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Unravelling Promising Diazotrophic Microbiota of *Rauvolfia tetraphylla* L. with Existing Conventional Nitrogen-Fixing Microbial Partners

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Abstract

Post green revolution witnessed excessive production of nitrogenous fertilizers and their application in farmers' fields, which although initially enhanced agricultural production, but later due to indiscriminate use, led to ecological perils including enhanced nitrogen footprint, emission of Green House gases, nitrate run off, eutrophication and several others. To address these concerns and promote sustainable agriculture, microbial inoculants, particularly based on diazotrophs or nitrogen-fixing bacteria like *Azotobacter, Azospirillum, Rhizobium* etc. were popularized. However, ecological incompatibility and sometimes inconsistent performance in stressed soils, necessitates exploring diverse diazotrophs other than the conventional ones for developing microbial inoculants. Besides, the use of chemical fertilizers have disrupted native soil microbiome and many rhizospheric bacteria with promising PGP traits might be lost even before their novelty is revealed and tapped. Bioprospecting for unconventional diazotrophs from resilient niches of medicinal plants like *Rauvolfia* must be prioritized to be utilized as microbial inoculants. In our study, ten diazotrophs with promising PGP traits were isolated from the rhizosphere of *Rauvolfia tetraphylla* L. The diazotrophs included members of *Bacilli*, Pseudomonads, *Lysobacter, Stenotrophomonas* and *Rhizobium.* Based on PGP traits *Stenotrophomonas maltophilia* RT2 was the most potent isolate and it was recommended as a promising contender for the development of bioinoculants.

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Introduction

The rampant use of agricultural fertilizers to meet the demand of food for burgeoning population has remained a contentious issue, especially when the world is faced with serious concerns like climate change, Greenhouse effect, depleting land resources and fossil fuels. Early 1950s witnessed pioneering efforts by Norman Boralug, who introduced the Green Revolution model to address food security concerns. This model among other tenets, emphasized on the adoption of nitrogenous fertilizers in the farmers' fields. Although the model became a pervasive aspect of agriculture, its excessive use proved unsustainable, because the application was undertaken without comprehensive and long-term assessment of their ecological, economic, and agronomic consequences.[1,2] The impact of green revolution in agriculture was overwhelming as the production enhanced and mortality rate decreased, but the sustainability of Green revolution itself was at stake because enhancing agricultural production remained the main focus, while other concerns like environmental sustainability and nutritional value of the produce were overlooked.[2,3] Among other environmental hazards, the nitrogen fertilizer industry and distribution chain of fertilizers contributed to 5 percent of the total nitrous oxide (a potent Greenhouse gas) emissions.[4] In 2023 alone, the emissions from pre and post agricultural production of fertilizer manufacturing was estimated to be 94.26kt, 32.37 kt, 2.01 kt and 0.423 kt for China, USA, Brazil and India respectively.[5] Nitrogenous fertilizers add nearly 150 million tons reactive nitrogen including HONO, NH_3 and NO (nitrous acid, ammonia and nitric oxide) globally, and this is expected to increase further to 600 million tons in the coming years.[6] Adding to the nitrogen footprint is also the nitrate runoff, that pollutes soil and water bodies and increases chances of blue baby syndrome or methemoglobinemia and carcinomas in children and adults respectively. The changes in the chemistry of the atmospheric air on the health of man and ecosystems costs approximately USD 340 billion to USD 3.4 trillion yearly (https://www.unep.org/news-and-stories/story/four-reasons-why-world-needs-limit-nitrogen-pollution, accessed on 29.11.2025). So, it is essential to bring down nitrogen foot print and for this, nitrogenous fertilizers must be substituted by other ecofriendly and sustainable options like biofertilizers or microbial inoculants.

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Nitrogen is the most abundant gas, comprising nearly 78 percent of the total atmospheric gases, contributing to vital component of biomolecules viz. amino acids, proteins, chlorophyll, nucleic acids, nitrogenous bases, alkaloids and numerous secondary metabolites in plants. However, plants are unable to access it directly from the environment. Diazotrophs are group of nitrogen-fixing microorganisms including bacteria, archaea and blue green algae, that play crucial role in reducing atmospheric nitrogen to plant usable forms like ammonium, referred as biological nitrogen fixation (BNF) through nitrogenase enzyme, a metalloprotein complex encoded by the *nifH* gene.[7] This process harnesses the physiological potential of nitrogen-fixers that find application in biofertilizer formulations. Diazotrophs have been isolated from the root nodules of leguminous plants, but recent years have seen their exploration from the non-legumes also. Most pioneering work on nitrogen-fixing bacteria was undertaken by Johanna Döbereiner, who originally hailed from Czechoslovakia, but later in 1953 moved to Brazil. Due to the contributions of Döbereiner and group, Brazil stands at the apex, surpassing many other countries for research on diazotrophic bacteria.[8] Although, conventional nitrogen fixers like *Rhizobium, Azotobacter, Azospirillum, Azolla-Anabaena* symbiosis etc. routinely find application as commercial inoculants, there are a vast number of other less explored diazotrophs with multiple PGP (plant growth promoting) traits, that can be developed into bioinoculants.[9] PGPR (plant growth promoting rhizobacteria) inoculants possess PGP traits like fixation of atmospheric nitrogen, solubilization of iron by siderophore production, solubilization of phosphate, synthesis of IAA etc.[10] The incorporation of diazotrophic bacteria into agricultural practices is ecofriendly and sustainable, and can reduce reliance on chemical fertilizers and therefore, nitrous oxide emissions during the production and distribution chain of nitrogenous fertilizers.[11]

*Rauvolfia tetraphylla (*barachandrika or devil pepper) belongs to Dogbane family/ Apocyanaceae and serves as a validated substitute to *Rauvolfia serpentina* or Sarpagandha due to their comparable alkaloid profile.[12,13] The plant originated in some parts of Northern and Southern America, and it was later introduced to India, China, Nepal, Taiwan, Vietnam and some parts of Australia.[14] *Rauvolfia* *serpentina* was popularized as an antihypertensive, antimicrobial and also for the treatment of insomnia, anxiety, etc. due to the presence of reserpine, a monoterpene indole alkaloid.[15] Because of its medicinal value, *Rauvolfia serpentina* was ruthlessly exploited by the pharmaceutical industry, which drastically brought down its numbers, resulting in its inclusion in the endangered category by the IUCN.[16] Recent *in silico* studies have shown that leaves of *R. tetraphylla* L. possess antiviral properties.[17] In the Bhagalpur district (25.2372° N, 86.9746° E) of Bihar, India *R. tetraphylla* L. is found growing abundantly in wild patches.

