How Do Different Nitrogen Application Levels and Irrigation Practices Impact Biological Nitrogen Fixation and its Distribution in Paddy System?

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Abstract

Background and aims

Biological nitrogen fixation (BNF) in paddy systems is impacted by nitrogen application levels and irrigation strategies, but the extents to which these factores influence BNF and its distribution in soil and rice were still largely unclear. This study investigates this influence.

Methods

An airtight transparent growth chamber ^{15}N-labelling system were used to investigate how different nitrogen application levels (0, 125, 187.5 and 250 kg N ha^{-1}) and irrigation strategies (flooding irrigation or intermittent irrigation) impact the amount of BNF and its distribution in soil and rice.

Results

Nitrogen application at 125 ~ 250 kg N ha^{-1} reduced the amount of BNF by 81% - 86%. The inhibition effect of nitrogen application on BNF at a soil depth of 1-15 cm was greater than that at 0-1 cm. Relative to the continuous flooding irrigation, intermittent irrigation enhanced rice growth and promoted the transfer of fixed nitrogen from 0-1 cm soil layer to rice, but it did not change the total amount of BNF.

Conclusions

This study indicated that BNF supplied little nitrogen for rice production at the high nitrogen application levels, but the intermittent irrigation could promote utilization of biologically fixed nitrogen.

Introduction

Nitrogen-fertilizer and irrigation practices are key factors affecting rice yields; however, they also have direct and indirect effects on biological nitrogen fixation (BNF) in paddy fields, which is a naturally-occurring process that can also affect rice yields. BNF itself can lead to moderate but constant yields when no chemical nitrogen is added (Roger and Watanabe 1996); making utilization of BNF an important process for sustainable agriculture. Since the invention of Haber Bosch process in 1910, nitrogen application greatly enhances crop production. However, overuse of synthetic nitrogen fertilizers from farms has led to several health and environmental problems, from fresh water eutrophication to global warming (Zhang et al. 2015). Therefore, increased utilization of naturally occurring BNF is of increasing interest, in order to reduce the usage of chemical nitrogen to ensure grain yield (Rosenblueth et al. 2018).

Nitrogen fixing microorganisms can not only obtain nitrogen source from air through BNF, but also absorb existing nitrogen sources from the external environment (Reed et al. 2011). Due to a high energy consumption of the nitrogen fixation process, nitrogen fixing bacteria prefer to use exogenous nitrogen (Bodelier et al. 2000). Some studies have reported that nitrogen application significantly changes the nitrogen fixing microbial community structure in the rice field (Knauth et al. 2005; Tan et al. 2003). By using the nitrogen balance method in a pot experiment planted with rice, Santiago-Ventura et al (1986) found that the low rate of N fertilizer (5.64 mg N kg^{-1} soil) did not depress BNF, while a high rate of N fertilizer (99.72 mg N kg^{-1} soil) eliminated the BNF. By using the acetylene (C_2H_2) reduction method,a range of lab-incubation studies showed that the free-living nitrogen fixation in environment with low nitrogen availability, whether soil, rhizosphere, or moss, is generally higher than that in environment with high nitrogen availability (Kox et al. 2016; Patra et al. 2007; Hobbs and Schimel 1984). However, at the field condition during the whole rice season, the extent of BNF reduction induced by different nitrogen fertilizer application as well as the distribution of fixed nitrogen in the soil layer and rice plants still remain elusive. We hypothesize that the amount of BNF could be gradually increased by reducing the amount of nitrogen fertilizer in the field environment.

Diazotrophs are known to be moisture sensitive (Smercina et al. 2019). Among these organisms, phototrophic bacteria and heterotrophic bacteria are the main contributors to BNF in the case of rice planting, and the contribution of these two types of microorganisms is about 50%, respectively (Bei et al. 2013). Phototrophic bacteria (e.g. blue green alga) usually inhabits flooded waters and soil surfaces, while heterotrophic bacteria lives in the rhizosphere of rice (Ladha and Reddy 2003). *Nostoc*, which is a genus of cyanobacteria with the strongest nitrogen fixation capacity in paddy soil (Ma et al. 2019b; Wang et al. 2020), can survive drought for a long time, and once there is a small amount of precipitation, they can wake up and activate nitrogen fixation (Gao 1998; Scherer et al. 1984). The activity of nitrogen fixing enzymes in rhizosphere soil was found to increase when the paddy soil was shifted from dry to wet soil conditions, but it would decrease from wet to dry soil conditions (Rao and Rao 1984).). We hypothesize in this study therefore that the amount of BNF under the flooding-moist irrigation practice pattern (intermittent irrigation) would be lower than that of continuous flooding irrigation.

