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Abundance of *nifH* gene copies in rhizosphere of rice as influenced by different N fertilization regimes and water management practices

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ABSTRACT

Biological nitrogen fixation is a significant source of fixed nitrogen in paddy soils and is influenced by the chemical N fertilizer application. The objective of this study was to quantify the abundance of *nifH* gene copies in different paddy soils and to characterize the impact of N fertilizers in the rhizosphere of rice as influenced by conventional flooding (CF) or direct seeded rice (DSR) during vegetative and flowering stages of plant growth. A microcosmic experiment was conducted with the amendment of three N concentrations (0, 10, and 100 mM) in six distinct paddy soils. Further, a field experiment was carried out by employing CF and DSR methods of rice cultivation with T1-RDF and T2-50% N as urea and KNO_3 at 75:25 with full PK. The *nifH* abundance ranged from 2.20×10^5 to 1.01×10^7 g^{-1} soil and diverged significantly among distinct soil types. Soils treated with 10 mM of N had 16 to 58% higher *nifH* abundance, while the addition of N at 100 mM reduced *nifH* gene copies by 4 to 8%. Similarly, the application of 50% N relative to the recommended fertilizer N dose, led to the enrichment of *nifH* in rice rhizosphere under both CF (9-25%) and DSR (11-29%) methods. Unlike the DSR (3.56 to 8.85×10^6 g^{-1} soil), conventionally flooded fields (6.14×10^6 to 1.01×10^7 g^{-1} soil) had higher *nifH* abundance in the rice rhizosphere. Furthermore, *nifH* abundance peaked during the flowering stage of plant growth and was 0.38 to 1.2 folds higher than at the vegetative stage. This study signifies that overuse of N fertilizers may restrict biological N fixation, while adequate N supply can enhance the abundance of *nifH* gene copies. Conventional flooding method of rice cultivation induced *nifH* abundance in the rice rhizosphere, which peaked during the flowering stage.

Key words: *Paddy, Nitrogen-fixation,* nifH, *qPCR, Conventional-flooded paddy, Direct-seeded rice*

Introduction

Rice is cultivated on 159 million acres of land globally, more under flooded soil conditions (130 million ha) than under upland (aerobic) conditions. Nitrogen transformations in flooded rice soils have distinct properties, including higher N fertility in flooded soils than in the upland soils, and increased accumulation of N on the surface of flooded soils via recycling or biological nitrogen fixation (Ladha and Reddy, 2003). Nitrogen fertilizers are intensively used to maintain soil fertility and rice crop productivity. However, overuse of N fertilizers has posed serious environmental threats particularly the escalated release of reactive N (Nr) species and nitrate leaching. Biological nitrogen fixation (BNF) is a sustainable process of N input in soil which accounts for nearly 52 to 130 Tg N $year^{-1}$ nitrogen inputs in

natural ecosystems (Davies-Barnard and Friedlingstein, 2020). Biological N_2 fixation is reported to contribute 22-30 kg N ha^{-2} $year^{-1}$ for rice (Herridge *et al.,* 2008). However, excessive N fertilizers can inhibit natural BNF process in paddy soils and have been reported to decrease microbial N acquisition by 55 % (Schleuss *et al.,* 2021). Moreover, diazotrophs are highly sensitive to moisture, and the flooded soils are demonstrated to be more conducive to BNF (Zhang *et al.,* 2021). Similarly, physico-chemical properties of soil including pH, soil texture, Fe-, Mo-, and P-content, and bulk density regulate the interaction and composition of diazotrophic community (Feng *et al.,* 2018; Yang *et al*., 2022). As Indian rice fields differ strikingly in soil characteristics, irrigation and crop management practices, it is vital to investigate how N fertilization and water management practices affect the activity of BNF in distinct paddy soil types. The *nifH* gene, which encodes for nitrogenase subunit protein, has been frequently used as the gene marker to study the diversity and abundance of diazotrophic communities. Hence, this study was conducted to examine the abundance of *nifH* gene copies in six distinct paddy soils, using quantitative PCR assay, and how N fertilization practices influence the *nifH* gene copies in the rhizosphere of rice cultivated under flooded or direct seeded rice method.