The seeds of *Rauvolfia tetraphylla* L. have poor germination inspite of 83.1% viability. Hence, multiplication through seeds and regeneration by stem cuttings is difficult due to occurrence of phenolic compounds in the seed and also limited rooting.[18] Besides, reserpine extraction from roots of *Rauvolfia tetraphylla* requires sacrificing the whole plant, making its sustainable utilization difficult. Therefore, it is necessary to bring this plant into cultivation and application of microbial inoculants based on diazotrophs with PGP traits like IAA synthesis, iron solubilization and phosphate solubilization can lead to plant growth promotion and also increase concentration of bioactives. Mishra and Kumar have critically reviewed the interactions of rhizospheric microbes and endophytes in *Rauvolfia* sp. and they could be of immense potential in sustainable agriculture.[19]

Among the major nitrogen-fixing bacteria is *Azotobacter,* which is frequently used in formulations of biofertilizers, but it suffers from several pitfalls like inconsistent performance due to variations in soil physicochemical parameters and hence may not be effective in enhancing alkaloid level and ameliorating growth. Hence, exploring rhizomicrobiota of *R. tetraphylla* L, other than the conventional ones can provide useful insights for the development of microbial inoculant/s specially designed for this medicinal plant.

Materials and Methods

Collection of Rhizospheric Soil

Healthy plants of *Rauvolfia tetraphylla* L. growing in the Botanical Garden of University Department of Botany, Tilka Manjhi Bhagalpur University were carefully uprooted. The plants were kept in sterile polybags, with their rhizospheric soil intact and taken to the Biolab at Bihar Agricultural University, Sabour within a few hours of collection. By vigourous shaking, bulk soil was removed, and the remaining soil adhering to the root (the rhizospheric soil) was used for the isolation of diazotrophs from the rhizosphere.

Isolation of Diazotrophic Bacterial Groups

The rhizospheric soil was obtained by vigorous shaking in 1 L 0.9 percent NaCl for 10 minutes. The contents were put on orbital shaker at 300 rpm, for 90 minutes and then centrifuged to homogenize bacterial content in the supernatant, while the soil particles pelleted at the bottom.[20] Using 1 mL of the supernatant, serial dilution was carried out until reaching 10^{-4} dilution. Usually 10^{-3} or 10^{-4} dilutions are taken since at higher dilutions colonies are crowded and might overlap, whereas at dilutions lesser than this, the colonies may be very few to count or almost absent from a plate. Ideally, the number of colonies on the plate should be from 30-300 for statistical accuracy. Hence 10^{-3} or 10^{-4} dilutions are preferred for enumeration and isolation of bacteria.[21,22]

Isolation was done on Jensen's agar media (procured from HiMedia) supplemented with bromothymol blue (BTB).[23] 100µL of each dilution was spread on the petriplates containing the sterile media in the laminar flow hood. After spreading the plates were incubated in inverted position in an incubator at 28°C till the appearance of visible colonies. The incorporation of BTB as a pH indicator is similar to that in NFb (nitrogen-free bromothymol blue) media for visual screening of diazotrophs.[24] Change in colour of the media around the colonies to blue colour is regarded as nitrogen-fixation.[25,26] Isolates were purified and preserved in 20% glycerol stocks until further study.

Screening for PGP Traits

Screening for Siderophore Production

For qualitative screening for siderophore production, the selected diazotrophs were spot inoculated on Chrome Azurol Sulfonate (CAS) agar media (procured from HiMedia) and incubated at 28°C for 3-5 days. Development of orange halos around bacterial colonies indicated positive siderophore production.[27]

Screening for IAA Production

50 µL of glycerol stock was inoculated in 5ml of nutrient broth medium containing 5 mM L-tryptophan for 48 h at 28°C. Thereafter, 500 µL of freshly

prepared Salkowski reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml 35% $HClO_4$) was added to each tube and incubated in dark for 30 minutes. Development of pink colour indicated positive IAA production.[28]

Screening for Phosphate Solubilization

This was done on Pikovskaya's agar medium (procured from HiMedia) containing tricalcium phosphate. Bacterial isolates were spot inoculated and incubated for 48 hours at a temperature of 28°C. Development of clear halo zones around isolates indicated positive phosphate solubilization.[29]

Morphological Characterization of Selected Isolates

Pure cultures (48-72 h old) were studied for their colony characteristics viz. colour, shape, size, margin etc. on Jensens agar media amended with BTB.