In this study, an airtight transparent growth chamber ^{15}N-labelling system was used (Bei et al. 2013) in the field environment to determine BNF under different nitrogen levels and irrigation practices in the whole growing season. The *nifH* gene of soil DNA was amplified by real-time fluorescent quantitative PCR to study the number of nitrogen fixing bacteria and its nitrogen fixation activity. The study is expected to reveal how nitrogen levels and water management impact biological nitrogen fixation and its distribution in the paddy field system.

Material And Methods

Site description, soil collection and soil properties

The experiment site is located in Xiaoji town, Jiangdu City, Jiangsu province, China (119°42'E, 32°35'N). The soil was classified as Inceptisol in the US soil taxonomy (or Shajiang Aquic Anthrosol based on Chinese soil taxonomy). The soil samples were randomly collected at several points along S shape from the plough layer (0-15cm) at the end of April. Visible plant materials in the soil were removed, and then the soils were air-dried, thoroughly homogenized asd sieved (< 5 mm). The soil had a pH value of 6.1

(soil:water = 1:5, w:v), and it contained 7.4% sand, 78.0% silt and 14.6% clay. More physio-chemical properties of the soil samples can be found in Wang et al. (2019).

Field $^{15}N_2$ labeling experiment

A field $^{15}N_2$ labeling experiment was conducted in the paddy field located in Xiaoji town, Jiangdu city, Jiangsu province, China from 23rd July to 23rd October in 2016. The airtight chambers (length × width × height = 1160 × 680 × 890 mm) (ITIGCN Crop., Ltd, Nanjing, China) in 3 replicates were treated with $^{15}N_2$ and installed in parallel in the rice field. The temperature and CO_2 concentration in the chambers were automatically controlled to keep in line with the ambient air temperature (ambient temperature ± 1 ℃) and CO_2 concentration (400 ± 20 ppm). Excessive O_2 generated by rice photosynthesis in the chambers was removed using a gas lighter keeping the O_2 concentration at 21%. In addition, three replicates of all treatments were placed outside the chambers as unlabelled controls. More detailed information about the design and control system of this airtight $^{15}N_2$-labeled chamber is described by Bei et al. (2013).

In this experiment, there were six treatments as follows: (1) no nitrogen application with continuous flooding irrigation (0N), (2) 125 kg N ha^{-1} with continuous flooding irrigation (125N), (3) 187.5 kg N ha^{-1} with continuous flooding irrigation (187.5N), (4) 250 kg N ha^{-1} with continuous flooding irrigation (250N), (5) no nitrogen application with intermittent irrigation (0N + II), (6) 250 kg N ha^{-1} with intermittent irrigation (250N + II) (250 kg N ha^{-1} is the local application level). Air-dried and screened (< 5 mm) soil (1.4 kg) was filled in each pot (length × width × height = 9 × 9 × 20 mm) to a depth of 15 cm. All treatments have three repetitions with each replication in each chamber, respectively. Before seedling transplantation, each pot was filled with water for 7 days, and the water surface was 1-2cm above the soil surface. Two rice seedlings (Oryza sativa L., cultivar Wuyunjing 23) were transplanted to each pot. Each pot was added with 116.37 mg KH_2PO_4 and 32.55 mg KCl (equivalent to 75 kg P_2O_5 ha^{-1} and 75 kg K_2O ha^{-1}) before rice transplantation. Nitrogen (N) was applied as urea: 50% before seedling transplantation, 10% at the tilling stage, and 40% at the heading stage. In the continuous flooding irrigation treatments, the water surface was kept 1-2cm above the soil surface until 10 days before rice sampling. In the intermittent irrigation treatments, the water was 1-2cm above the soil surface at the first 10 days, and then was kept moist. The strategy for keeping constant moisture pattern was practiced as follows: stop watering until small cracks appear between the soil and the wall of the pot, then add water to the soil to 1-2cm above the soil surface. Theis moisture protocol was repeated until 10 days before sampling.

Right after the preparation of each air-tight chamber with the rice pots of different treatments moving inside of it, 40 L of the air in each chamber was replaced by 40 L of $^{15}N_2$ (99 atom% ^{15}N). $^{15}N_2$ was produced and purified following the method of Ohyama and Kumazawa (1981), and detailed information on $^{15}N_2$ preparation was described by Ma et al. (2019b). The ^{15}N enrichment of N_2 within the three chambers was monitored by taking gas samples once a week during the 90-day labeling period.

Sampling and ^{15}N determination

After 90 days of $^{15}N_2$ continuous labelling, the growth chambers were opened for soil and plant sampling. In each pot, rice plants were separated into aboveground and belowground parts (roots), The soil samples was divided into 0–1 cm and 1–15 cm. The subsample of a soil was stored at -80 °C for further molecular analysis. The remaining soil sample was dried and ground using a Retsch MM 400 mixer mill (Retsch, Haan, Germany), A Thermo Finnigan Delta plus Advantages Mass Spectrometer coupled with an elemental analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for analysis of total N content and ^{15}N-enrichment in soil and plants.

