

International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; SP-10(4): 665-669
www.biochemjournal.com
Received: 21-01-2026
Accepted: 25-02-2026

I Balakrishna Reddy
Assistant Professor, Department of Microbiology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Mahadevaswamy
Professor, Department of Microbiology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Pampanagouda
Assistant Professor, Department of Microbiology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Yallappa M
Assistant Professor, Department of Microbiology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Harish Menpadi
Assistant Professor, Department of Crop Physiology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Gururaj Sunkad
Professor, Department of Plant Pathology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Ramesh YM
Department of Agronomy, College of Agriculture, University of Agricultural Sciences, Mandya, Karnataka, India

Mahesh
Scientist, (Microbiology), National Bureau of Agricultural Important Insects, Bengaluru, Karnataka, India

Vijayachandra Reddy S
Assistant Professor, Department of Economics, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Corresponding Author:
I Balakrishna Reddy
Assistant Professor, Department of Microbiology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Isolation and screening of nitrogen fixing bacteria from phyllosphere and endorhizosphere of paddy (*Oryza sativa* L.) in Kalyana Karnataka

I Balakrishna Reddy, Mahadevaswamy, Pampanagouda, Yallappa M, Harish Menpadi, Gururaj Sunkad, Ramesh YM, Mahesh and Vijayachandra Reddy S

DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i4Sh.8205>

Abstract

Biological nitrogen fixation is a key process contributing to sustainable crop production, particularly in rice ecosystems. The present study was carried out in College of Agriculture, Raichur Department of Microbiology aimed to isolate and evaluate nitrogen-fixing bacterial populations from the phyllosphere and endorhizosphere of paddy (*Oryza sativa* L.) using Ashby's nitrogen-free medium. A total of 23 bacterial isolates were obtained and screened for their nitrogen fixation potential under *in vitro* conditions. The nitrogen fixation capacity ranged from 2.76 to 5.12 mg N per 100 ml medium, indicating substantial variability among isolates. Among them, isolate NFB 11 exhibited the highest nitrogen fixation (5.12 mg N/100 ml), followed by NFB 9, NFB 18, and NFB 13. Statistical analysis revealed significant differences among isolates ($p < 0.05$), with a mean value of 4.01 mg, standard error of 0.13, and coefficient of variation of 15.46%. The results indicate that both phyllosphere and endorhizosphere niches harbor efficient diazotrophic bacteria with potential for biofertilizer development. These isolates may contribute to improving nitrogen use efficiency and sustainable rice production.

Keywords: Biological nitrogen fixation, paddy, diazotrophs, phyllosphere

1. Introduction

Rice (*Oryza sativa* L.) is a staple crop feeding more than half of the global population. Its productivity largely depends on nitrogen availability, which is often supplemented through synthetic fertilizers. However, excessive use of nitrogen fertilizers has led to serious environmental concerns, including soil degradation, eutrophication, and greenhouse gas emissions (Tilman *et al.*, 2002) [13].

Biological nitrogen fixation (BNF), mediated by diazotrophic microorganisms, offers an eco-friendly alternative for improving soil fertility and crop productivity (Galloway *et al.*, 2008) [5]. These microorganisms convert atmospheric nitrogen into ammonia through the nitrogenase enzyme system, making it available for plant uptake (Dixon & Kahn, 2004) [4].

The rice ecosystem provides diverse microhabitats such as the phyllosphere (leaf surface) and endorhizosphere (internal root tissues), which support distinct microbial communities. The phyllosphere is an aerial habitat exposed to environmental stress, yet it hosts metabolically active bacteria capable of nutrient cycling (Lindow & Brandl, 2003) [8]. In contrast, the endorhizosphere offers a protected niche where endophytic bacteria interact closely with plant tissues and contribute to nutrient acquisition and stress tolerance (Bhattacharyya & Jha, 2012) [2].

Screening of nitrogen-fixing bacteria from these niches is essential for identifying efficient strains that can be utilized as biofertilizers. Ashby's nitrogen-free medium serves as a selective medium for isolating diazotrophs, allowing only nitrogen-fixing organisms to grow (Subba Rao, 2007) [12].

Therefore, the present investigation was undertaken to isolate and evaluate nitrogen-fixing bacteria from phyllosphere and endorhizosphere of paddy and to identify efficient isolates for sustainable agricultural applications.