Biochemical and Molecular Characterization of Selected Diazotrophs

Isolates showing *in vitro* nitrogen-fixation by their visible blue colour on Jensens agar media amended with BTB were screened for citrate utilization, lysine and ornithine utilization, urease detection, phenylalanine deamination, nitrate reduction, H_2S production, and carbohydrate metabolism tests (glucose, adonitol, lactose, arabinose, and sorbitol) using HiAssorted™ Biochemical Test Kit KB002 procured from HiMedia.[30]

Extraction of Genomic DNA From Bacterial Isolates

From glycerol stocks, the bacterial isolates were revived in nutrient broth medium till the appearance of turbidity/ visible growth. The cultures were centrifuged at 10,000 rpm for 10 minutes. Following centrifugation, the supernatant was discarded, and bacterial cells were pelleted down. From the pellets, extraction of genomic DNA was performed using HiPurA® Genomic DNA Purification Kit. The protocol provided by the manufacturer was followed, and upon isolation, the quality of separated DNA sequences was checked using agarose gel electrophoresis. The genomic DNA of the isolates in the gel were visualized by UV transilluminator.

Amplification of 16S rRNA Gene with 16S Universal Primers

Using purified DNA as template,16S rRNA of the isolates were amplified using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT3'). The resulting amplicons were resolved on a 1.5% TAE-agarose gel. Thereafter, the amplicons were purified and sequencing was performed using ABI 3500 XL Genetic Analyzer.[31] The generated sequences were allowed to overlap to get a concatenated sequence using CodonCode Aligner (Version 12.0.4). Thereafter, the assembled sequences were converted into FASTA format files and subjected to alignment in public database using the EzBioCloud server (last updated on 21.04.2025) for identification of closely related species.[32] Later, comparisons were carried on NCBI BLAST server to determine the closest phylogenetic neighbour and the sequences were deposited in NCBI GenBank using the submission portal to obtain accession numbers. Alignment was done using Clustal W and phylogenetic tree was made using MEGA 12 software (version 12.0.14).[33]

Results and Discussion

Isolation and Screening For Nitrogen Fixation

Qualitative screening for nitrogen fixation was done on BTB containing Jensens media. The production of distinct blue colour within 2-5 days of incubation by the isolates, was taken as confirmatory test for nitrogen fixation. The study yielded 79 isolates and they were designated with codes RT1 to RT79. Among them, ten isolates produced distinct blue colour on BTB containing Jensens media, but the fixation was higher among the isolates RT2, RT34, 40A,RT71,RT77, RT78 and RT79. Fig 1. Showing high nitrogen fixation by isolates RT71, RT77 and 78 on BTB containing Jensens media. Depicted the results for *in vitro* screening for nitrogen fixation and PGP traits are highlighted in Table 1.

The isolate RT2 showed strong nitrogen fixation changing the colour of the Jensens medium containing BTB from forest green to blue and then to yellow and this aligns well with the earlier findings in *Stenotrophomonas maltophilia*.[34] *Stenotrophomonas maltophilia* belongs to Gammaproteobacteria and earlier research has implicated their role in cystic fibrosis and also nosocomial infections, frequently leading to morbidity.[35,36,37] In sharp contrast to these studies, several strains of *S. maltophilia* harbour the rhizosphere and possess PGP traits like nitrogen fixation, phosphate solubilization, production of plant growth regulators, siderophore production etc. and are also involved in biofortification and degradation of xenobiotic compounds.[38] *S. maltophilia* have also been isolated from the roots of *Arachis hypogea,* where they have shown to regulate physiological and biochemical responses, and improve tolerance to stress, under limiting nitrogen conditions.[39] Earlier the biofertilizer and biocontrol properties of *S. maltophilia* in sustainable cultivation of wheat has also been reported .[40]

The first report of isolation of *Bacillus rugosus* was done from a marine sponge.[41] In another study in rice, although not reported as a diazotroph, *B. rugosus* occurred as an endophyte and it was found to provide tolerance to multiple stress.[42] *B. rugosus* RT34 has shown positive for nitrogen fixation on BTB containing Jensens media, however further validation for nitrogen fixation would require amplification of *nif* gene. Among the *Bacilli* members isolated in this study, *Bacillus stercorsis* RT40A was earlier isolated from *Magnaporthe oryzae* in rice and tomato wilts respectively, and they were found effective in controlling blast fungus in rice and Fusarial wilts in tomato.[43,44] Although not shown to be a diazotroph in previous studies, *B. stercorsis* RT 40A showed *in vitro* nitrogen fixation on BTB containing Jensens agar medium. Another PGPR, *Bacillus cabrialesii* was isolated from Mexican wheat varieties but its diazotrophic potential has not been reported yet.[45] In sharp contrast, the diazotrophic capacity of *Bacillus cabrialesii* RT51 on Jensens media containing BTB has been evidenced in our study. Another member, *Lysobacter prati* RT71 has shown high nitrogen fixation activity and this aligns with the earlier observation in E4 *Lysobacter strain*.[46] The earliest report of isolation of *Lysobacter prati* was from plateau meadow.[47] The observation of nitrogen fixation by *R. oryzihabitans* aligns with the previous reports for the same being isolated from rice endosphere.[48] Previously, *Metapseudomonas lalkuanensis*, was isolated from contaminated soil in Lalkuan in India .[49] Although literature does not mention it as a nitrogen fixer, but our studies have shown isolate *Metapseudomonas lalkuanensis* RT76 to be moderately fixing nitrogen. Nitrogen-fixing ability of *Pseudomonas hunanensis* RT77 aligns with the nitrogen-fixing ability of the same isolate from soybean.[50] *Pseudomonas hunanensis* SPT26 was earlier reported to secrete antifungal substances that could control wilt diseases in tomato and was proposed as promising rhizobacteria for the formulation of microbial inoculant.[51] In the present study the release of siderophores by *Pseudomonas hunanensis* RT77 may be effective in controlling phytopathogens. *Pseudomonas mosselii* isolated from rice rhizosphere is a promising biocontrol agent against rice blast fungus *Magnaporthe oryzae* and it was also reported to enhance yield and nutrient acquisition in rice.[52,53] *P. mosselii* isolated from durian rhizosphere was also reported as a nitrogen fixer and produced IAA from rhizosphere of durian rhizosphere.[54] In our *study. P. mosselii* RT78 did not solubilize phosphate but showed nitrogen fixation and siderophore production in the *in vitro* assay. *Bacillus tequilensis* RT79 although showing strong nitrogen fixation in our study, has not been reported as a diatroph from previous studies.

