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Umakant Banjare
Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, B.H.U., Varanasi, Uttar Pradesh, India

Arun Kumar Patel
Department of Botany, Institute of Science, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Ashish Kumar Pandey
Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, B.H.U., Varanasi, Uttar Pradesh, India

Santosh Kumar
ICAR – Indian Agricultural Research Institute, Jharkhand, India

Sandeep Kumar Gupta
Department of Botany, Institute of Science, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Ramesh Kumar Singh
Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, B.H.U., Varanasi, Uttar Pradesh, India

Corresponding Author:
Umakant Banjare
Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, B.H.U., Varanasi, Uttar Pradesh, India

Morphological & biochemical characterization of root Nodulating Endosymbionts of lentil (*Lens culinaris* Medik L.)

Umakant Banjare, Arun Kumar Patel, Ashish Kumar Pandey, Santosh Kumar, Sandeep Kumar Gupta and Ramesh Kumar Singh

Abstract
Lentil (*Lens culinaris* Medik. L.) is a legume & cool season crop. Mature lentil seeds are usually rich in proteins although they produce these proteins during their growth. Lentil seeds have a high amount of protein between 20.6 and 31.4 g/100 g. It maintains soil fertility through symbiotic nitrogen fixation in association with *Rhizobium leguminosarum* bv. Viciae. This study was carried out to characterize *Rhizobium* species isolated from root nodules of lentil. The isolates were identified and characterized on the basis of colony morphology and biochemical traits *viz*. gram staining, catalase, oxidase tests, KOH and nitrate reductase test. The *Rhizobium* isolates were white, mucilaginous, translucent, circular & fast grower on YEMA plates. They were rod-shaped, gram negative, positive for catalase, oxidase, KOH & nitrate reductase tests. Out of twenty isolates two isolates were found to be gram positive by showing violet/purple staining in gram reaction indicated their non-*Rhizobium* characteristic.

Keywords: Morphological, biochemical Nodulating, Endosymbionts, *Lens culinaris* Medik

Introduction
Lentil (*Lens culinaris* Medik.) is a leguminous crop with agricultural and economic significance. It is rich in protein, grown mainly in developing countries in nutrient limited soils and often subjected to intermittent drought (Gahoonia *et al*., 2005) [11]. The most significant attribute of the lentil is its ability for (N_2) fixation symbiotically in agro ecological system. To fix (N_2) symbiotically with growth promoting rhizobacteria, pulses are different because they are known to improve the nitrogen status in the soil by trapping the atmospheric nitrogen into root nodules of crop in the form of ammonia (NH_3) and ultimately increase the soil fertility and crop productivity (Riah *et al*., 2014) [30]. Rhizobia are gram-negative bacteria present in the root nodules of legumes (Ngakou *et al*., 2009) [20]. These soil bacteria are aerobic, non-sporulating, and rod-shaped (Chaintreuil *et al*., 2000) [6]. A symbiotic relationship termed mutualism develops between legumes and rhizobia, wherein both organisms benefit from each other. Rhizobia produce nitrogen-containing ammonium which can be easily taken up by plants (Kiers *et al*., 2002) [23]. Legume-rhizobia symbiosis accounts for 60% of the total BNF on the earth (Zou *et al*. 2016; Dong & Song 2020) [39, 10]. Rhizobia may live in two states: as free-living bacteria in the soil or as symbiotic bacteroids inside legume nodules (Quelas *et al*. 2013) [28]. Formation of root nodules involves two separate processes, the infection by rhizobia and the organogenesis of the nodule (Clúa *et al*. 2018) [7]. The absence of effective nitrogen-fixing strains of rhizobia in the soil and root nodules, results in a crop decline and yield loss of legumes that gain their nitrogen mainly from the process of BNF (Hassen *et al*. 2018). Bacterial strains appear in diff erent colour, texture and morphology on yeast extract mannitol agar plates (Varun *et al*., 2017) [35]. Crop yield enhances by bacteria (endophytes) which are used as bioinoculant, Batra *et al*. (2018) [1]. Seed inoculation of diff erent *Rhizobium* showed signifcant results on lentil crop (Das *et al*., 2017). Recently, some R. leguminosarum bv. viciae strains are considered as plant growth promoting rhizobacteria (PGPR), since (Sommers and Vanderleyden, 2004). PGPR stimulate plant growth directly either by synthesizing phytohormones such as indole-3-acetic acid (IAA) or by promoting nutrition processes such as phosphate solubilization and siderophore production, which facilitate phosphorus and iron uptake, respectively from soil (Lippmann *et al*., 1995) [25].