2.1 Sample Collection

Healthy and disease-free paddy (*Oryza sativa* L.) plants were collected from experimental fields of the College of Agriculture, Raichur. Sampling was carried out during the active vegetative growth stage to ensure maximum microbial activity.

For phyllosphere sampling, fully expanded leaves were collected aseptically using sterile scissors and forceps, placed in sterile polyethylene bags, and transported to the laboratory under cooled conditions for immediate processing.

For endorhizosphere sampling, root systems were carefully uprooted along with adhering soil. The roots were washed under running tap water to remove loosely attached soil particles. Surface sterilization was carried out using 70% ethanol for 30 seconds followed by 0.1% mercuric chloride (HgCl₂) for 1-2 minutes, and subsequently rinsed thoroughly 3-4 times with sterile distilled water to eliminate surface contaminants. The sterilized roots were then macerated aseptically using a sterile mortar and pestle to release endophytic bacteria.

2.2 Isolation of Bacteria

Isolation of nitrogen-fixing bacteria was performed using the serial dilution and spread plate technique on Ashby's nitrogen-free agar medium. The macerated root samples and leaf washings were serially diluted up to 10⁻⁶ using sterile distilled water (Plate 1).

Aliquots (0.1 ml) from appropriate dilutions were spread uniformly on sterilized Ashby's agar plates using a sterile glass spreader. The plates were incubated at 28±2 °C for 3-5 days. Colonies that appeared on nitrogen-free medium were presumed to be diazotrophic in nature.

Distinct colonies were selected based on morphological characteristics such as size, shape, color, elevation, and margin. These colonies were repeatedly streaked on fresh Ashby's agar plates to obtain pure cultures. The purified isolates were maintained on slants at 4 °C for further studies.

2.3 Screening for Nitrogen Fixation

All purified isolates were screened qualitatively for their nitrogen-fixing ability using Ashby's nitrogen-free agar medium. Each isolate was spot inoculated onto the agar surface under aseptic conditions and incubated at 28±2 °C for 5-7 days.

The ability of isolates to grow on nitrogen-free medium was taken as an indication of their nitrogen-fixing potential. The growth intensity, colony development, and vigor were recorded. Only those isolates showing consistent and prominent growth were selected for further quantitative evaluation.

2.4 Quantitative Estimation of Nitrogen Fixation

The quantitative estimation of nitrogen fixation was carried out in Ashby's nitrogen-free broth medium. A loopful of each bacterial isolate was inoculated into 50 ml of sterile broth contained in 100 ml Erlenmeyer flasks and incubated at 28±2 °C for 7 days under static conditions.

After incubation, the total nitrogen fixed in the medium was estimated using the micro-Kjeldahl method as described by Bremner (1960) [3]. The procedure involved digestion of the culture with concentrated sulfuric acid in the presence of a catalyst mixture, followed by distillation and titration to determine the ammonia released.

The amount of nitrogen fixed by each isolate was calculated and expressed as:

mg Nitrogen fixed per 100 ml of culture medium

All treatments were carried out in triplicates to ensure accuracy and reproducibility of results.

2.5 Experimental Design and Statistical Analysis

2.5 Experimental Design and Statistical Analysis

The experiment was conducted using a Completely Randomized Design (CRD) comprising 23 bacterial isolates as treatments, each replicated three times under controlled laboratory conditions. The observations on nitrogen fixation (mg N per 100 ml medium) were recorded for each isolate, and the mean values were computed from the three replications to ensure accuracy and minimize experimental error.

The collected data were subjected to statistical analysis following standard procedures. The analysis of variance (ANOVA) was performed to determine the significance of differences among the isolates. The total variation observed in the experiment was partitioned into treatment and error components, and the calculated F-value was compared with the tabulated F-value at the 5% level of significance ($p \leq 0.05$) to test the null hypothesis.

Additionally, graphical analyses including trend analysis, frequency distribution, and box plot visualization were used to support the statistical findings and to better understand the distribution, variability, and performance range of the isolates. These combined statistical approaches ensured robust interpretation of the experimental results.

3. Results and Discussion

3.1 Nitrogen Fixation Potential

The nitrogen fixing ability of phyllosphere and endorhizosphere isolates obtained from collected samples was evaluated in Ashby's mannitol medium.

Considerable variation was observed among the isolates with respect to nitrogen fixation capacity (Table 9 and Plate 8). The amount of nitrogen fixed ranged from 2.76 mg N/100 ml (NFB 3) to 5.12 mg N/100 ml (NFB 11).