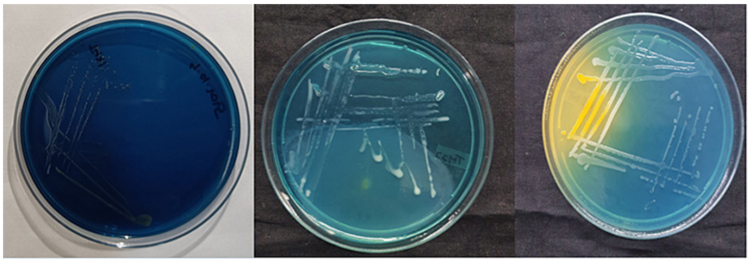

Fig. 1: Petriplates showing pure cultures of RT71, RT77 and RT78 showing nitrogen fixing ability on Jensens media containing BTB

Screening of Rhizospheric Diazotrophic Bacteria for Plant Growth-Promoting Trait IAA Synthesis

The principal auxin, Indole-3-acetic acid (IAA) is an important phytohormone regulating plant growth and development by cell enlargement and division, tissue differentiation, and responses to light, and also controls many aspects of plant development including stimulation of root growth and nutrient acquisition from soil. The capacity to produce the phytohormone indole-3-acetic acid

(IAA) is widespread among bacteria that inhabit diverse environments such as soils, fresh and marine waters, and plant and animal hosts. Among the ten diazotrophic isolates, all the isolates produced IAA, but it was significantly higher for RT2, RT34, RT76 and 79. This was revealed by the intensity of colour produced using Salkowski's reagent, followed by incubation in dark. Therefore, it is expected that incorporating any of these isolates in a biofertilizer formulation will provide IAA to the host plants and improve root architecture and branching and also help in facilitating accumulation of bioactive. Enhanced IAA production by RT34 is consistent with *Bacillus rugosus* isolated from *Arachis hypogea*.[55] Similarly RT2 showed higher production of IAA and this observation is in line with IAA released by *S. maltophilia* isolated from rhizosphere of chilli.[56] *Stenotrophomonas,* isolated from poplar also showed auxin production in the *in vitro* condition similar to RT2, but there it existed as an endophyte, and in this study it was isolated from the rhizosphere.[57] Higher production of IAA by *Metapseudomonas lalkuanensis* RT76 in this study has not been reported in any previous study. IAA production by *P. mosselii* RT78 aligns with the production of IAA by the same isolate from *Agave americana.*[58] Similarly, *Bacillus tequilensis* from soybean rhizosphere was shown to produce IAA ,which is similar to *B. tequilensis* RT79 from *R. tetraphylla*.[59] Growth responses of IAA producing rhizobacteria on dicots and monocots by pot experiments would be further required to validate their potential in agriculture.

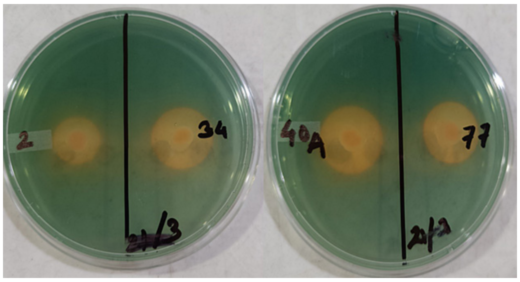

Fig. 2: Plates highlighting iron solubilization on CAS media by RT2, RT34, RT40A and RT77.

Screening of Diazotrophs for Siderophore Production

In soils, iron occurs mostly as Fe^{3+} oxy-hydroxides and must be reduced to Fe^{2+} to be easily assimilated by plants and microorganisms. In agriculture, this is usually accomplished by synthetic chelates, which are costly and sometimes may even lead to toxicity when used in excess.[60] Besides this, alkalinity of soils in arid regions limit iron availability to plants leading to reduced crop yields, chlorosis etc. by disrupting the photosynthetic machinery. Certain rhizospheric bacteria release siderophores which help in enhancing iron availability to plants. The siderophores complexes with iron, diminishing its availability to pathogens like *Fusarium oxysporum* and *Rhizoctonia solani,* thereby exerting their plant growth promoting effect. Qualitative screening for siderophore production relied on visual examination of the halo zone around orange-yellow colonies produced by the isolates on CAS media followed by incubation for 3-5 days at 28°C. All the nitrogen fixing isolates, except RT51 and RT71 produced siderophores. *S. maltophilia* RT 2 showed efficient iron solubilization and probably it was useful in scavenging iron from the soil.[61] *Rhizobium oryzihabitans* RT74 also showed orange halo on CAS plate confirming siderophore production, however efficiency of solubilization is lesser as compared to other diazotrophs, and this observation is similar to earlier reports regarding siderophore production, in addition to IAA and ACC deaminase.[48] *Bacillus rugosus* RT34 is also positive for siderophore release, and this observation is in line with the observation in rice, where the same isolate also provides tolerance to salinity and drought stress.[62] Another *Bacilli* isolate, *Bacillus tequilensis* RT79 from *R. tetraphylla* was evidenced to solubilize iron and this observation was in line with earlier research focussing on biocontrol properties of this isolate against *Fusarium* and it also showed production of IAA and siderophore.[63] Members of *Pseudomonas* generally produce siderophores under iron limiting conditions.[64] *M. lalkuanensis* RT76 has been shown to produce siderophore *P. mosselii* RT78 produced siderophore and this was also observed in *P. mosselii* from durian rhizosphere.[54] Rhizobacteria producing siderophores control phytopathogens by secreting antimicrobial compounds.[65] "In Fig. 2:Plates highlighting iron solubilization on CAS media by isolates RT2, RT34, RT40A and RT77 are shown.