Materials and Method

Site of sample collection

Root nodule samples of lentil were obtained from Durg, Dhamtari, Rajnandgaon, Balod & Raipur districts of Chhattisgarh & Mau, Mirzapur & Varanasi districts of Uttar Pradesh. The root nodule samples collected from 45-60 days old lentil plants. A total of 57 isolates from 42 places were obtained out of which 20 isolates were retained on the basis of their morphological & biochemical properties and maintained in the P.G.P.R. Laboratory of Prof. Ramesh Kumar Singh, Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University Varanasi-India. Details of bacterial isolates have been shown in table 1.

Table 1: Description of site and source of bacterial isolates

S.N.	Isolates	Host	Place of sample collection
1	LUP2	Root nodule of lentil	Mirzapur, Uttar Pradesh
2.	UP4N	Root nodule of lentil	Mirzapur, Uttar Pradesh
3.	LN2	Root nodule of lentil	Sankara, balod chhattisgarh
4.	19N	Root nodule of lentil	Bhanpuri, Dhamtari, Chhattisgarh
5.	20NII	Root nodule of lentil	Sarseewa, Dhamtari, Chhattisgarh
6.	LUP4	Root nodule of lentil	Shivpur, Varanasi, Uttar Pradesh
7.	18N	Root nodule of lentil	Bundeli, Rajnandgaon, Chhattisgarh
8.	1N	Root nodule of lentil	Chandkhuri, Durg, Chhattisgarh
9.	UP6N	Root nodule of lentil	Mau, Uttar Pradesh
10.	LN4Y	Root nodule of lentil	Mahoba, Rajnandgaon, Chhattisgarh
11.	LN3	Root nodule of lentil	Jangera, balod, chhattisgarh
12.	LN4P	Root nodule of lentil	Jagnathpur, balod, chhattisgarh
13.	LCG6	Root nodule of lentil	Devkar, Bemetara, Chhattisgarh
14.	26N	Root nodule of lentil	Hanoda, Durg, Chhattisgarh
15.	LCG5	Root nodule of lentil	Abhanpur, Raipur, Chhattisgarh
16.	UP8N	Root nodule of lentil	Mau, Uttar Pradesh
17.	27N	Root nodule of lentil	Hanoda, Durg, Chhattisgarh
18.	UP2N	Root nodule of lentil	RGSC, BHU, Barkachha, Mirzapur, Uttar Pradesh
19.	LCG2	Root nodule of lentil	Dharampura, Raipur, Chhattisgarh
20.	LNBHU	Root nodule of lentil	Agriculture farm BHU, Varanasi

Sterilization of samples

Healthy roots were carefully selected, washed under tap water to remove soil. Nodules were collected in separate beaker, washed in distilled water and drained. Fresh root nodules of lentil plants were sterilized with 70% ethanol for 4-5 min and 0.1% $HgCl_2$ for 1 min. After sterilization, root nodules were washed thrice with sterile distilled water and dried in laminar air flow. Surface disinfection parameters like selection of disinfectant, its strength, duration of immersion in disinfectant were optimized prior to experimentation.

Streaking of samples on culture media

After sterilization, nodules were crushed on the sterilized slides in sterile distilled water and then streaked on yeast extract Mannitol agar (YEMA) plates and incubated at 28±2° C for 3 days (Bhattacharya and chandra, 2013) [4]. Growth of bacteria was carefully observed. At the end of incubation period, bacteria with white, mucilaginous & translucent colonies were selected. These colonies were purified to get single colonies by repeated streaking on YEMA plates and used for further studies. Glycerol stock was prepared to store the cultures for a longer time at refrigerated conditions (-80 °C). Short-term storage for further characterization was on YEMA slants at 4 °C also sub-culturing was done continuously throughout the period of investigation.

Morphological diversity

For the primary identification and characterization, morphological and biochemical tests are still one of the key criterions utilized (Rai *et al.*, 2014) [29]. In this study, morphological characterization of the 20 isolates was done to determine the colony characteristics of the bacterial isolates. The purified bacterial isolates were examined for the morphological and colony characteristics like, shape, size, margin, growth pattern etc. On the basis of morphological and biochemical attributes studied these isolates were grossly identified as *Rhizobium* defined by Somasegaran and Hoben (1994). Twenty isolates used under this study are: LUP2, UP4N LN2, 19N, LUP4, 1N, UP6N, LN4Y, LN3, LN4P LCG6, 26N, LCG5, UP8N, 27N, UP2N, LCG2 & LNBHU.