Materials and Methods

Microcosms design and incubation

The soil samples were collected from the paddy fields of six different agro-ecological zones of India, including; Aduthurai (ADU), Tamil Nadu; (ii) Indian Agricultural Research Institute (IARI), New Delhi; (iii) Karnal (KAR), Haryana; (iv) Kuttanad (KUT), Kerala; (v) Bundi, Rajasthan (RAJ); and (vi) Umiam (UMI), Meghalaya. The physico-chemical characteristics of these soils are presented in Table 1.

The microcosms were constructed in serum bottles using the 1.25:1 volumetric ratio of soil enrichment medium to 10 g of different soil samples. The soil enrichment medium contained: NaCl (1 g l^{-1}), $MgCl_2 \cdot 6H_2O$ (0.4 g l^{-1}), $CaCl_2 \cdot 2H_2O$ (0.1 g l^{-1}), KCl (0.5 g l^{-1}), $MgSO_4.7H_2O$ (5 g l^{-1}), $NaNO_2$ (0.1 mM), and KH_2PO_4 (0.2 g l^{-1}) with 1 ml each of the non-chelated trace elements solution and the vitamin solution (De La Torre *et al.,* 2008). The stock solutions of urea as N source was prepared using deionized water and were then added in the soil enrichment medium to have the microcosms with three different nitrogen (N) concentrations (0, 10 and 100 mM). The soil samples were collected after 14 days of incubation and were stored at –20 °C until subsequent molecular analyses.

Field experiment and soil sampling

The field experiment was conducted at the research farm of ICAR-Indian Agricultural Research Institute, New Delhi (28° 40'N and 77°12'E). The mean annual temperature of the site was 25 °C, and the mean annual rainfall was about 650 mm. Pusa Basmati 1509 was cultivated using conventional flooding (CF) and direct seeded rice (DSR) methods having two treatments as; (i) T1-the recommended dose of fertilizers (RDF; 110, 60, 50 NPK kg ha^{-1}, as prilled urea, single super phosphate, and muriate of potash, respectively), (ii) T2-50% N with urea and KNO_3 at a ratio of 75:25 with full doses of PK. Urea was given in two splits; the first application of 55 kg N ha^{-1} for the T1 and 27.5 kg N ha^{-1} for T2 as basal dose while the second split was given during tillering stage. In the CF plots, the rice field was kept flooded with 5-6 cm water during the entire growth period while only necessary irrigation was given in DSR to saturate the soils. The rhizosphere soil samples were collected at the vegetative stage, 3-d after the second split application of urea, and the flowering stage. Soil samples were stored at -80 °C for extracting total DNA and further *nifH* quantification.

Table 1. Physico-chemical properties of soils collected from different regions

Collection area	Soil type	pH	Organic carbon	Available N (g kg^{-1}) (mg kg^{-1})
Aduthurai, Tamil Nadu (ADU)	Typic Haplustert	7.40	10.1	95.2
IARI, New Delhi (IARI)	Typic Haplustepts	7.70	6.8	89.6
Karnal, Haryana (KAR)	Typic Natrustalf	10.6	2.51	20.8
Kuttanad, Kerala (KUT)	Typic Sulfaquent	4.47	32.0	112.0
Bundi,Rajesthan (RAJ)	Typic Chromustart	8.10	5.69	276.8
Umiam, Meghalaya (UMI)	Typic Hapludalf	4.27	17.6	100.8

DNA Extraction and absolute quantification of *nifH*

Total soil DNA was extracted using the Nucleopore G-DNA soil kit (Genetix, New Delhi, India) and DNA concentration was determined using Nanodrop 3300 spectrofluorometry (Waltham, Massachusetts, USA). The quantitative PCR using Fast SYBR® Green dye was employed to determine thegene copies of *nifH*in tested soils, using the Light Cycler® 96 Real-Time PCR System (Roche Diagnostics Corp., Indianapolis, Indiana, USA). Soil DNA was used as a template (3µl) in the 20µl reaction volume with the forward (*5′*-TGCGAYCCSAARGCBGACTC-*3′*) and reverse (*5′*-ATSGCCATCATYTCRCCGGA-*3′*) primers specific for *nifH*, the SYBR Green I Master Mix (10 µl), bovine serum albumin (1 µL of 10 mg ml^{-1}), and the nuclease-free water (5 µl) (Poly *et al*., 2001). The 10-fold serial dilutions of purified genomic DNA were prepared to generate the standard curve and the C_T values were plotted against the gene copies. The concentrations of genomic DNA and cloned plasmid were determined using Nanodrop 3300 spectrofluorometry and the gene copy numbers were then calculated and expressed in per gram of soil.