DNA extraction and real-time quantitative PCR (qPCR)

Soil microbial DNA from the growth chamber was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, Cleveland, OH, USA), The concentration and purity of the extracted DNA were analyzed using a Ultramicro ultraviolet visible spectrophotometer (NanoVue Plus,USA). The primer pairs polF (TGCGAYCCSAARGCBGACTC) / polR(ATSGCCATCATYTCRCCGGA) (Poly et al. 2001) was used for quantifying *nifH* gene copies. More detailed information about the Real-time qPCR can be found in Ma et al. (2019b).

Calculation and statistical analysis

The amount of ^{15}N fixed by BNF in the rice soil system during the experiment was calculated as:

$$^{15}N_{excess\ sample}(\%) = {}^{15}N_{chamber\ sample}(\%) - {}^{15}N_{ambient\ sample}(\%) \quad [1]$$

$$^{15}N_{excess\ gas}(\%) = {}^{15}N_{chamber\ gas}(\%) - {}^{15}N_{ambient\ gas}(\%) \quad [2]$$

$$\%N_{fixed} = \frac{^{15}N_{excess\ sample}(\%)}{^{15}N_{excess\ gas}(\%)} \times 100 \quad [3]$$

$$N_{fixed} = \sum_{1}^{i} \{N_{i\ sample} \times \%N_{fixed}\} \quad [4]$$

$$Total\ N_{fixed} = N_{fixed\ in\ soil} + N_{fixed\ in\ rice\ plant} \quad [5]$$

$$Average\ nitrogen\ fixation\ activity = \frac{N_{fixed(0-1cm\ soil)}}{nifH\ gene\ copy\ number_{(0-1cm\ soil)}} \quad [6]$$

Where $\%N_{fixed}$ is the percentage of N derived from BNF in rice plant and soil. $N_{i\ sample}$ is the amount of N in rice plant and soil in the $^{15}N_2$-labeled chamber.

Statistical differences of the data was analysed using the one-way ANOVA and Tukey's honestly significant difference (HSD) test in SPSS. Values with $P < 0.05$ were recognized as significantly different.

Results

$^{15}N_2$-labelling growth chamber performance

The abundance of $^{15}N_2$ in the growth chambers decreased gradually from 8.26–1.99% over the rice season, with the average value at 5.44% (Fig. 1A). The CO_2 concentration in the chambers remained at 400 ± 20 μL/L during daytime, but it accumulated to a maximum of approximately 2000 μL/L at night due to lack of photosynthesis (Fig. 1B). The temperature in the growth chambers was consistent with the field temperature (Fig. 1C). The relative humidity in the chambers was between 50% and 90% (Fig. 1D). The oxygen produced by photosynthesis in the chambers was eliminated by a gas lighter, and the concentration was maintained at about 21% (Fig. 1E)

Nitrogen application levels on nitrogen fixation

The $^{15}N_{excess\ sample}$ in the 0-1cm and 1-15cm soil layers of the 0N treatment was significantly higher than that in nitrogen fertilizer application treatments, Also in each treatment, the $^{15}N_{excess\ sample}$ in the 0-1cm soil layer was significantly higher than that in the 1-15cm soil layer (Table 1). The N_{fixed} in the 0-1cm and 1-15cm soil layers in the 0N treatment were significantly higher than those in the nitrogen application treatments, with the total N_{fixed} in soil in 0N treatment being 6.5–8.3 times higher than those of the N application treatments (125, 187.5 and 250 kg N ha^{-1}) (Table 1). Among the treatments of 125N, 187.5N and 250N, there were no significant differences of the N_{fixed} in soil between each other either in the 0–1 cm or the 1–15 cm soil layers, respectively. The N_{fixed} was mainly in the 0-1cm soil layer for these treatments as the 0N. The proportion of the N_{fixed} in the 0-1cm soil layer to that in the whole soil were 70%, 82%, 88%, 98% in the 0N, 125N, 187.5N, 250N treatments, respectively (Table 1).

Table 1

Dry weight, total N, $^{15}N_{excess\ sample}$ and N_{fixed} at various soil depths and different plant parts in the $^{15}N_2$-labelled chamber after labelling for 90 days with different nitrogen application rates