Biochemical characterization

Pure cultures of the isolates were made from single colonies and then subjected to Gram reaction. The Gram negative isolates were further subjected to biochemical tests including catalase, oxidase, potassium hydroxide solubility (KOH) and nitrate reductase for confirmation of the *Rhizobium* strains.

Gram staining

A fresh culture of bacterium was smeared on a different clean glass slide, air dried and fixed with slight heating. The slide was flooded for 1 min in crystal violet solution (2 g crystal violet, 1 g ammonium oxalate, in 100 ml distilled water). It was washed gently with tap water for 3–4 s and then kept with iodine solution (100 ml distilled water added to 1 g iodine, 2 g potassium iodide and stored in dark bottle) for one min. The slide was rinsed again gently and then de-stained with 95% ethanol to the point where no more stain runs off the slide. The slide was rinsed again and counterstained for 10 second with safranin (safranin, stock solution is a 2.5% solution in 95% ethanol, diluted tenfold before using), then rinsed and blotted dried. Gram-positive cells showed blue appearance, Gram-negative cells are pink.

Catalase activity

Fresh culture (24 h old) of bacteria was placed on a glass slide

and one drop of H_2O_2 (3%) was dropped on the colony; appearance of gas bubbles indicated the catalase activity and was recorded as weak (+), moderate (++), and strong (+++) respectively (Mac Faddin, J. F. 1980) [26].

Oxidase test

It was done on freshly grown cultures from YEMA with 1% glucose onto a filter paper moistened with a fresh 1% aqueous solution of tetramethyl-p-phenylenediamine dihydrochloride. A purple reaction in 10 seconds is positive, purple color appearance after 60 seconds is considered a delayed positive, and any reaction after 60 seconds or no reaction at all is negative (Klement *et al.*, 1990) [24].

Potassium hydroxide test *(*KOH)

One drop of 3% KOH was taken on a clean microscopic slide and a loop was used to transfer a generous number of bacteria (cultured for 24-48 hrs.) to the drop of KOH carefully and mixed properly. After mixing, the loop was elevated to observe for the formation of mucoid string within 30 seconds indicating the gram-negative bacteria.

Nitrate reductase activity

The isolates were inoculated into 10 ml of sterile nitrate broth in test tubes and incubated at 28°C. After 24 hours, culture broth was tested by adding equal amounts (3-4 drops) of sulfanilic acid and alpha naphthylamine. Development of red color indicated the positive result in which nitrate had been reduced to nitrite. The nitrate broth tubes in which red color didn't appear were added with pinch of zinc dust. The tubes in which the color changes to red after addition of zinc dust were considered as negative while the tube without any change in colour were considered as positive as in such tubes the nitrate has been reduced to nitrogen gas.

Result and Discussion

Morphological characterization: On the basis of morphological observations on Yeast extract mannitol agar (YEMA) medium, colonies of all the strains appeared mucilaginous, smooth and gummy in nature. The color of the bacterial colonies was whitish for all the strains except LN3, 19N, LCG6 & 26N (Creamy) and LN4P (Yellowish). The size of the colonies formed was small for the eight strains *viz*. LUP2, LN2, 18N, 1N, LN4Y, UP2N, LCG2 & LNBHU. Two strains LN3 and 26N formed larger colonies in comparison to the other strains used in this study. Rest of the ten strains formed medium sized colonies. All the strains showed round appearance of their colonies. Thus, the diversity observed in the colony morphology suggests the existence of genetic diversity among the different isolates. The variation in the appearance of the colony due to the different genes has been reported by several workers (Liu, W. Y. Y. 2014 & Muthini *et al.*, 2014) [18, 19]. Colony morphology are often involved in protein sorting and epigenetic regulation. Few genes are likely to play a direct role in establishing colony morphology, *e.g. FLO11*, a gene encoding a cell-surface adhesin that has already been implicated in colony morphology, biofilm formation, and invasive and pseudo hyphal growth (Voordeckers *et al.*, 2012) [36]. The *R. leguminosarum* plasmids pRL12JI and pRleVF39f carry auxotrophic markers, and that the plasmid pR1eVF39c carries genes which affect colony morphology (Hynes *et al*., 1989) [14]. Bacterial strains showed moderate to very fast growth which indicated the feature of *Rhizobium* along with other endosymbionts. Initial growth was started on 2nd day and usually took 4-5 days for the complete growth on the YEMA media plate. These results were in agreement with Begom *et al*., (2021) [3] who reported Fast grower nature of *Rhizobium* which can nodulate lentil for biological N_2 fixation. Similar findings were also reported by Keyser *et al.*, (2022) [16] who reported Fast grower nature of *Rhizobium* which can nodulate soybean for biological N_2 fixation. The details of morphological characteristics and growth habit of bacterial isolates are mentioned in Table 2 and Figure 1.

