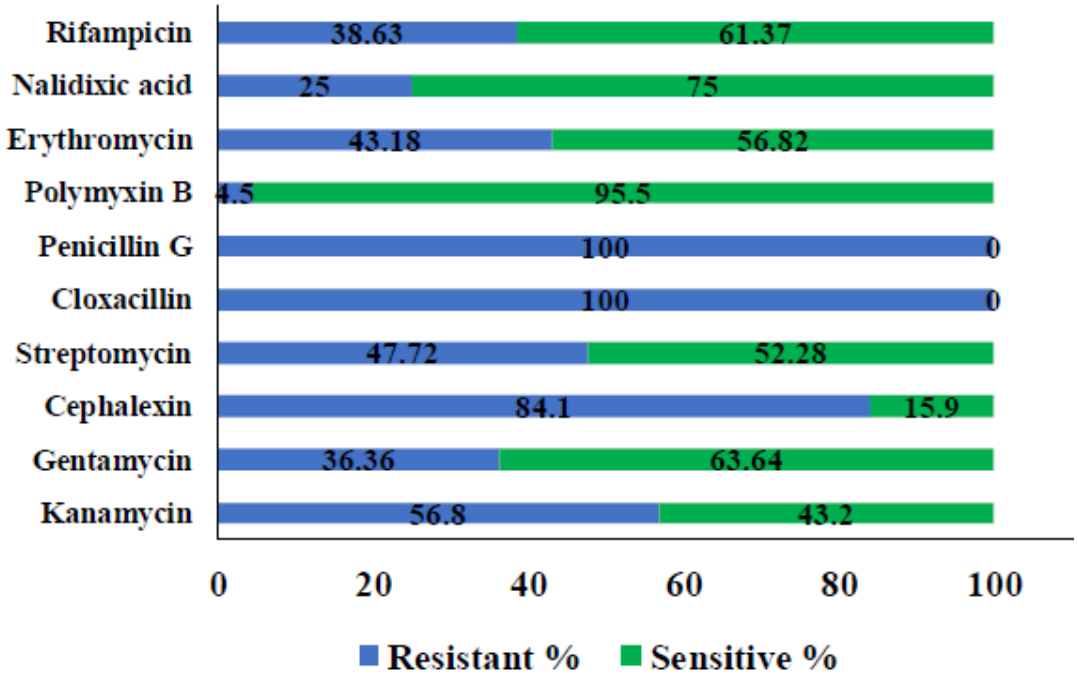


**Figure 2.** Antibiotic susceptibility patterns of *Rhizobium* species.

isolates R3, R5, R7, R8, R10, R12, R17, R18, R24, R32, R35 and R41.

Nitrogenase enzyme, encoded by *nif* gene, reduced nitrogen to ammona and occuerd in only nitrogen fixing organisms (Singh et al., 2013). The majority of the strains gave PCR amplified product with an approximate length of 781 bp for the *nifH* gene. The *nodC* gene with the help of Nod factors initiated the root nodule development in