Treatment	**Soil depth or Plant part**	**Dry weight (g)**	**Total N (g pot^{-1})**	**$^{15}N_{excess\ sample}$**	**N_{fixed} (mg pot^{-1})**
	Soil depth (cm)				
0N	0–1	104.92 ± 5.60bA	0.12 ± 0.03bA	0.184 ± 0.039aA	9.06 ± 2.01aA
	1–15	1297.01 ± 25.15aA	1.67 ± 0.05aA	0.008 ± 0.006bA	3.94 ± 1.13bA
	Whole soil	1401.93 ± 19.72A	1.79 ± 0.02A		13.01 ± 4.13A
125N	0–1	73.61 ± 22.45bA	0.10 ± 0.03bA	0.040 ± 0.006aB	1.40 ± 0.09aB
	1–15	1317.66 ± 10.61aA	1.73 ± 0.04aA	0.001 ± 0.000bB	0.31 ± 0.01bB
	Whole soil	1391.27 ± 25.37A	1.83 ± 0.06A		1.72 ± 0.37B
187.5N	0–1	87.51 ± 25.51bA	0.13 ± 0.04bA	0.049 ± 0.006aB	1.77 ± 0.79aB
	1–15	1334.27 ± 18.19aA	1.85 ± 0.05aA	0.001 ± 0.000bB	0.23 ± 0.17bB
	Whole soil	1421.78 ± 33.65A	1.99 ± 0.06A		2.01 ± 0.67B
250N	0–1	84.01 ± 6.74bA	0.120 ± 0.01bA	0.050 ± 0.021aB	1.53 ± 0.34aB
	1–15	1349.03 ± 18.40aA	1.84 ± 0.03aA	0.001 ± 0.000bB	0.04 ± 0.03bB
	Whole soil	1433.04 ± 20.22A	1.96 ± 0.030A		1.56 ± 0.78B
	plant part	Dry weight (g)	Total N (g pot-1)	15N atom% excess	N fixed (mg pot^{-1})
0N	Root	10.85 ± 1.35bA	0.01 ± 0.00bA	0.060 ± 0.018aA	0.16 ± 0.01bA

Note: mean ± standard error (n = 3). The same small letters in the same group (soil or rice) of the same column indicate that differences in different parts of (soil depth or rice parts) each treatment is not significant (P > 0.05). The same capital letters in the same group (soil or rice) of the same column indicate that differences among the different treatments is not significant (P > 0.05).

Treatment	Soil depth or Plant part	Dry weight (g)	Total N (g pot^{-1})	$^{15}N_{excess\ sample}$	N_{fixed} (mg pot^{-1})
	Aboveground	23.58 ± 2.56aA	0.10 ± 0.00aA	0.044 ± 0.011aA	2.43 ± 0.38aA
	Whole plant	34.43 ± 1.31A	0.11 ± 0.01A		2.59 ± 0.39A
125N	Root	10.33 ± 3.17bA	0.01 ± 0.00bA	0.038 ± 0.023aA	0.14 ± 0.09bA
	Aboveground	22.39 ± 3.22aA	0.11 ± 0.02aA	0.020 ± 0.009aA	0.76 ± 0.14aB
	Whole plant	32.72 ± 6.11A	0.12 ± 0.02A		0.90 ± 0.23B
187.5N	Root	6.58 ± 1.54bA	0.01 ± 0.00bA	0.023 ± 0.006aA	0.04 ± 0.02bA
	Aboveground	31.95 ± 0.46aA	0.09 ± 0.02aA	0.022 ± 0.021aA	0.88 ± 0.17aB
	Whole plant	38.53 ± 1.91A	0.10 ± 0.02A		0.92 ± 0.39B
250N	Root	6.55 ± 2.470bA	0.01 ± 0.01bA	0.024 ± 0.004aA	0.06 ± 0.04bA
	Aboveground	20.79 ± 3.23aA	0.10 ± 0.04aA	0.026 ± 0.005aA	0.54 ± 0.12aB
	Whole plant	27.34 ± 5.10A	0.11 ± 0.05A		0.60 ± 0.23B

Note: mean ± standard error (n = 3). The same small letters in the same group (soil or rice) of the same column indicate that differences in different parts of (soil depth or rice parts) each treatment is not significant (P > 0.05). The same capital letters in the same group (soil or rice) of the same column indicate that differences among the different treatments is not significant (P > 0.05).

The N_{fixed} in the rice plant in the 0N treatment was 2.8–4.3 times higher than that in the 125N, 187.5N and 250N treatments (Table 1). Nitrogen application significantly reduced N_{fixed} in the whole rice plant and the aboveground plant, but there was no significant difference in the roots. With the increase of nitrogen application rate, the $^{15}N_{excess\ sample}$ of rice roots and the aboveground biomass decreased, but there was no statistical significance among the different N application treatments. No significant difference was observed in the whole rice biomass and the total nitrogen contained in rice among all the treatments (Table 1).

Irrigation practices on nitrogen fixation

In either 0N or 250N application treatments (Table 2), the N_{fixed} in the 0-1cm soil layer, 1-15cm soil and the total soil from the continuous flooding treatments was higher than those from the intermittent irrigation treatment. In each treatment, the total nitrogen of 0-1cm soil layer was significantly lower than that of 1-15cm soil layer, while the N_{fixed} and the $^{15}N_{excess\ sample}$ in 0-1cm soil layer were significantly higher than those in 1-15cm soil layer.