Table 2: Colony morphology of bacterial isolates on YEMA plates

S.N.	Strains	Colony characteristics on YEMA plate	Growth habit
1.	LUP2	Mucilagenous, whitish, opaque, small, circular, smooth, flat surface	Fast grower
2.	UP4N	Mucilagenous, whitish, opaque, medium, circular, raised, entire margin	Fast grower
3.	LN2	Mucilagenous, whitish, opaque, small, circular, flat surface.	Fast grower
4.	19N	Mucilaginous, smooth, creamy, opaque, medium, circular, raised, entire margin	Fast grower
5.	20NII	Mucilagenous, whitish, medium, circular, opaque, flat surface, entire margin	Fast grower
6.	LUP4	Mucilagenous, whitish, opaque, medium, circular, slightly raised, entire margin	Fast grower
7.	18N	Mucilagenous, whitish, opaque, small, circular, drop like, slightly raised	Fast grower
8.	1N	Mucilagenous, whitish, opaque, small, circular, slightly raised, entire margin	Fast grower
9.	UP6N	Mucilagenous, whitish, opaque, medium, circular, raised and entire margin	Fast grower
10.	LN4Y	Dry, whitish, opaque, small, circular, punctiform, flat	Fast grower
11.	LN3	Mucilagenous, creamy, opaque, large, circular, convex, entire margin	Fast grower
12.	LN4P	Mucilagenous, yellowish, opaque, medium, circular, convex, entire margin	Fast grower
13.	LCG6	Mucilagenous, creamy, opaque, medium, circular, convex, entire margin	Fast grower
14.	26N	Mucilagenous, creamy, opaque, large, circular, umbonate, entire margin	Fast grower
15.	LCG5	Mucilagenous, whitish, opaque, medium, circular, convex, entire margin	Fast grower
16.	UP8N	Mucilagenous, whitish, opaque, medium, circular, raised, entire margin	Fast grower
17.	27N	Dry, whitish, opaque, medium, circular, flat, entire margin	Fast grower
18.	UP2N	Dry, whitish, opaque, small, circular, flat, entire margin	Fast grower
19.	LCG2	Mucilagenous, whitish, opaque, small, circular, raised, entire margin	Fast grower
20.	LNBHU	Mucilagenous, whitish, opaque, small, irregular, raised, entire margin	Fast grower

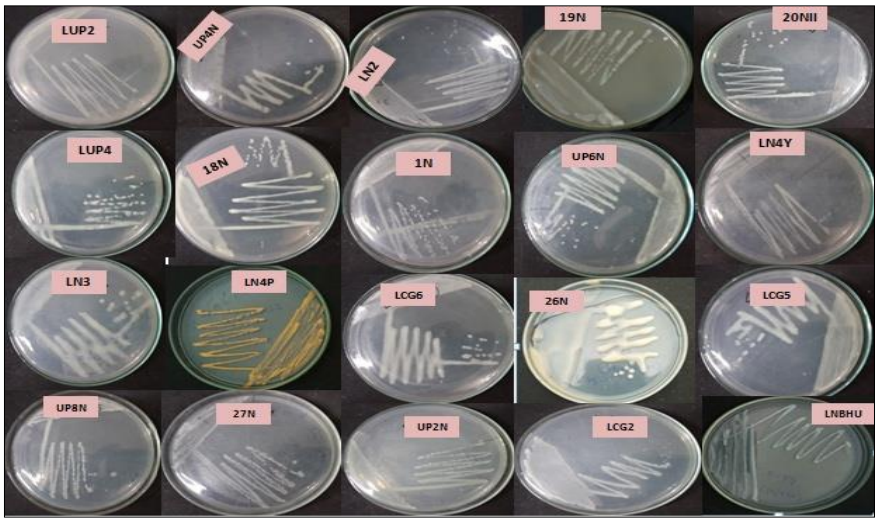

Fig 1: Morphological characterization of twenty bacterial isolates obtained from root nodule of lentil