Screening of Diazotrophs for Phosphate Solubilization

After nitrogen, phosphorus is the second most important macronutrient that are needed by plants for growth and development. But sometimes, phosphorus availability to plants is also limited because it occurs in combined form with metal complexes. Phosphate-solubilizing bacteria (PSB) are applied as inoculants in fields to improve bioavailability of phosphate from the soil by converting organic and insoluble forms of phosphorus into soluble forms. Some diazotrophs, in addition to fixing nitrogen, also contribute to phosphorus solubilization through the release of organic acids and enzymes, further contributing to plant growth. It has been suggested that several nitrogen-fixing strains of *Azotobacter vinelandii* have demonstrated promising phosphate-solubilizing capabilities and they could be used as superior candidates for biofertilizer development.[66] In rice, phosphate-solubilizing diazotrophic bacteria like *Herbaspirillum* and *Burkholderia* were isolated and they were found to improve nitrogen use efficiency too.[67] In our study, diazotrophic isolates RT2, RT40A, and RT51 showed solubilization zone around colonies on Pikovskaya's agar and the efficiency of phosphate solubilization was almost comparable.

The ability of *Stenotrophomonas maltophilia* RT2 to solubilize phosphate aligns with previous research, where this isolate was also used for reclamation of wastes.[68] *S. maltophilia* was earlier isolated from Porang, and it was able to fix nitrogen and also solubilize phosphate.[69] Among the *Bacilli* isolates, *Bacillus stercoris* RT40A and *B. cabrialessi* RT51 have shown phosphate solubilization but this PGP trait is not shown by the other two *Bacilli* isolates *B. rugosus* and *B. tequilensis.* The production of IAA and phosphate solubilization by RT40A, have previously been reported in *Bacillus stercoris.*[44] *Pseudomonas hunanensis* RT77 has shown no phosphate solubilization in our study, however the same isolate has shown other PGP traits in addition to phosphate solubilization in earlier studies.[70] Although none of the *Pseudomonas* isolates have shown phosphate solubilization in our study, nevertheless phosphate solubilizing bacteria including *Bacillus, Pseudomonas, Serratia* and *Enterobacter* find application in the development of microbial inoculants and can be used to boost plant growth and development.[71] In Fig.: 3 Phosphate solubilization by isolates on Pikovskaya's agar medium are shown.

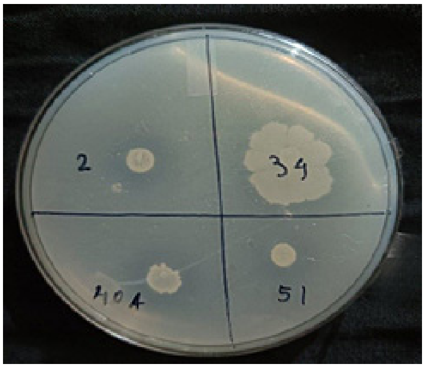

Fig. 3: Plates showing screening for phosphate solubilization on Pikovskaya's agar medium.

Note : Although RT34 forms a big colony but the halo is missing, so it is considered negative for phosphate solubilization.

Carbon Utilization Pattern of Selected Nitrogen-Fixing Bacterial Isolates

Different isolates showed varying responses for the utilization of different carbohydrates. Except RT34, RT40A and RT74, none of the isolates were capable of utilizing glucose. Adonitol utilization was shown by isolate RT 74 only. None of the isolates utilized lactose except RT74. Isolates RT34, RT40A, RT74, RT77 and RT78 utilization of arabinose. Only one isolate RT74 was capable of utilizing sorbitol. The results for carbon utilization pattern is highlighted in table 2.

Stenotrophomonas maltophilia RT2 was found to be non-fermentative and this is consistent with the results of Swapna and Audipudi.[56] Glucose and arabinose utilization by *B. rugosus* is consistent with the findings in *Arachis hypogea* by Rabari *et al*.[55] *B. rugosus* RT34 was not able to utilize lactose, but *B. rugosus* from *Arachis hypogea* was found to utilize lactose.[55] One of the plausible reasons for this could be that certain bacteria prefer using simpler sugars, and may require ATP to breakdown disaccharide and therefore sticks to the former. Studies on adonitol utilization revealed that RT34 was not able to utilize adonitol, however previous studies have not revealed anything on the utilization of adonitol by this isolate. Figueroa-Brambila *et al*., have reported glucose fermentation by *B. cabrialessi* but this is not the case with *B*. cabrialesii RT51.[72] Previous studies have not explicitly mentioned studies on sugar utilization by *B. cabrialessi*. *Lysobacter prati* RT71 did not utilize sorbitol, arabinose and lactose and these results are consistent with the previous findings.[47] The ability of *Bacillus stercorsis* RT40A for citrate utilization and glucose fermentation is consistent with earlier findings while negative nitrate reduction is contrary to the findings reported so far.[73] *R. oryzihabitans* RT74 was found to ferment arabinose, sorbitol, lactose, adonitol and glucose is in sync with the findings.[48] *Metapseudomonas lalkuanensis* RT76 did not utilize sorbitol, any sugar in our study , but it showed utilization of glucose, arabinose, lactose, adonitol, and sorbitol as earlier reports.[49] *Pseudomonas hunanensis* RT77 and *Pseudomonas. mosselli* RT78 utilized only arabinose and not any other sugars and this is a characteristic for several Pseudomonads. *Bacillus tequilensis* RT79 did not utilize any of the carbon sources tested including glucose, sorbitol, adonitol, lactose and arabinose and this observation is in sharp contrast to earlier studies indicating that phenotypic variability could be common.[74]

Table 1: The table highlights PGP traits of the diazotrophs. The efficiency of each bacterial partner is indicated by + (low), ++ (moderate), or +++ (high) or ++++(very high) and the absence of PGP trait is indicated by '– ' sign.