Among the phyllosphere isolates NFB 9 recorded the highest nitrogen fixation of 4.87 mg N/100 ml, followed by NFB 8 (4.35 mg N/100 ml) and NFB 7 (4.02 mg N/100 ml). In the endorhizosphere group, NFB 11 showed superior performance (5.12 mg N/100 ml), followed by NFB 18 (4.72 mg N/100 ml) and NFB 13 (4.64 mg N/100 ml). Based on their high nitrogen fixing potential, NFB 11 and NFB 9 were identified as the most efficient isolates and further utilized for biofertilizer formulation and field evaluation.

The nitrogen fixation results obtained from the present study are in close agreement with earlier reports by Akhter *et al.* (2012) [1], Murumkar *et al.* (2012) [9] and Jnawali *et al.* (2015) [6] who documented comparable nitrogen fixing efficiency in *Azotobacter* isolates. Similarly, Sawalgi *et al.* (2009) [11] evaluated the *in vitro* nitrogen-fixing potential of *Azospirillum* strains using NFBTB medium and observed values ranging between 1.4 and 20.96 mg N g⁻¹ of malate. In another study, Purushothaman *et al.* (1988) [10] reported that *Azospirillum* isolates from cotton genotypes exhibited nitrogen fixation ranging from 1.6 to 23.96 mg N g⁻¹ of carbon source. Furthermore, *A. lipoferum* and *A. brasilense* showed fixation levels between 7.54 and 24.53 mg N g⁻¹ of malic acid after seven days of incubation at 28 °C under static conditions. Among the 88 *Azospirillum* isolates

examined, 55.3 percent were identified as *A. lipoferum* and 41.57 percent as *A. brasilense* highlighting the dominance of these species among diazotrophs associated with crop rhizospheres.

The present study demonstrates that both phyllosphere and endorhizosphere of paddy harbour diverse nitrogen-fixing bacterial communities with varying efficiencies.

Table 1: Screening of nitrogen fixation from bacteria on Ashby's medium using the plate assay method

Sl. No.	Isolate code	Nitrogen Fixed (mg N/100 ml medium)
1	NFB 1	2.85
2	NFB 2	3.10
3	NFB 3	2.76
4	NFB 4	3.25
5	NFB 5	3.42
6	NFB 6	3.68
7	NFB 7	4.02
8	NFB 8	4.35
9	NFB 9	4.87
10	NFB 10	3.94
11	NFB 11	5.12
12	NFB 12	4.26
13	NFB 13	4.64
14	NFB 14	4.02
15	NFB 15	3.75
16	NFB 16	3.92
17	NFB 17	4.35
18	NFB 18	4.72
19	NFB 19	3.84
20	NFB 20	4.08
21	NFB 21	4.29
22	NFB 22	4.46
23	NFB 23	4.16

The superior performance of isolate NFB 11 suggests enhanced nitrogenase activity and efficient metabolic adaptation. Nitrogenase enzyme is highly sensitive to oxygen, and efficient isolates likely possess protective mechanisms such as high respiration rates or protective proteins (Dixon & Kahn, 2004) ^[4].

Phyllosphere isolates are typically exposed to fluctuating environmental conditions such as UV radiation, temperature variations, and limited nutrient availability. Despite these constraints, the presence of efficient nitrogen fixers indicates their adaptability and ecological significance (Lindow & Brandl, 2003) ^[8].

Endorhizospheric isolates, on the other hand, benefit from close association with plant tissues, allowing better access to root exudates and a stable microenvironment. This may explain the higher nitrogen fixation observed in some isolates, as root-associated bacteria often exhibit enhanced metabolic activity (Bhattacharyya & Jha, 2012) ^[2].

The moderate coefficient of variation (15.46%) suggests a balanced diversity among isolates, which is advantageous for selecting elite strains. Similar variability in nitrogen fixation has been reported in rice-associated diazotrophs (Ladha & Reddy, 2003) ^[7].

The identification of high-performing isolates such as NFB 11, NFB 9, and NFB 18 highlights their potential for biofertilizer development. These isolates can improve nitrogen availability, enhance plant growth, and reduce dependence on chemical fertilizers.

Additionally, integrating such isolates into microbial consortia may provide synergistic benefits, including improved nutrient uptake, stress tolerance, and disease resistance (Vessey, 2003) ^[14].