Biochemical characterization

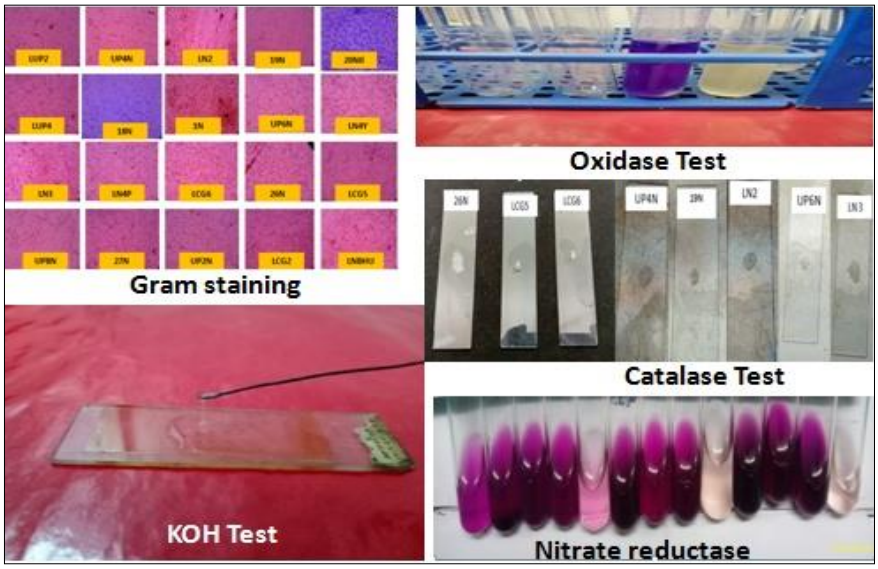

Fig 2: Bacterial isolates showing different biochemical tests

Table 3: Bacterial isolates showing Gram reaction, oxidase, and catalase, KOH & nitrate reductase test

S. No.	Strains	Gram reaction	Oxidase	Catalase	KOH test	Nitrate reductase test
1.	LUP2	Negative	Positive	Positive	Positive	Positive
2.	UP4N	Negative	Positive	Positive	Positive	Positive
3.	LN2	Negative	Positive	Positive	Positive	Positive
4.	19N	Negative	Positive	Positive	Positive	Positive
5.	20NII	Positive	Positive	Negative	Negative	Positive
6.	LUP4	Negative	Positive	Positive	Positive	Positive
7.	18N	Positive	Positive	Positive	Negative	Positive
8.	1N	Negative	Positive	Negative	Positive	Positive
9.	UP6N	Negative	Positive	Positive	Positive	Positive
10.	LN4Y	Negative	Positive	Negative	Positive	Positive
11.	LN3	Negative	Positive	Positive	Positive	Positive
12.	LN4P	Negative	Positive	Positive	Positive	Positive
13.	LCG6	Negative	Positive	Positive	Positive	Positive
14.	26N	Negative	Positive	Positive	Positive	Positive
15.	LCG5	Negative	Positive	Positive	Positive	Positive
16.	UP8N	Negative	Positive	Positive	Positive	Positive
17.	27N	Negative	Positive	Positive	Positive	Positive
18.	UP2N	Negative	Positive	Positive	Positive	Positive
19.	LCG2	Negative	Positive	Positive	Positive	Positive
20.	LNBHU	Negative	Positive	Negative	Positive	Positive

Gram staining

Gram Staining is a technique proposed by Christian Gram (1884) to distinguish the two types of bacteria based on the difference in their cell wall structures. This process distinguishes bacteria by identifying peptidoglycan that is found in the cell wall of the gram-positive bacteria. The cell walls for Gram-positive microorganisms have a higher peptidoglycan and lower lipid content than gram-negative bacteria. The result showed all the bacteria to be Gram negative except the strains 20NII & 18N which were Gram positive (Table 3 & figure 2). Similar results were found by Hameed *et al*., (2004) [13] who reported that most of the *Rhizobium* and *Brady Rhizobium* (class of the Alpha proteobacteria, order of the Rhizobiales) are Gram-negative nitrogen-fixing bacteria that occur either as free-living soil bacteria or in interaction with the roots of leguminous plants. Similar findings were also obtained by (Datta *et al*., 2015 & Tanim *et al*., 2019) [9, 33], they found that all isolated strains shown to be gram negative and rod shaped and motile. Ismail *et al*., (2022) [15] also reported that isolated strains were found to be gram negative as the cells appeared pink in color after gram staining.