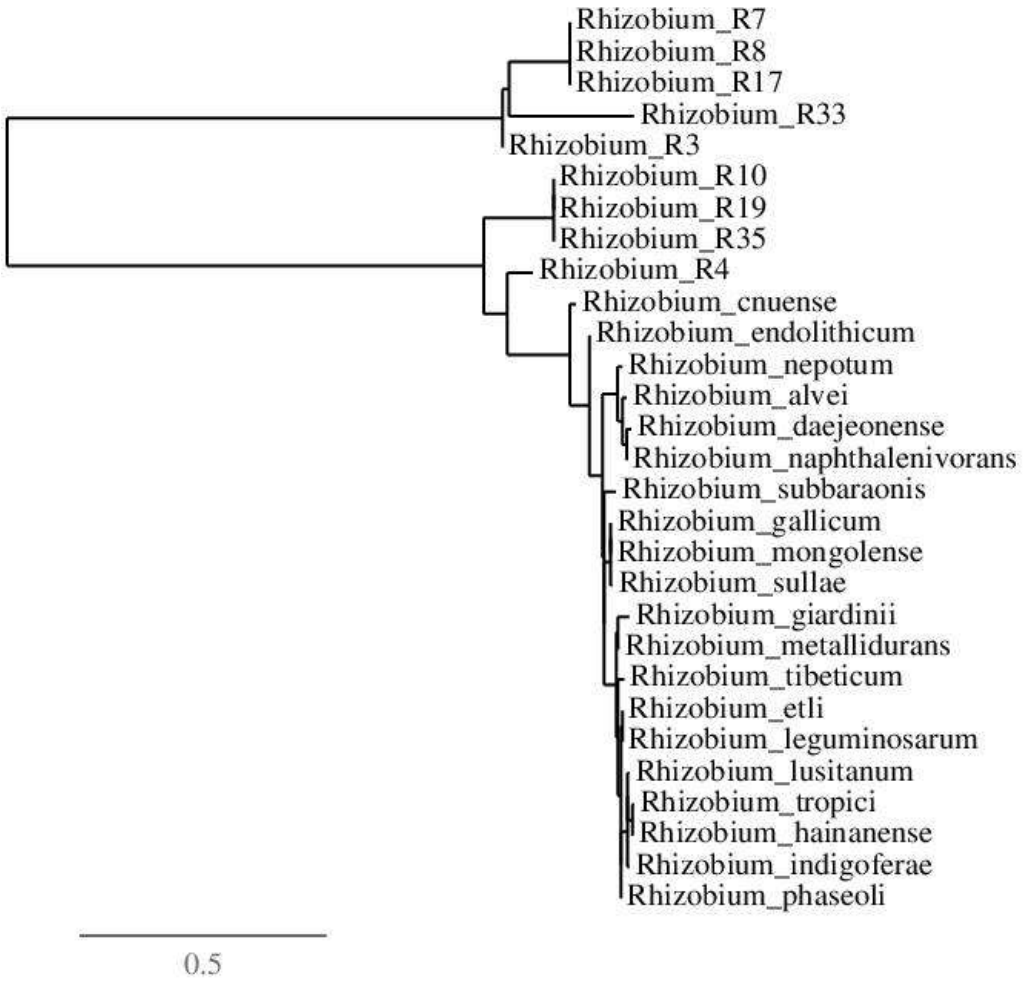


**Figure 3.** Dendrogram constructed by the complete linkage method base on 16S rRNA sequences of *Rhizobium* species.

leguminous plants (Barny et al., 1996). When DNA was amplified with *nodC* specific primer, an amplicon of 500 bp was observed. Not all the strains harboured both *nifH* and *nodC* genes. The absence of the amplicon product was probably due to some nucleotide mismatches in *nifH* and *nodC* gene which agreed with the result of Laguerre et al. (2001). Some strains (R1, R14, R15, R16, R30, R31, R37 and R39) showed positive result in *nodC* gene amplification but failed to form nodule in the plant infection test. We concluded that pot experiment might have failed to form nodule for certain limitations such as temperature, humidity etc. which were controlled in the laboratory experiment.

The 16S rRNA sequence of nine isolates were compared with 20 species from the NCBI data base. The sequences were aligned and dendrogram was constructed (Figure 3). In the 16S rRNA sequencing, two distinct clusters were seen. In the first cluster, the isolates R7, R8, R17, R33, R3 were grouped together. On the other hand, the isolates R10, R19, R35 and R4 were clustered together with the 20 reference strains. So, first cluster was phylogenetically distinct from the reference strains and might have evolved from a distinct lineage. Based on the 16S rRNA sequences, the isolates R7, R8 and R17 were indistinguishable from each other. A similar conclusion was applicable for the isolates R10, R19, R35. It could also be inferred that the isolates R10, R19 and R35 exhibited a convincing degree of dissimilarity as compared to the reference strains and hence branched off to form a different cladogroup. The isolate R4 was placed adjacent to *Rhizobium cnuense*. It is concluded that the findings may represent new species of *Rhizobium*. Menna et al. (2006) also observed new rhizobial isolates based on the sequencing of 16S rRNA.