Bacterial Isolate	Nitrogen Fixation	Siderophore Production	Phosphate Solubilization	IAA Production
RT2	++++	+++	++	+++
RT34	+++	+++	-	+++
RT40A	+++	+++	++	+
RT51	++	-	++	++
RT71	++++	-	-	+
RT74	++	+	-	+
RT76	++	+	-	+++
RT77	+++	+++	-	+
RT78	+++	++++	-	+
RT79	+++	++++	-	+++

Table 2 : Shows the ability of diazotrophs to utilize various carbon sources.

	Glucose	Adonitol	Lactose	Arabinose	Sorbitol
RT 2	-	-	-	-	-
RT 34	+	-	-	+	-
RT 40A	+	-	-	+	-
RT 51	-	-	-	-	-
RT 71	-	-	-	-	-
RT 74	+	+	+	+	+
RT 76	-	-	-	-	-
RT 77	-	-	-	+	-
RT 78	-	-	-	+	-
RT 79	-	-	-	-	-

Biochemical Characterization of the Selected Diazotrophs

All the isolates were capable of utilizing lysine as the sole nitrogen source except RT71. Citrate utilization was shown by the isolates RT34, RT40A, RT71, RT74, RT76, RT77, RT78 and RT79. All the isolates utilized ornithine except RT71 and RT79. All the isolates showed urease activity except RT71. None of the isolates were capable of phenylalanine deamination. Nitrate reduction was shown only by the isolates RT74 and RT78. Only RT77 produced H_2S. Overall, the biochemical profiling of the isolates highlight their functional potential in the rhizosphere and are in agreement with the earlier findings, and minor variations have also been observed. In Table 3 biochemical characterization of the selected nitrogen fixers are mentioned.

Table 3 : Shows biochemical characterization of the selected nitrogen fixers

	Citrate utilization	Lysine Utilization	Ornithine utilization	Urease Activity	Phenylalanine Deamination	Nitrate Reduction	H_2S Production
RT2	-	+	+	+	-	-	-
RT34	+	+	+	+	-	-	-
RT40A	+	+	+	+	-	-	-
RT51	-	+	+	+	-	-	-
RT71	+	-	-	-	-	-	-
RT74	+	+	+	+	-	+	-
RT76	+	+	+	-	-	-	-
RT77	+	+	+	+	-	-	+
RT78	+	+	+	+	-	+	-
RT79	+	+	-	+	-	-	-

S. *maltophilia* was found positive for nitrate reduction, but in our study it did not show nitrate reduction.[75] Further, urease positive result by RT2 is found to be consistent with earlier studies. Similarly, positive responses to lysine, ornithine, urease activity and negative for phenylalanine deaminase utilization is consistent with the results.[56] While negative nitrate reduction in RT2 is in sharp contrast to positive tests for nitrate reduction by *Stenotrophomonas*, probably indicating that nitrate reduction may not be a useful feature of all *Stenotrophomonas* isolates.[56] Citrate utilization by *Bacillus stercorsis* RT40A is consistent while negative nitrate reduction is contrary to the earlier findings reported.[73]

B. *cabrialesii* RT51 in sharp contrast to previous studies was neither able to reduce nitrite to nitrate, nor able to use citrate, which possibly may be due to strain variations.[45] Negative lysine utilization, ornithine utilization and urease activity is consistent, while negative nitrate reduction of *L. prati* RT71 is not in sync with the observation of Fang *et al*.[47] *R*. *oryzihabitans* RT74 showing ornithine utilization, urease activity, nitrate reduction is similar to the observation.[48] *Metapseudomonas lalkuanensis* RT76 utilized citrate as earlier but did not show nitrate reduction in sharp contrast to earlier studies by Thorat *et al.* where it was positive for nitrate reduction.[49] *P. hunanensis* RT77 showed positive citrate utilization, negative nitrate reduction and this was consistent with the reports of Gao *et al*.[76] *P. mosselii* RT78 showed similar traits to *P. hunanensis* except negative nitrate reduction and positive hydrogen sulfide production for the former, and vice versa for former. *Bacillus tequilensis* RT79 utilized citrate, ornithine, lysine, showed urease activity and nitrate reduction, and this is similar to the observation of Gatson *et al*.[74]

Table 4 : Shows morphological characteristics of the selected diazotrophs.

	Colony shape	Colony size	Margin of colony	Colony elevation	Chromogenesis	Surface of Colony	Texture	Cell Shape	Grams Staining
RT 2	Round	>1mm	Entire	Raised	White to pale yellow	Smooth	Mucoid	Rod	Negative
RT34	Round	>1mm	Entire	Convex	yellow	Rugose	Butyrous	Rod	Positive
RT40 A	Round	>1mm	Undulate	Convex	Creamy white	Wrinkled	Rough and dry	Rod	Positive
RT51	Round	>1mm	Entire	Convex	White	Rough	Slimy	Rod	Positive
RT71	Round	<1mm	Entire	Raised	Off white	Smooth and shiny	Mucoid	Rod	Negative
RT74	Round	>1mm	Entire	Raised	Yellow	Smooth and shiny	Slimy	Rod	Negative
RT76	Round	>1mm	Entire	Flat	Round	Smooth and moist	Mucoid	Rod	Negative
RT77	Round	>1mm	Entire	Flat	White	Smooth and moist	Slimy	Rod	Negative
RT78	Round	>1mm	Entire	Flat	White	Smooth and moist	Slimy	Rod	Negative
RT79	Round	>1mm	Entire	Convex	White	Rough	Mucoid	Rod	Positive

Morphological Traits of Selected Isolates

For all the isolates, colonies were round with smooth consistency, shape and margins. for Certain isolates exceeded 1mm in diameter, and for some they were less than 1mm. Colour of colonies were generally white except RT34 and RT74, which were yellow in colour. The isolates were all rod shaped, and Gram negative, except *Bacilli* isolates RT34, RT40A, RT51 and RT79, which were Gram positive. The colonies of the diazotrophs were mostly slimy or mucoid, probably a mechanism to prevent nitrogenase enzyme from inactivation due to oxygen. Colony morphology and Grams reaction were similar to previous findings for all the isolates. The morphological characters of selected diazotrophs are mentioned in Table 4.