Statistical analyses

The statistical analysis was done using OriginPro® 2022 (OriginLab, Massachusetts, USA). Data from the microcosmic experiment were analyzed by Two-way ANOVA to assess the significant differences in the *nifH* abundance of different soil types and to test the influence of N application at different concentrations. Field data were subjected to a three-way ANOVA to determine the significant differences ($p<0.05$) due to cultivation method (CF and DSR), N treatments, and growth stages and further, a multi-comparison was done by Tukey's HSD.

Results and Discussion

Abundance of nitrogen-fixing gene (nifH) in different paddy soils as influenced by inorganic N inputs

The *nifH* gene copies in tested soil types ranged from 2.20×10^5 and 7.64×10^6 g^{-1} soil. The soils from ADU (Typic Haplustert), KUT (Typic Sulfaquent), and UMI (Typic Hapludalf) had a relatively higher abundance of *nifH* which varied from 4.14 to 6.93 × 10^6, 5.95 to 7.64 × 10^6, and 3.22 to 4.80 × 10^6 g^{-1} soil respectively. On the other hand, soils from IARI (Typic Haplustepts), KAR (Typic Natrustalf), and RAJ (Typic Chromustart) possessed lower gene copies of *nifH* that were in the range of 2.20 to 6.76 × 10^5 g^{-1} soil (Fig. 1). These observations suggest that the population densities of diazotrophic microorganisms varied among different soil types. Our results agree with Shu *et al.* (2012) and Goel *et al.* (2021), who reported nearly 10^6 copies of *nifH* per gram of rice soil. The highest abundance of *nifH* gene copies was found in the KUT soil, while the soil from KAR possessed the least copies. For instance, KUT soil showed 20 to 26 folds higher *nifH* gene copies than the KAR soil at all N concentrations. This difference can be partly explained by the higher organic C and N contents of KUT soil (Table 1) which can support the microbial activity and energy-intensive biological nitrogen fixation (Zhang *et al.*, 2021). Our observationof significantly higher copies of *nifH* in the ADU and the UMI soils also supports this hypothesis as these two soils had substantially higher C and N content than the other soils tested (IARI, KAR, and RAJ). Moreover, differences in *nifH* gene copies can also be due to the variations in the struc-

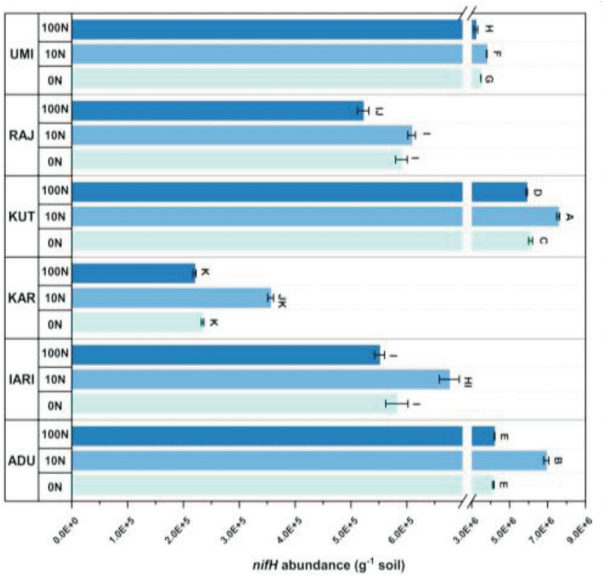

Fig. 1. Absolute abundance of *nifH* in six different paddy soils treated with 0, 10, and 100 mM nitrogen. Horizontal bars represent the mean copy number of *nifH* (n=3) and error bars represent standard error ± 0.05. Means followed by different alphabets (as per Tukey's HSD) are significantly ($p<0.05$) different among the different soil types and treatments. SS-U; 3 d after second split of urea.

ture of the diazotrophic community (Wu *et al.,* 2021). Indeed, soil texture and pH have been identified as the two most crucial shaping factors of the diazotrophic microbiome in paddy soils (Yang *et al.,* 2022). Higher *nifH* abundance in ADU, KUT, and UMI soil can also be explained by the fact that these soils receive considerably lower N-fertilizer inputs (Sreelatha and Shylaraj, 2017), while intensive cultivation of rice with nitrogenous fertilizers in IARI, KAR, and RAJ probably led to inhibition of BNF in these soils (Ayuni *et al.,* 2015).