## Conclusion

Most of the isolates found in this study were fast grower and were able to utilize different carbohydrates. Antibiotic

susceptibility test showed that isolates were resistant to antibiotics antibiotics that might help them to survive in antibiotic stress environment. Isolates harbouring *nifH* and *nodC* genes might be useful to increase soil fertility. The sequencing results revealed the genetic variability of the isolates. The goal of the present study was to raise the worldwide knowledge of the biodiversity of soil rhizosphere and identified the usefulness of rhizobial population so that isolates can be used as biofertilizers. To identify the genetic diversity of *Rhizobium* populations, molecular characterization such as fingerprinting techniques are needed. Different environmental stress conditions such as temperature, pH, heavy metals and pesticidal effects needs to be checked prior to the release of *Rhizobium* among the field populations so that it can survive under the adverse environmental condition and improve soil productivity.

## CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

## ACKNOWLEDGEMENTS

The authors thank the Head of the Department of Microbiology, Prof. Humaira Akhter and the Ministry of Science and Technology, Government of the Peoples Republic of Bangladesh (MOST) for granting the fellowship which made this study possible.

### REFERENCES

Agrawal PK, Agrawal S, Singh U, Katiyar N, Verma SK (2012). Phenotypic characterization of rhizobia from legumes and its application as a bioinoculant. Int. J. Agric. Technol. 8(2):681-692.

Arora DR (2003). The textbook of Microbiology new Delhi. CBS publisher: pp. 41-48.

Barny MA, Schoonejans E, Economou A, Johnston AW, Downie JA (1996). The C-terminal domain of the *Rhizobium leguminosarum* chitin synthase *nodC* is important for function and determines the orientation of the N-terminal region in the inner membrane. Mol. Microbiol. 19(3):443-53.

Belachew T (2010). Intrinsic antibiotic resistance, survival of *Rhizobium leguminosarum* strains and fixation potential of peavarieties (*Pisum sativum L*.) in southeast Ethiopia. Int. J. Microbiol. Res. 2:75-79.

Bishop PE, Guevara JG, Engelke JA, Evans HJ (1976). Relation between glutamine synthetase and nitrogenase activities in the symbiotic association between *Rhizobium japonicum* and *Glycine max*. Plant Physiol. 57(4):542-546.

Cappuccino JG, Sherman N (2008). Microbiology: a laboratory manual. Boston, MA: Pearson/Benjamin Cummings. P. 9.

Christensen WB (1946). Urea decomposition as a means of differentiating *Proteus* and paracolon organisms from each other and from *Salmonella* and *Shigella* types. J. Bacteriol. 52:461-466.

Cole MA, Elkan GH (1973). Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. Antimicrob. Agents Chemother. 4:248-253.

Cole MA, Elkan GH (1979). Multiple antibiotic resistance in *Rhizobium japonicum.* Appl. Environ. Microbiol. 37:867-870.

El-Idrissi MM, Aujjar N, Belabed A, Dessaux Y, Filali-Maltouf A (1996). Characterization of rhizobia isolation from Carob tree (*Ceratonia siliqua*). J. Appl. Bacteriol. 80:165-173.

Fred EB, Baldwin IL, McCoy E (1932). Root nodule bacteria and leguminous plants. University of Wisconsin Studies in Science P 5.

Frioni L, Rodrıguez A, Meerhoff M (2001). Differentiation of rhizobia isolated from native legume trees in Uruguay. Appl. Soil Ecol. 16:275-282.

Gemell L, Roughley R (1993). Counting rifampicin-resistant rhizobia when a minor component of the soil rhizobial flora. Soil Biol. Biochem. 25:539-544.

Glenn AR, Dilworth MJ (1981). The uptake and hydrolysis of disaccharides by fast- and slow-growing species of *Rhizobium*. Arch. Microbiol. 129:233-239.

Graham PH, Parker CA (1964). Diagnostic features in the characterisation of the root-nodule bacteria of legumes. Plant Soil 20(3):383-396.