Oxidase test

Oxidase test was performed to determine the presence of oxidase enzyme in different strains of bacterial isolates. Kovac's reagent (1% N, N, N.N tetramethyl- p-phenylenediamine) was dissolved in warm water and stored in dark bottle. A strip of filter paper was dipped in this reagent and air-dried and put into one-day-old Rhizobial colonies from agar plates. All the twenty bacterial isolates were examined for the presence of cytochrome c oxidase enzyme. After the test all the bacterial isolates were found to turn the reagent blue/purple hence proved to be oxidase positive (Table 3 & figure 2). Singh, N. P., & Sewak, S. (2013) [31] observed positive results for oxidase activity. Wani and Khan (2013) and Bedi, M. K., & Naglot, A. (2011) [37, 2] also reported that *Mesorhizobium* isolates were positive for oxidase test.

Catalase test

All the strains showed positive catalase activity except 20NII, 1N, LN4Y & LNBHU. (Table 3 and figure 2). Catalase test demonstrates the presence of catalase, an enzyme that catalyzes the release of oxygen from hydrogen peroxide (H_2O_2). Hydrogen peroxide, the product of dis-mutation, is degraded by catalases (CAT), which play a crucial role in the symbiosis during both host plant infection and nitrogen fixation processes (Orikasa *et al.*, 2010) [21]. Panek and Brian (2004) reported that aerobically grown *Bradyrhizobium japonicum* cells express a single catalase activity. GSH (gamma-L-glutamyl-L-cysteinyl-glycine) plays a critical role by regulating redox homeostasis, and appears to be essential for initiation and development of the root nodules (Bianucci *et al*., 2008; Yin *et al*., 2018) [5, 38].

KOH test

Gram staining was confirmed by the potassium hydroxide solubility test which was recorded positive for all the strains except for 20NII & 18N which showed negative KOH test (Table 3 and figure 2). The purpose of the potassium hydroxide test (KOH test) is to identify gram negative bacteria. KOH dissolves the thin layer of peptidoglycan of the cell walls of gram negative bacteria, but does not affect gram positive cell walls. Disintegration of gram negative cell wall lyses the cell and release its contents, including the DNA. As a result, a viscid chromosomal material is released which causes the bacterial suspension to become thick and stringy. Upadhyay *et al.,* (2015) also reported the positive KOH test for the *Rhizobium* strains isolated from nodules of lentil and pea.

Nitrate reductase

The inhibitory effect of nitrate on nitrogenase activity in root nodules of legume plants has been known for a long time. The presence of nitrate may cause changes in the resistance to O_2 diffusion. All the twenty strains tested were positive for nitrate reductase activity. Among twenty strains based on visual observation for color intensity four strains (26N, LCG6, LN4P & LCG5) showed very strong (++++) nitrate reductase activity, followed by six strains (UP4N, LN2, LN3, 1N, LCG2 & UP6N) which were observed for strong (+++) nitrate reductase activity, six strains showed moderate (++) nitrate reductase activity whereas four strains exhibited average (+) activity (Table 3 & figure 2). Kang, M., & Seo, T. (2022) [22] reported *Rhizobium setariae* sp. isolated from Green Foxtail which was able to reduce nitrate. Ligero *et al*., (1987) [17] also reported nitrate reductase activity in strains of *Rhizobium leguminosarum* isolated from pea nodules.

Conclusion

Morphological and biochemical tests are essential criterion for primary identification of *Rhizobium* species. In the present study, 20 rhizobial isolates from lentil plant from different agroclimatic zones were obtained and their morphological and biochemical characterization was done. Based on morphological characterization on YEMA plates, different size, shape, color, elevation & texture were observed. Colonies on YEMA plates were observed to be smooth, translucent, mucilaginous, white and with concave/convex/flat surface. Biochemical characterization revealed that most of the strains were gram negative, catalase positive, oxidase positive, KOH positive & had ability to reduce nitrate into nitrite. Growth behavior of isolates on YEMA plates & their biochemical characterization may help in identification of the species on a preliminary basis.