Molecular Phylogenetic Identification of Diazotrophs by 16S rRNA gene Sequencing

Molecular phylogenetic diversity studies of diazotrophs by 16S r RNA gene sequencing, confirmed four species of *Bacilli,* three Pseudomonads, one each of *Lysobacter, Stenotrophomonas* and *Rhizobium,* showing 100% sequence similarity with the corresponding strains on NCBI. Fig 4: showed amplification of conserved 16S rRNA gene of the rhizospheric isolates, with M indicating 1 kb marker. Table 5 below highlights 16S rRNA based identification of diazotrophs using EzBiocloud and NCBI. NCBI Accession numbers of the isolates are also mentioned. Fig 5 – In fig 5 phylogenetic tree showing isolated diazotrophic strains from the rhizosphere of *R. tetraphylla* L. have been depicted. Bootstrap values are indicated on the branches and scale bar showing the number of substitutions per site is indicated below the tree.

Tree was made by the Neighbouring joining approach using MEGA 12. The numbers indicated on the branch indicate the bootstrap confidence values which means how many times this grouping was repeated when the tests were replicated 1,000 times. GenBank accession numbers are also indicated before the type strains. The phylogenetic distances were calculated by the Kimura 2-parameter method and are shown as units of substitution of bases at each site, modelled using gamma distribution (shape parameter = 1.00). Construction of phylogenetic tree revealed that bootstrap values ranged from 75-100%, with 75% considered reliable and 100% representing very strong statistical support for the grouping of clades.

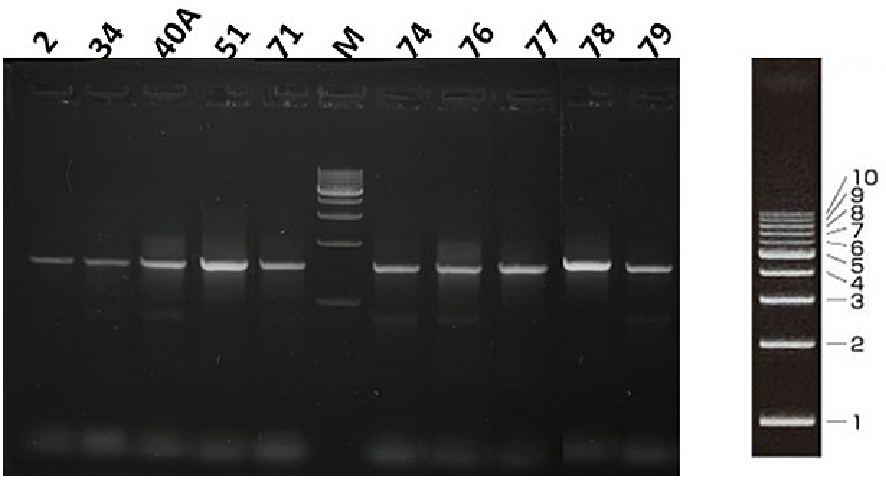

Fig. 4: Amplification of 16S rRNA gene of the diazotrophs, with M indicating marker of 1 kb DNA plus ladder are shown.

Table 5: Molecular identification of diazotrophs based on EzBiocloud type strains and NCBI reference sequences.

Isolate code	Homologous microorganism (% identity) (Accession no. of NCBI reference seq.)	Identified organism	GenBank Accession Number
RT2	*Stenotrophomonas maltophilia* M 15 (100%) (LT222224.1)	*Stenotrophomonas maltophilia* RT2	PX363496.1
RT34	*Bacillus rugosus* strain SPB7(100%) (NR_181236.1)	*Bacillus rugosus* RT34	PX363497.1
RT40A	*Bacillus stercoris* strain JCM 30051 (100%) (MN536904.1)	*Bacillus stercoris* RT40A	PX363498.1
RT51	*Bacillus cabrialesii* strain TE3 (100%) (MK462260.1)	*Bacillus cabrialesii* RT51	PX363499.1
RT71	*Lysobacter prati* SYSU H10001 (Type) (100%) (MN181427)	*Lysobacter prati* RT71	PX363500.1
RT74	*Rhizobium oryzihabitans* strain M15 (100%) MT023790.1	*Rhizobium oryzihabitans* RT74	PX363501.1
RT76	*Metapseudomonas lalkuanensis* strain PE08(100%) (MF943158.1)	*Metapseudomonas lalkuanensis* RT76	PX363502.1
RT77	*Pseudomonas hunanensis* strain LV(100%) (JX545210.1)	*Pseudomonas hunanensis* RT77	PX363503.1
RT78	*Pseudomonas mosselii* strain CIP 105259 (100%) (AF072688.2)	*Pseudomonas mosselii* RT78	PX363504.1
RT79	*Bacillus tequilensis* KCTC13622 (100%) (AYTO01000043.1)	*Bacillus tequilensis* RT79	PX363505.1