Table 2

Dry weight, total N, $^{15}N_{excess\ sample}$, and N_{fixed} at various soil depths and different plant parts in the $^{15}N_2$-labelled chamber after labelling for 90 days with different irrigation practices

Treatment	**Soil depth or Plant part**	**Dry weight (g)**	**Total N (g pot^{-1})**	**$^{15}N_{excess\ sample}$**	**N_{fixed} (mg pot^{-1})**
	Soil depth (cm)				
0N	0–1	104.92 ± 5.60bA	0.12 ± 0.03bA	0.184 ± 0.039aA	9.06 ± 2.01aA
	1–15	1297.01 ± 25.15aA	1.67 ± 0.05aA	0.008 ± 0.006bA	3.94 ± 1.13bA
	Whole soil	1401.93 ± 19.72A	1.79 ± 0.02A		13.01 ± 4.13A
0N + II	0–1	86.44 ± 9.92bA	0.10 ± 0.01bA	0.158 ± 0.005aA	7.50 ± 0.93aA
	1–15	1345.61 ± 14.74aA	1.62 ± 0.09aA	0.009 ± 0.004bA	3.23 ± 0.80bA
	Whole soil	1432.05 ± 23.99A	1.72 ± 0.10A		10.72 ± 1.36A
250N	0–1	84.01 ± 6.74bA	0.12 ± 0.01bA	0.050 ± 0.021aA	1.53 ± 0.34aA
	1–15	1349.03 ± 18.40aA	1.84 ± 0.02aA	0.000 ± 0.000bB	0.04 ± 0.04bB
	Whole soil	1433.04 ± 20.22A	1.96 ± 0.03A		1.56 ± 0.78A
250N + II	0–1	90.57 ± 12.33bA	0.10 ± 0.02bA	0.008 ± 0.002aB	0.23 ± 0.08aB
	1–15	1326.81 ± 31.68aA	1.60 ± 0.03aB	0.000 ± 0.000bB	0.03 ± 0.02bB
	Whole soil	1417.44 ± 21.91A	1.70 ± 0.05B		0.27 ± 0.10B
	Plant part	Dry weight (g)	Total N (g pot^{-1})	^{15}N atom% excess	N fixed (mg pot^{-1})
0N	Root	10.85 ± 1.35bB	0.01 ± 0.00bA	0.060 ± 0.018aA	0.16 ± 0.01bB

Note: mean ± standard error (n = 3). The same small letters in the same group (soil or rice) of the same column indicate that differences in different parts of (soil depth or rice parts) each treatment is not significant ($P > 0.05$). The same capital letters in the same group (soil or rice) of the same column indicate that differences between continuous flooding irrigation and intermittent irrigation is not significant ($P > 0.05$).

Treatment	Soil depth or Plant part	Dry weight (g)	Total N (g pot^{-1})	$^{15}N_{excess\ sample}$	N_{fixed} (mg pot^{-1})
	Above ground	23.58 ± 2.56aB	0.10 ± 0.00aA	0.044 ± 0.011aA	2.43 ± 0.38aB
	Whole plant	34.43 ± 1.31B	0.11 ± 0.01A		2.59 ± 0.39B
0N + II	Root	13.14 ± 2.37bB	0.02 ± 0.00bA	0.056 ± 0.014aA	1.15 ± 0.17aA
	Above ground	27.65 ± 2.76aB	0.19 ± 0.06aA	0.039 ± 0.003aA	4.34 ± 0.24aA
	Whole plant	40.79 ± 1.38A	0.21 ± 0.05A		5.49 ± 0.35A
250N	Root	6.55 ± 2.470bB	0.01 ± 0.01bA	0.024 ± 0.004aB	0.06 ± 0.04aA
	Above ground	20.79 ± 2.23aB	0.10 ± 0.04aB	0.026 ± 0.005aB	0.54 ± 0.12aB
	Whole plant	27.34 ± 5.10B	0.11 ± 0.05B		0.60 ± 0.23B
250N + II	Root	21.25 ± 2.19bA	0.03 ± 0.01bA	0.017 ± 0.003aB	0.13 ± 0.04bA
	Above ground	31.04 ± 3.75aA	0.21 ± 0.00aA	0.020 ± 0.000aB	1.12 ± 0.20aA
	Whole plant	52.29 ± 2.76A	0.24 ± 0.01A		1.25 ± 0.20A

Note: mean ± standard error (n = 3). The same small letters in the same group (soil or rice) of the same column indicate that differences in different parts of (soil depth or rice parts) each treatment is not significant (P > 0.05). The same capital letters in the same group (soil or rice) of the same column indicate that differences between continuous flooding irrigation and intermittent irrigation is not significant (P > 0.05).