References

1. Batra P, Barkodia M, Ahlawat U, Sansanwal R, Sharma T, Wati L. Endophytes: An environmental friendly Bacteria for plant growth promotion. Int. J Curr. Microbiol. Appl. Sci. 2018;7(2):1899-1911.
2. Bedi MK, Naglot A. Characterization of Rhizobium isolated from root nodules of Trifolium alexandrinum. Journal of Agricultural Technology. 2011;7(6):1705-1723.
3. Begom MF, Ahmed MGU, Sultana R, Zahurul M. Isolation, identification and characterization of Rhizobium from lentil root nodule. 2021.
4. Bhattacharjya S, Chandra R. Effect of inoculation methods of Mesorhizobium ciceri and PGPR in chickpea (*Cicer areietinum* L.) on symbiotic traits, yields, nutrient uptake and soil properties. Legume Research-An International Journal. 2013;36(4):331-337.
5. Bianucci E, Tordable MDC, Fabra A, Castro S. Importance of glutathione in the nodulation process of

peanut (*Arachis hypogaea*). Physiologia Plantarum. 2008;134(2):342-347.
6. Chaintreuil C, Giraud E, Prin Y, Lorquin J, Bâ A, Gillis M, *et al*. Photosynthetic bradyrhizobia are natural endophytes of the African wild rice Oryza breviligulata. Applied and environmental microbiology. 2000;66(12):5437-5447.
7. Clúa J, Roda C, Zanetti ME, Blanco FA. Compatibility between legumes and rhizobia for the establishment of a successful nitrogen-fixing symbiosis. Genes. 2018;9(3):125.
8. Das K, Rajawat MVS, Saxena AK, Prasanna R. Development of Mesorhizobium ciceri-based biofilms and analyses of their antifungal and plant growth promoting activity in chickpea challenged by Fusarium wilt. Indian journal of microbiology. 2017;57(1):48-59.
9. Datta A, Singh RK, Tabassum S. Isolation, characterization and growth of Rhizobium strains under optimum conditions for effective biofertilizer production. Int. J Pharm. Sci. Rev. Res. 2015;32(1):199-208.
10. Dong W, Song Y. The significance of flavonoids in the process of biological nitrogen fixation. International journal of molecular sciences. 2020;21(16):5926.
11. Gahoonia TS, Ali O, Sarker A, Rahman MM. Root traits, nutrient uptake, multi-location grain yield and benefit–cost ratio of two lentil (*Lens culinaris*, Medikus.) varieties. Plant and soil. 2005;272(1):153-161.
12. Gram C. The differential staining of Schizomycetes in tissue sections and in dried preparations. Fortschitte der Medicin. 1884;2(6):185-189.
13. Hameed S, Yasmin S, Malik KA, Zafar Y, Hafeez FY. Rhizobium, Bradyrhizobium and Agrobacterium strains isolated from cultivated legumes. Biology and Fertility of Soils. 2004;39(3):179-185.
14. Hynes MF, Quandt J, O'Connell MP, Pühler A. Direct selection for curing and deletion of Rhizobium plasmids using transposons carrying the Bacillus subtilis sacB gene. Gene. 1989;78(1):111-120.
15. Ismail EA, Ali SM, Khaled AGA, EL-Sherbeny GAR. Genetic diversity and symbiotic compatibility among Rhizobium trifolii-clover isolated from Sohag governorate. SVU-International Journal of Agricultural Sciences. 2022;4(1):168-178.
16. Keyser HH, Sadowsky MJ, Bohlool BB. Fast-growing soybean rhizobia. In World Soybean Research Conference III: Proceedings. CRC Press. 2022 Feb, 926-934.
17. Ligero F, Lluch C, Olivares J, Bedmar EJ. Nitrate reductase activity in nodules of pea inoculated with hydrogenase positive and hydrogenase negative strains of Rhizobium leguminosarum. Physiologia Plantarum. 1987;69(2):313-316.
18. Liu WYY. Characterisation of rhizobia and studies on N_2 fixation of common weed legumes in New Zealand: A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy (Doctoral dissertation, Lincoln University). 2014.
19. Muthini M, Maingi JM, Muoma JO, Amoding A, Mukaminega D, Osoro N, *et al*. Morphological assessment and effectiveness of indigenous rhizobia isolates that nodulate P. vulgaris in water hyacinth compost testing field in Lake Victoria basin. British Journal of Applied Science & Technology. 2014;4(5):718.
20. Ngakou A, Megueni C, Ousseni H, Massai A. Study on the isolation and characterization of rhizobia strains as biofertilizer tools for growth improvement of four grain legumes in Ngaoundéré-Cameroon. International Journal of Biological and Chemical Sciences, 2009, 3(5).
21. Orikasa Y, Nodasaka Y, Ohyama T, Okuyama H, Ichise N, Yumoto I, *et al*. Enhancement of the nitrogen fixation efficiency of genetically-engineered Rhizobium with high catalase activity. Journal of Bioscience and Bioengineering. 2010;110(4):397-402.
22. Kang M, Seo T. Rhizobium setariae sp. nov., an Indole-3-Acetic Acid-Producing Bacterium Isolated from Green Foxtail, Setaria viridis. Current Microbiology. 2022;79(6):1-8.
23. Kiers ET, West SA, Denison RF. Mediating mutualisms: Farm management practices and evolutionary changes in symbiont co-operation. Journal of Applied Ecology. 2002;39(5):745-754.
24. Klement Z. Inoculation of Plant Tissues. In. Z. Klement, K. Rudolph, and DC Sands. Methods in Phyto Bactriology. 1990.
25. Lippmann B, Leinhos V, Bergmann H. Influence of auxin producing rhizobacteria on root morphology and nutrient accumulation of crops, pt. 1: changes in root morphology and nutrient accumulation in maize (*Zea mays* L.) caused by inoculation with indole-3-acetic acid (IAA) producing Pseudomonas and Acinetobacter strains or IAA applied exogenously. Angewandte Botanik (Germany). 1995.
26. Mac Faddin JF. Biochemical tests for identification of medical bacterial (No. 616.07581 M3), 1980.
27. Panek HR, O'Brian MR. KatG is the primary detoxifier of hydrogen peroxide produced by aerobic metabolism in *Bradyrhizobium japonicum*. Journal of bacteriology. 2004;186(23):7874-7880.
28. Quelas JI, Mongiardini EJ, Pérez-Giménez J, Parisi G, Lodeiro AR. Analysis of two polyhydroxyalkanoate synthases in Bradyrhizobium japonicum USDA 110. Journal of bacteriology. 2013;195(14):3145-3155.
29. Rai R, Bantawa P, Sur S. Trends in biochemical and molecular characterization of rhizobia and their nitrogen fixation mechanism: a review. Biology of useful plants and microbes (ed. By A. Sen), Narosa Publishing House, New Delhi, India, 2014, 61-119.
30. Riah N, Béna G, Djekoun A, Heulin K, de Lajudie P, Laguerre G. Genotypic and symbiotic diversity of Rhizobium populations associated with cultivated lentil and pea in sub-humid and semi-arid regions of Eastern Algeria. Systematic and applied microbiology. 2014;37(5):368-375.
31. Singh NP, Sewak S. Global perspective of chickpea research. AICRP on chickpea, 2013, 8-13.
32. Somers E, Vanderleyden J, Srinivasan M. Rhizosphere bacterial signalling: A love parade beneath our feet. Critical reviews in microbiology. 2004;30(4):205-240.
33. Tanim MTI, Chowdhury MMK, Bari L, Rahaman MM, Rahman SR, Rahman MM. Genetic diversity of *Rhizobium* spp. isolated from soil samples of Bangladesh. Bangladesh Journal of Microbiology. 2019;36(1):7-10.
34. Upadhayay SP, Pareek N, Mishra G. Isolation and biochemical characterization of Rhizobium strains from