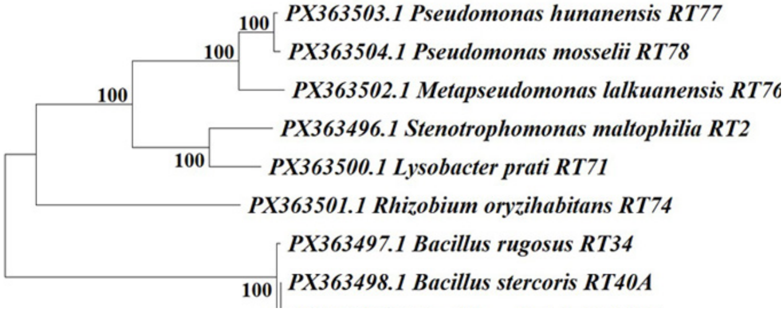

Fig. 5: Phylogram/ phylogenetic tree showing relationship of isolated diazotrophic strains from the rhizosphere of *R. tetraphylla L.*

The earliest derived lineage is represented by the *Bacilli* clade, while the other taxa were derived later. Basal taxon is *Bacillus tequilensis* RT79, meaning it diverged earliest as compared to other sister taxa. The diazotrophs based on *Bacilli* (Phylum – Firmicutes) clustered together in the same clade, while the Pseudomonads (Phylum - Gammaproteobacteria) clustered in a separate clade. Two other diazotrophs, *Stenotrophomonas maltophilia* RT2 and *Lysobacter prati* RT71, belonging to Gammaproteobacteria have also been isolated and they form a separate clade showing 100% bootstrap value. Two *Pseudomonas isolates* RT77 and RT78 clustered together in the same clade showing 100% bootstrap values. *Metapseudomonas lalkuanensis* RT76 and the two isolates *Pseudomonas hunanensis* RT77 and *P. mosselli* RT78 were derived from a common ancestor, again showing consistent 100% bootstrap value. *Rhizobium* oryzihabitans RT74 (Alphaproteobacteria) and all the members of Gammaproteobacteria/ Pseudomonads evolved from a common ancestor and this is supported by a strong bootstrap value of 100%.

Conclusion

The rhizosphere of *R. tetraphylla* L. harboured immense diversity of diazotrophs showing multiple PGP traits. A total of 79 rhizospheric isolates were obtained on Jensen's agar media and among these 10 isolates showed positive nitrogen-fixing capacity indicated by their ability to change forest green colour to blue on BTB containing Jensens media. The rhizospheric isolates from *R. tetraphylla* L. belonged to *Bacilli,* Pseudomonads, *Lysobacter, Stenotrophomonas and Rhizobium*. A careful analysis of Table 1 indicates that RT2 and 40A are showing all the PGP traits including nitrogen fixation, siderophore production, phosphate solubilization and IAA synthesis. Although RT2 and RT40A showed comparable PGP traits, but IAA production by RT2 is comparatively higher than RT40A. Hence, based on PGP traits highlighted in table 1, we recommend that *Stenotrophomonas maltophilia* RT2 is the best isolate and it can be further studied for its colonization and plant growth promoting effects in *in vivo* conditions.

Stenotrophomonas maltophilia RT2 strain is expected to provide increased levels of fixed nitrogen to *Rauvolfia* plants that can be assimilated into biomolecules including monoterpene indole alkaloids(reserpine), nitrogenous bases, amino acids, proteins and nucleic acids, enhancing root and shoot biomass and also accommodating elevated concentration of alkaloids. Iron is an important micronutrient in plants, and also a component of electron transport system (cytochromes), chlorophyll and ferredoxin etc., and plants need to maintain iron homeostasis. Enhanced IAA release by RT2 in the rhizosphere of *R. tetraphylla* is further expected to increase root branching and its architecture, to provide home for the biosynthesis and accumulation of alkaloids. Hence, developing a microbial inoculant based on RT2 is expected to manifest overall health benefits to *Rauvolfia.* Screening for phosphate solubilization on Pikovskaya's agar medium has explicitly revealed the potential of RT2 for phosphate solubilization, and therefore developing inoculants based on RT2 can help in improving phosphate flux into molecules like ATP, NADPH, phospholipids, nucleic acid backbone etc. Overall such studies can be useful in addressing sustainable cultivation of not only medicinal plants but also other crops. Whole genome sequencing studies of RT2 should also be performed to know about the presence of gene/s and gene products responsible for PGP activities. Besides, since the isolate has previously been reported to cause nosocomial infections, it would be necessary to carry out functional genomic studies to ascertain its pathogenicity in humans. Besides, annotation of genes in whole genome of the organism would be useful in identifying other potential uses of this bacteria like production of secondary metabolites. Further studies are required to test the efficiency of this isolate by the acetylene reduction assay. Various combinations of isolates can be made into microbial consortia to study microbial synergies in plant growth promotion. Besides this, assessment of increase in alkaloid level following inoculation into tissue culture raised *R. tetraphylla* L. plants to ascertain increment in the alkaloid level could be beneficial to the pharmaceutical industry, which at present faces scarcity of raw materials due to their over exploitation and switching to substitutes. Thus, isolation and identification of diazotrophs particularly unconventional ones, with excellent PGP traits, from the rhizosphere of *R. tetraphylla* points out that they could be complementing the conventional nitrogen-fixing microbial partners by adapting to diverse ecological niches. These endeavours would be valuable in addressing SDG 2030 by reducing excessive reliance on chemical fertilizers, and promote ecological sustainability in the production of medicinal plants and bioactives.

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Conflict of Interest

The authors do not have any conflict of interest.

Data Availability

16S rRNA gene sequences of the various diazotrophs have been deposited in the GenBank database and can be retrieved from their accession numbers.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Permission to reproduce material from other sources

Not Applicable

Author Contributions

- **Manisha Mishra:** Conceptualization, Study Design, Methodology, Data Analysis, Investigation Writing-Original Draft, Revision and Editing
- **Sandeep Kumar:** Study Design, Methodology, Data Analysis, Revision and Editing
- **Arvind Kumar:** Study Design, Methodology and Supervision

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