The N_{fixed} in rice root, aboveground plant and the whole rice plant under intermittent irrigation was significantly higher than those under continuous flooding irrigation (Table 2). As compared with the flooding irrigation, the intermittent irrigation did not significantly change the $^{15}N_{excess\ sample}$ and total nitrogen of rice, but it significantly increased the dry weight of the aboveground plant, root and the whole rice plant. In each treatment, The N_{fixed} and dry weight of the aboveground plant were significantly higher than those in rice root, but there was no significant difference of $^{15}N_{excess\ sample}$ between the aboveground plant and rice roots (Table 2).

Proportion of fixed nitrogen in soil and rice

The proportion of N_{fixed} in the 0-1cm soil layer to the total N_{fixed} increased with increasing nitrogen application rates (Table 3). The ratio for the 250N treatment was significantly higher than that in the 0N and 125N treatments. Along with the increase of nitrogen application rate, the proportion of N_{fixed} in the 1-

15cm soil layer to the total N_{fixed}, as well as the proportion of N_{fixed} in the whole soil to the total N_{fixed} decreased, with the proportion in the 0N treatment significantly higher than that in the 187.5N and 250N treatments. As compared to the 0N treatment, nitrogen application didn't change the proportion of N_{fixed} in root to the total N_{fixed}, but it increased the proportion of N_{fixed} in aboveground plant to total N_{fixed}, as well as the proportion of N_{fixed} in whole rice to total N_{fixed}. As compared with the continuous flooding irrigation, intermittent irrigation reduced the proportions of the N_{fixed} in 0-1cm soil or in the whole soil to total N_{fixed}. On the contrary, intermittent irrigation increased the proportions of N_{fixed} in root, aboveground plant and whole rice plant to the total N_{fixed} (Table 3).

Table 3
The proportion of fixed nitrogen in the 0-1cm soil layer, the 1-15cm soil layer, total soil, rice root, rice aboveground, total rice, relative to total biological nitrogen fixation under different nitrogen application rates and irrigation practices

Treatments	**($N_{fixed\ in\ 0–1cm\ soil}$)/($N_{fixed\ in\ pot}$)**	**($N_{fixed\ in\ 1–15cm\ soil}$)/($N_{fixed\ in\ pot}$)**	**($N_{fixed\ in\ rice\ root}$)/($N_{fixed\ in\ pot}$)**	**($N_{fixed\ in\ rice\ above\ ground}$)/($N_{fixed\ in\ pot}$)**	**($N_{fixed\ in\ total\ soil}$)/($N_{fixed\ in\ pot}$)**	**($N_{fixed\ in\ total\ rice}$)/($N_{fixed\ in\ pot}$)**
Nitrogen rates						
0N	58.33% ± 1.43%b	23.27% ± 4.13%a	1.09% ± 0.19%a	17.30% ± 4%b	81.6% ± 2.15%a	18.4% ± 2.15%b
125N	59.29% ± 2.42%b	13.4% ± 2.67%ab	4.30% ± 2.01%a	23.00% ± 1.48%ab	72.7% ± 1.82%ab	27.3% ± 1.82%ab
187.5N	63.20% ± 1.40%ab	6.07% ± 2.65%b	1.37% ± 0.37%a	29.12% ± 2.82%a	69.5% ± 2.60%b	30.5% ± 2.60%a
250N	68.89% ± 1.20%a	1.89% ± 0.49%b	2.49% ± 0.42%a	26.73% ± 1.11%ab	70.8% ± 2.80%b	29.2% ± 2.80%a
Irrigation practices						
0N	58.33% ± 1.43%A	23.27% ± 4.13%A	1.09% ± 0.19%B	17.31% ± 4%A	81.6% ± 4.2%A	14.8% ± 4.2%B
0N + II	46.39% ± 1.76%B	19.69% ± 1.03%A	7.1% ± 0.37%A	26.82% ± 1.61%A	66.1% ± 2.0%B	33.9% ± 2.0%A
250N	68.89% ± 3.28%A	1.89% ± 0.49%A	2.49% ± 0.42%B	26.73% ± 3.21%B	70.8% ± 2.8%A	29.2% ± 2.8%B
250N + II	14.77% ± 4.84%B	2.52% ± 0.94%A	9.57% ± 2.69%A	73.14% ± 2.35%A	17.3% ± 5.6%B	82.7% ± 5.6%A

Note: mean ± standard error (n = 3), the same small letters of the same column indicate that differences among different nitrogen application rates treatments is not significant ($P > 0.05$); the same capital letters in the same nitrogen application treatments indicate that differences between continuous flooding irrigation and intermittent irrigation is not significant ($P > 0.05$)