nodules of lentil andpea in Tarai agro-ecosystem, Pantnagar, India. Jurnal Nasional. 2015;7(2):73-76.
35. Varun A, Patel A, Banjare U, Kumar S, Singh RK. Standardization of Isolation Technique of Rhizobium from Root Nodules of Lentil. Int. J. Curr. Microbiol. App. Sci. 2017;6(11):132-134.
36. Voordeckers K, De Maeyer D, van der Zande E, Vinces MD, Meert W, Cloots L, *et al*. Identification of a complex genetic network underlying Saccharomyces cerevisiae colony morphology. Molecular microbiology. 2012;86(1):225-239.
37. Wani PA, Khan MS. Isolation of multiple metal and antibiotic resistant mesorhizobium species and their plant growth promoting activity. Research Journal of Microbiology. 2013;8(1):25.
38. Yin C, Tang Y, Li X, Yang Z, Li J, Li X, e al. A Single Composition Architecture- Based Nanoprobe for Ratiometric Photoacoustic Imaging of Glutathione (GSH) in Living Mice. Small. 2018;14(11):1703400.
39. Zou L, Chen YX, Penttinen P, Lan Q, Wang K, Liu M, *et al*. Genetic diversity and symbiotic efficiency of nodulating rhizobia isolated from root nodules of faba Bean in one field. Plos one. 2016;11(12):e0167804.