The addition of inorganic nitrogen at three different concentrations (0, 10, and 100 mM) considerably changed the *nifH* copies in the tested soils (Fig. 1). Interestingly, the gene copies of *nifH* invariably increased with the addition of N at 10 mM in all the soils tested. However, the addition of N at 100 mM decreased the gene copies of *nifH*, relative to the addition of N at 10mM. For example, compared to 0 mM N, KUT soil treated with 10 mM N showed a 23% increase in the abundance of *nifH* gene copies, while adding 100 mM N reduced itscopies by 21%. Similarly, the KAR soil treated with 10 and 100 mM N displayed 52% higher and 6% lower copies, respectively, than the untreated KAR soil. Our results indicate that high N inputs depressed the abundance of *nifH* in paddy soils, with the possibility of reducing soil fertility. In general, the nitrogen-fixing bacteria prefer to utilize external nitrogen due to the high energy need of the BNF process (Bodelier *et al.*, 2000; Zhang *et al*., 2021). Induction of BNF after soil treatment with 10 mM N can likely be attributed to the stimulation of tiazotrophic community by exogenous N inputs by eliminating the N-limitation for protein (nitrogenase) synthesis. Hence, the results of our microcosmic experiment imply that the abundance of *nifH* varied considerably with different soil types, altering their nitrogen fixation potentials. Further, excess N application to the soils restricts the BNF while adequate supply may facilitate the BNF process by alleviating the limitation of N for the growth of diazotrophs.

Effects of N management practices and rice cultivation methods on the abundance of nifH in the rhizosphere of rice

Earlier, Tan *et al*. (2003) showed that a high dose of fertilizer N altered the structure of diazotrophs associated with rice roots. Zhang *et al*. (2021) also showed that high nitrogen application (125–250 kg N ha^{-1}) reduced biological N fixation by 81–86%. A field experiment was conducted to test the effects of N application levels and different water management practices on *nifH* abundance in the rhizosphere during distinct rice growth stages. The *nifH* copies varied with the lowest copies (3.56×10^6 g^{-1} soil) in the rhizosphere of T1 of the DSR method at the vegetative stage, while the rhizosphere of T2 of the CF method had the highest copies (1.01×10^7 g^{-1} soil) at the flowering stage (Fig. 2). Our findings of *nifH* abundance corroborate with the report of Jia *et al.* (2020) who observed about 10^5 to 10^7 copies of *nifH* g^{-1} soilin the flooded paddy fields. Hence, the biological N fixation potential was inherently high in this soil, and the microbial community performing this process did respond substantially to the additions of N. In the rice fields,anoxygenic phototrophs, cyanobacteria, sulfate-reducing bacteria and other diazotrophs contribute to the overall N budget (Ladha and Reddy, 2003). Our results corroborated the previous findings of higher *nifH* copies in the rhizosphere of rice (Goel *et al.,* 2021; Xu *et al*., 2023). Application of 50% less N (T2) than the recommended N dose (T1-RDF) led to the enrichment of *nifH* copies in the rice rhizosphere under both cultivation (CF and DSR) methods. The rhizosphere of T2 under the DSR method had 26%, 29%, and 11% higher copies of *nifH* during vegetative, 3 d

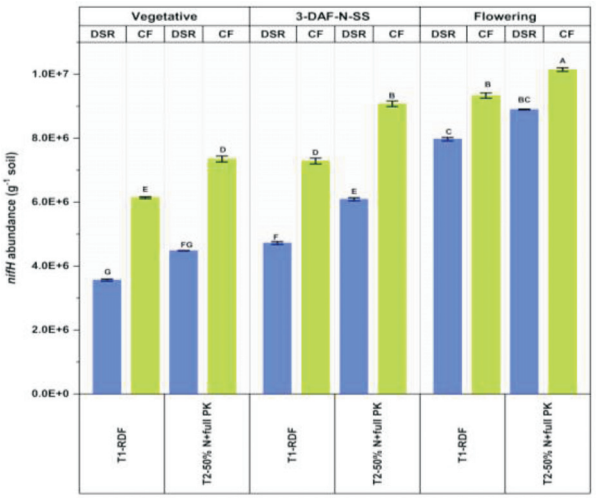

Fig. 2. Copies of *nifH*as influenced by different N management practices in the rice rhizosphere during different plant growth stages. The mean copies (n=3) of *nifH* are shown by vertical bras and error bars represent standard error ± 0.05. Means followed by different alphabets (as per Tukey's HSD) are significantly ($p<0.05$) different among the different N-fertilization treatments and stages. SS-U stand for 3 d after second split of urea.