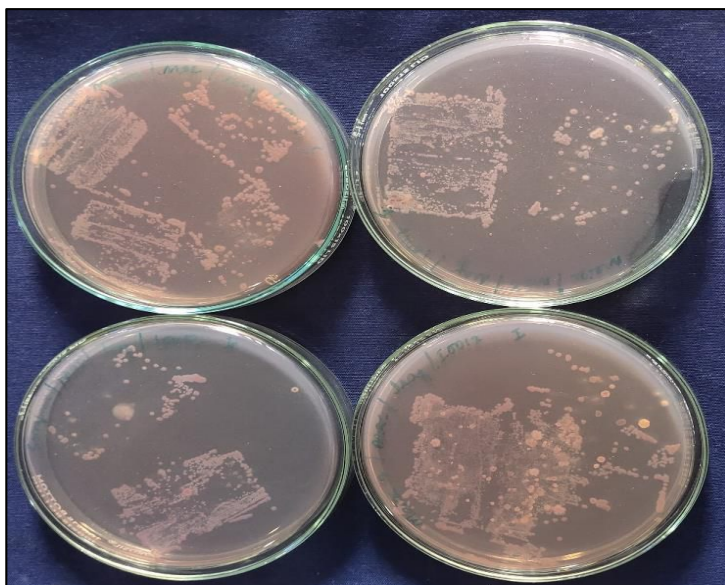


Plate 1: Isolation of nitrogen fixing bacteria from phyllosphere and endorhizosphere samples

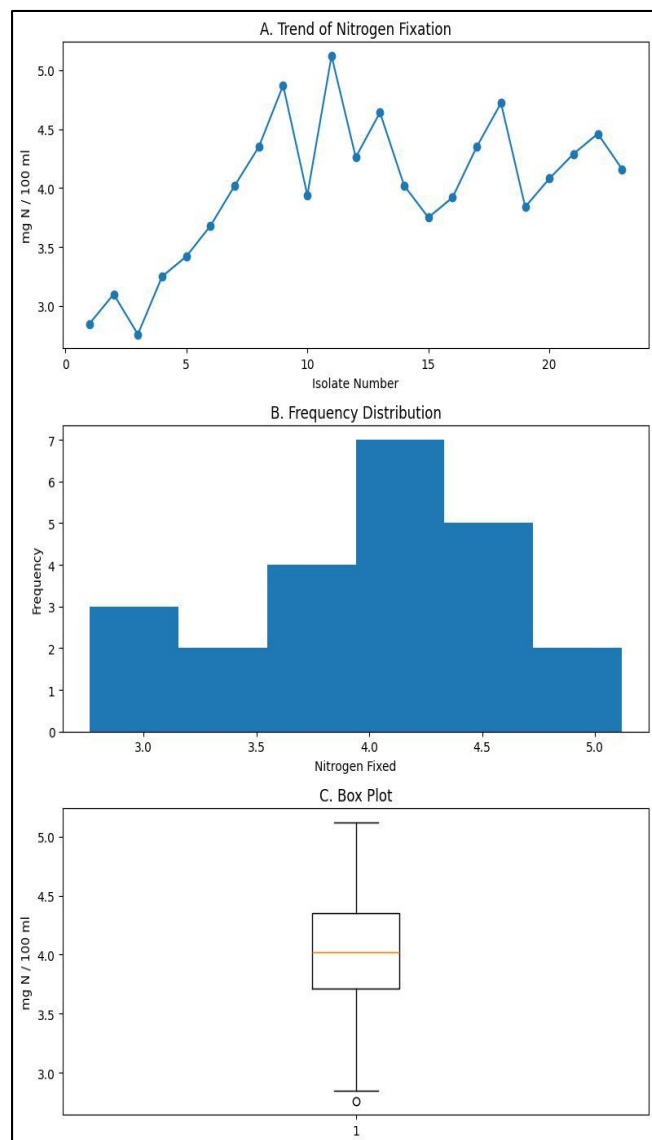


Fig 1: The multi-panel graphical analysis revealed a clear trend in nitrogen fixation among isolates. The line graph (Fig. 1A) showed a gradual increase in nitrogen fixation from initial isolates, reaching a peak at NFB 11, followed by stabilization. The histogram (Fig. 1B) indicated that most isolates fall within the moderate range (3.5–4.5 mg N), while only a few exhibited high nitrogen fixations. The box plot (Fig. 1C) demonstrated a median around 4.0 mg and identified NFB 11 as an upper extreme value.