Abundance of nifH gene and nitrogen fixation activity

The total N_{fixed} of the 0N, 125N, 187.5N, 250N treatments were 15.59, 2.61, 2.93, and 2.16 mg N pot^{-1} (equivalent to 19.25, 3.22, 3.61, 2.67 kg N ha^{-1}), respectively (Fig. 2 − 1). Nitrogen application reduced the total N_{fixed} at 12.66–13.43 mg N pot^{-1}(equivalent to 15.63–16.58 kg N ha^{-1}), which accounted for 81.21% − 86.15% of total N_{fixed} in the 0N treatment. On the contrary, the *nifH* gene copy numbers increased with the increase of nitrogen application rate, ranging from 1.16×10^5 to 3.23×10^5 copies g^{-1} dry soil. The average nitrogen fixation activity of 0N treatment was significantly higher than those of nitrogen fertilizer treatments, which was by 15.49–10.76 times (Fig. <link rid="fig2">2</link>–2). The total N_{fixed} of the 0N, 0N + II, 250N, 250N + II treatments was 15.59, 16.21, 2.93, and 1.53 mg N pot^{-1} (equivalent to 19.25, 20.02, 2.67, 1.89 kg N ha^{-1}), respectively (Fig. 3 − 1). There were no significant difference in total N_{fixed} and *nifH* gene copy number between continuous flooding irrigation and intermittent irrigation. Similarly, there was no significant difference in the average nitrogen fixation activity between the continuous flooding irrigation and intermittent irrigation regimes (Fig. 3 − 2).

Discussion

Nitrogen application greatly reduced the total N_{fixed} by 81.21% − 86.15%,but along with the increase of nitrogen application from 125N to 250N there was no further decrease of total N_{fixed} (Fig. 2 − 1). These results is broadly in accordance with our hypothesis that nitrogen application gradually decreases the amount of BNF. This implies that under large applications of nitrogen fertilizer, BNF contributes much less nitrogen than anticipated 22 kg ha^{-1} to rice fields (Ladha et al. 2016). BNF has been found to be suppressed in rhizosphere samples after ammonium sulphate treatments at 40 kg N ha^{-1}, and even more so at 80 kg N ha^{-1} (Charyulu et al. 1981). This may be due to nitrogen applications significantly reducing the activity of nitrogen fixing bacteria (Fig. <link rid="fig2">2</link>–2). Nitrogen application also affects the distribution of fixed nitrogen in soil. The proportion of N_{fixed} in the 0-1cm soil layer to total BNF increased with the increase of the nitrogen application rate, but the proportion of N_{fixed} in the 1-15cm soil layer to total N_{fixed} decreased with the increase of nitrogen application rate. This may be related to the distribution and retention time of the urea added to soil. Firstly, the NH_4^+ derived from the urea on the soil surface easily diffuse into the atmosphere through the water layer (Inubushi and Watanabe, 1986). Therefor, as compared with the 1–15 cm soil layer, the retention time of NH_4^+ in 0-1cm soil layer is relatively shorter, thus the nitrogen fixation activity of nitrogen fixing bacteria in the 0–1 cm soil layer may be less inhibited by NH_4^+. In addition, ammonia deposition in this closed system may lead to an underestimation of the amount of BNF in the 0N treatment. Secondly, there is still a certain oxidation environment on the surface of soil (Knauth et al. 2005; Irisarri et al. 2001), where part of NH_4^+ may be transformed into NO_3^- by nitrifying bacteria (Yamamuro, 1986). Cyanobacteria was reported to be the main nitrogen fixing bacteria on the surface of soil (Ma et al. 2019b; Wang et al. 2020). Großkopf (2012) found that the presence of high nitrate concentrations (up to 800 mM) had no inhibitory effect on growth and nitrogen fixation activity of cyanobacteria. Since nitrogen fixing bacteria may need more energy to absorb and assimilate NO_3^--N than NH_4^+-N (Norman and Friesen 2017), the nitrogen fixing bacteria in the surface soil (0-1cm) may be unable to directly use enough applied N fertilizer, thus being more active to assimilate the atmospheric N_2. In comparison, in the deeper soil layer (1-15cm) of the rice paddy fields, the potential higher inhibition effect of nitrogen fertilizer application may lead to less N_{fixed}.