after second N split, and flowering stage, respectively, than that in the T1 rhizosphere. Similarly, rice grown by the conventional flooding method showed a 9 to 25% increase in the *nifH* copies in the rhizosphere of T2 over the T1 during all growth stages of rice. This could be due to the inhibitory effect of inorganic N inputs on the biological fixation of N by diazotrophs that preferably utilize labile nitrogen over N fixation. These results are parallel to a recent quantitative synthesis that showed that free-living N-fixation was substantially inhibited by nitrogen fertilization in terrestrial ecosystems (Dynarski and Houlton 2018). Similarly, Ayuni *et al.* (2015) and Tang *et al*. (2017) also demonstrated that overuse of N fertilization suppressed diazotrophic population and nitrogenase activity in rice paddies.

The abundance of *nifH* was significantly higher during the flowering stage under both cultivation methods ($p<0.05$). For example, compared to the vegetative stage, *nifH* abundance was stimulated at the flowering stage, more under the DSR (88 to 124% increase) than that of CF (38 to 52%) method. This increase might be explained by the fact that the flowering stage is the vigorous period of plant growth, during which higher rhizodeposition, root abscission, and root exudates provide the carbon-rich substrates and energy for microbial activity and N-transformations. This observation corroborated with findings of Xu *et al.* (2023), who reported that the abundance of *nifH* gene copies in the rhizosphere increased by 5.19 folds during the flowering stage compared to the vegetative stage of rice. Furthermore, the gene copies of *nifH* were more abundant in the rhizosphere of rice grown under the CF method than the DSR method during the growth stages tested ($p<0.05$). For instance, at the vegetative stage, the rhizosphere of rice under the DSRhad 3.56 to 4.48 × 10^6 copies, whereas therhizosphere under the CF method had 6.14 to 7.35 × 10^6*nifH*gene copies g^{-1} soil. Likewise, the rhizosphere of rice under the CF method had 14 to 18% higher copies of *nifH* than the rhizosphere under the DSR method at the flowering stage. Higher *nifH* population under the CFmethod can be due to the sensitivity of diazotrophs to moisture and anaerobic conditions (Smercina *et al.,* 2019). In the case of rice cultivation, phototrophic bacteria and heterotrophic bacteria are the principal nitrogen-fixing organisms, with each contributing approximately 50% to BNF (Bei *et al.,* 2013). Flooded paddy fields are also suitable habitats for phototrophic bacteria (blue-green algae), leading to higher nitrogen-fixing activity of them, relative to the DSR fields. Zhang *et al.* (2021) demonstrated that when paddy soil was switched from dry to wet soil conditions, the activity of nitrogenase enzymes in the rhizosphere soil increased. Hence, continuous flooding method can enrich the gene copies of *nifH* in the rhizosphere of rice.

Conclusion

The abundance of *nifH* ranged from 10^5 to 10^7 copies g^{-1} soil and varied significantly among the paddy soils tested. Excessive N application (100 mM N or T1; RDF) significantly reduced the *nifH* abundance, while adequate N supply (10 mM N or 50% less N than RDF) facilitated the BNF process by alleviating the N-limitation of diazotrophs. Compared with the DSR method, conventionally flooded fields had higher abundance of *nifH* in the rhizosphere of rice. In addition, the gene copies of *nifH* peaked during the flowering stage of rice under both DSR and CF methods of cultivation.

Authorship Contribution Statement

Babanpreet Kour: Conducting experiments, Sample processing, qPCR analysis, Data collection, Statistical analysis, Data interpretation and representation, and Writing-Original draft.
Prasanta Kumar Prusty: Primer designing, Data collection, and Manuscript proofreading.
Sagar S.P.: Data collection and manuscript reviewing.
Yashbir Singh Shivay: Experimental planning, Conceptualization, supervision, reviewing and editing manuscript.
B Ramakrishnan: Conceptualization, supervision, Resources, Methodology, Data Validation, Reviewing and Editing of the manuscript.
* All authors reviewed the manuscript.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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