Graham PH, Sadowsky MJ, Keyser HH, Barnet YM, Bradley RS, Cooper JE, Deley DJ, Jarvis BDW, Roslycky EB, Strijdom BW, Young JPW (1991). Proper minimal standards for the description of new genera and species of root and stem-nodulating bacteria. Int. J. Syst. Bacteriol. 41:582-587.

Jacob TW, Egelhoff TT, Long SR (1985). Physical and genetic map of *Rhizobium meliloti* nodulation gene region and nucleotide sequence of *nodC*. J. Baceriol. 162:469-476

Ji X, Shen Q, Liu F, Ma J, Xu G, Wang Y, Wu M (2012). Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai. China J. Hazard. Mater. 235:178-185.

Kovacs N (1956). Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature (London) 178:703.

Ladha JK, Pareek RP, Becker M (1992). Stem-nodulating legume-*Rhizobium* symbiosis and its agronomic use irr lowland rice. Adv. Soil Sci. 20:147-192.

Laguerre G, Nour SM, Macheret V, Sanjuan J, Drouin P, Amarger N (2001). Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. Microbiol. 147(4):981-993.

Lowe GH (1962). The rapid detection of lactose fermentation in paracolon organisms by the demonstration of beta-D-galactosidase. J. Med. Lab. Technol. 19(1):21-5.

Menna P, Hungria M, Barcellos FG, Bangel EV, Hess PN, Martínez-Romero E (2006). Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. Syst. Appl. Microbiol. 29(4):315-332.

Mihaylova S, Genov N, Moore E (2014). Susceptibility of environmental strains of *Rhizobium radiobacter* to antimicrobial Agents. World Appl. Sci. J. 31(5):859-862.

Mulligan JT, Long SR (1985). Induction of *Rhizobium meliloti nodC* expression by plant exudate requires *nodD*. Proc. Natl. Acad. Sci. 82(19):6609-6613.

Naamala J, Jaiswal SK, Dakora FD (2016). Antibiotics resistance in *Rhizobium*: type, process, mechanism and benefit for agriculture. Curr. Microbiol. 72(6):804-816.

Naz I, Bano A, Hassan TU (2009). Morphological, biochemical and molecular characterization of rhizobia from halophytes of Khewra Salt Range and Attock. Pak. J. Bot. 41(6):3159-3168.

Ndoye I, Dreyfus B (1988). NP fixation by *Sesbania rostrata* and *Sesbania sesban* estimated using N-15 and total N difference methods. Soil Biol. Biochem. 20:209-213.

Oldroyd GE (2013). Speak, friend, and enter: signaling systems that promote beneficial symbiotic associations in plants. Nat. Rev. Microbiol. 11:252-263.

Perret X, Staehelin C, Broughton WJ (1998). Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. 64:180-201.

Perret X, Staehelin C, Broughton WJ (2000). Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. 64:180-201.

Prasuna ML (2014). Characterisation of *Rhizobium* isolates associated with wild legumes on the basis of antibiotic resistance. Indian J. Sci. Res. 4(1):22-24.

Sadowsky MJ, Keyser HH, Bohlool BB (1983). Biochemical characterization of fast- and slow-growing rhizobia that nodulate soybeans. Int. J. Syst. Bacteriol. 33(4):716-722.

Saidi S, Ramırez-Bahena MH, Santillana N, Zuniga D, Alvarez-Martınez E, Peix A, Mhamdi R, Velazquez E (2014). *Rhizobium laguerreae* sp.

nov. nodulates Vicia faba on several continents. Int. J. Syst. Evol. Microbiol. 64:242-247.

Singh D, Sharma A, Saini GK (2013). Biochemical and molecular characterisation of the bacterial endophytes from native sugarcane varieties of Himalayan region. 3 Biotech. 3(3):205-212.

Singh NK, Luthra DU, Desai DN (2013). Phenotypic and genotypic characterization of *Rhizobium* species isolated from the root nodules of *Sesbania sesban* found in Mumbai and its suburban areas. Indian J. Appl. Res. 3:2249-555.