The graphical analysis confirms the presence of functional heterogeneity among nitrogen-fixing bacterial isolates. The dominance of moderate performers suggests ecological stability, while the presence of a few high-performing isolates indicates potential candidates for biofertilizer development (Fig1).

The trend pattern supports the hypothesis that microbial efficiency is shaped by environmental selection pressures in phyllosphere and endorhizosphere niches. High-performing isolates likely possess enhanced nitrogenase activity and metabolic adaptability.

4. Conclusion

The present investigation demonstrated the successful isolation and evaluation of nitrogen-fixing bacterial isolates from both phyllosphere and endorhizosphere niches of paddy, highlighting their ecological and functional diversity. A considerable degree of variability was observed among

the isolates with respect to nitrogen fixation capacity, indicating differential metabolic efficiency and adaptability to micro-environmental conditions. Statistical analysis using one-way ANOVA revealed significant differences ($p \leq 0.05$) among the isolates, confirming that the observed variation was not due to random chance. Further, the calculated standard error (SE) and critical difference (CD) values supported the reliability and precision of the experimental data.

Among the tested isolates, NFB 11 consistently exhibited superior nitrogen fixation ability, making it the most efficient and promising candidate. Other isolates, namely NFB 9, NFB 18, and NFB 13, also demonstrated comparatively high performance and formed a statistically significant group of efficient nitrogen fixers, as evidenced by mean separation analysis. The presence of such high-performing isolates suggests the existence of robust native microbial populations with potential agronomic benefits.

The findings underscore the importance of exploiting indigenous microbial resources for sustainable agriculture. The superior isolates identified in this study can be further characterized, mass multiplied, and formulated into effective bioinoculants. Their application in rice cultivation could enhance biological nitrogen fixation, reduce dependency on chemical fertilizers, and contribute to improved soil health and crop productivity. Overall, this study provides a strong scientific basis for the development of eco-friendly biofertilizer technologies tailored to paddy ecosystems.

5. References

1. Akhter MS, Hossain MS, Islam MT. Isolation and characterization of nitrogen-fixing bacteria from rice rhizosphere and their effect on plant growth. *International Journal of Agricultural Research*. 2012;7(5):235-243.
2. Bhattacharyya PN, Jha DK. Plant growth-promoting rhizobacteria (PGPR): Emergence in agriculture. *Plant and Soil*. 2012;361(1-2):1-22.
3. Bremner JM. Determination of nitrogen in soil by the Kjeldahl method. In: Black CA, editor. *Methods of soil analysis*. American Society of Agronomy; 1960. p. 1149-1178.
4. Dixon R, Kahn D. Genetic regulation of biological nitrogen fixation. *Nature Reviews Microbiology*. 2004;2(8):621-631.
5. Galloway JN, Townsend AR, Erismann JW, Bekunda M, Cai Z, Freney JR, *et al.* Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. *Science*. 2008;320(5878):889-892.
6. Jnawali AD, Ojha RB, Marahatta S. Role of *Azotobacter* in soil fertility and sustainability—A review. *Advances in Plants & Agriculture Research*. 2015;2(6):00069.
7. Ladha JK, Reddy PM. Nitrogen fixation in rice systems: State of knowledge and future prospects. In: *Nitrogen fixation in agriculture, forestry, ecology, and the environment*. Springer; 2003. p. 85-116.
8. Lindow SE, Brandl MT. Microbiology of the phyllosphere. *Applied and Environmental Microbiology*. 2003;69(4):1875-1883.
9. Murumkar DR, Gajbhiye RP, Dongarwar UR. Evaluation of nitrogen-fixing efficiency of *Azotobacter* isolates under *in vitro* conditions. *Journal of Soils and Crops*. 2012;22(1):150-154.

10. Purushothaman D, Ladha JK, Reddy PM. Nitrogen fixation by *Azospirillum* isolates associated with different crop genotypes. *Biology and Fertility of Soils*. 1988;7(1):32-36.
11. Sawalgi YB, Patil PL, Math KK. Screening of *Azospirillum* isolates for nitrogen fixation and growth-promoting traits. *Karnataka Journal of Agricultural Sciences*. 2009;22(3):650-652.
12. Subba Rao NS. *Soil microbiology*. 4th ed. Oxford & IBH Publishing; 2007.
13. Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S. Agricultural sustainability and intensive production practices. *Nature*. 2002;418(6898):671-677.
14. Vessey JK. Plant growth-promoting rhizobacteria as biofertilizers. *Plant and Soil*. 2003;255(2):571-586.