As compared with the continuous flooding irrigation, the intermittent irrigation had no significant effect on the total N_{fixed}, but it changed the distribution of N_{fixed} in soil and rice plants. Intermittent irrigation reduced the proportion of N_{fixed} in the 0–1 cm soil layer and whole soil to total N_{fixed}, but it increased the proportion of N_{fixed} in root, aboveground plant and whole rice plant to total N_{fixed}. This may be due to the change of soil moisture status altering soil microbial abundance and community structure (Lu et al. 2006; Yoshida and Ancajas 1971). When the paddy soil is insufficient of water, some anaerobic nitrogen fixing bacteria will die and release nitrogen-containing compounds which will be absorbed by rice (Valiente et al. 2000). As compared with the 1-15cm soil layer, the 0-1cm soil layer is more likely to be subjected to the water shortage, but N_{fixed} is mainly concentrated in the 0–1 cm soil layer (Table 1) (Bei et al. 2013; Ma et al. 2019a). Therefore, more N_{fixed} was transferred from the 0-1cm soil layer to rice than from the 1-15cm soil layer (Table 3). For the whole growing season, intermittent irrigation did not change the total N_{fixed}, which may be due to the decrease of N_{fixed} in water shortage period was offset by the potential increase of N_{fixed} in flooding period. When the paddy soil changed from flooded state to water deficient state, the death or deactivation of some anaerobic nitrogen-fixing bacteria and the inhibition of oxygen on nitrogen fixation could reduce the N_{fixed}. Cyanobacteria, which was supposed as the main nitrogen-fixing bacteria (Ma et al. 2019b; Wang et al. 2020), would not fix nitrogen when water is deficient (Siegfried Scherer 1984). When the paddy soil changed from water deficient state to flooded state, soil oxygen content and redox potential (EH) would decrease rapidly, the soil pH would tend to be neutral, and soil available carbon may increase due to the potential increase of soil organic matter decomposition (Zeng et al. 2011). These changes provide better environmental conditions and energy sources beneficial for BNF than the continuous flood condition (Bais et al. 2006). Therefore, the BNF activity during the flooding period within the intermittent irrigation pattern was supposed to be higher than that of the continuous flooding pattern. In general, as compared with the continuous flooding irrigation, although intermittent irrigation did not change the total N_{fixed}, but it promoted the transfer of N_{fixed} from the 0–1 cm soil layer to rice, which was supposed to increase the nitrogen use efficiency.

The *nifH* gene copy numbers increased with the increase of nitrogen application rate (Fig. 2), although nitrogen application decreased the amount of BNF. Soil nitrogen fixing bacteria can use a variety of nitrogen sources to support their growth. They can access inorganic N through direct uptake (Norman and Friesen 2017). Therefore, when the inorganic nitrogen in soil is sufficient, it is no need for them to consume energy to obtain nitrogen source through biological nitrogen fixation. On the other hand, Fan et al (2019) found that long-term fertilization reduces N fixation and specific groups of N fixers. In this study the soil used were collected from the paddy soil with long-term nitrogen fertilization of 250 kg N ha^{-1}, but we didn't find that reduction of nitrogen application increased the number of nitrogen fixing bacteria. Nitrogen application enhanced the number of nitrogen fixing bacteria, but it inhibited activity of nitrogen fixation (Fig. 2).

Conclusion

Nitrogen application at 125 kg N ha^{-1} greatly reduced the amount of BNF, and as nitrogen application increases further, no more reduction was observed. Inhibition of BNF by nitrogen application in 1–15 cm soil was greater than that in 0–1 cm soil. As compared with the continuous flooding pattern, the intermittent irrigation enhanced rice growth and promoted the transfer of N_{fixed} from 0–1 cm soil layer to rice, but it did not change the total N_{fixed}. Nitrogen application promoted the growth of diazotrophs but it decreased their activity to fix the atmospheric N_2.

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References

1. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. The Annual Review of Plant Biology 57:233–266. doi:10.1146/annurev.arplant.57.032905.105159
2. Bei QC, Liu G, Tang HY, Cadisch G, Rasche F, Xie ZB (2013) Heterotrophic and phototrophic $^{15}N_2$ fixation and distribution of fixed ^{15}N in a flooded rice–soil system. Soil Biol Biochem 59:25–31. doi:10.1016/j.soilbio.2013.01.008
3. Bodelier PLE, Roslev P, Henckel T, Frenzel P (2000) Stimulation by ammonium-based fertilizers of methane oxidation in soil around rice roots. Nature 403:421–424
4. Charyulu PBBN, Nayak DN, Rao VR (1981) $^{15}N_2$ incorporation as influence of rice variety, organic matter and combined nitrogen. Plant Soil 59:399–405
5. Fan KK, Delgado-Baquerizo M, Guo XS, Wang DZ, Wu YY, Zhu M, Yu W, Yao HY, Zhu YG, Chu HY (2019) Suppressed N fixation and diazotrophs after four decades of fertilization. Mirobiome 7:143
6. Gao KS (1998) Chinese studies on the edible blue-green alga, Nostoc flagelliforme: a review. J Appl Phycol 10:37–49
7. Großkopf T, LaRoche J (2012) Direct and Indirect Costs of Dinitrogen Fixation in Crocosphaera watsonii WH8501 and Possible Implications for the Nitrogen Cycle. Front Microbiol 3:236. doi:10.3389/fmicb.2012.00236

8. Hoobs NT, Schimel D (1984) Fire Effects on Nitrogen Mineralization and Fixation in Mountain Shrub and Grassland Communities. Journal of Range Mamagement 37:402–405
9. Inubushi K, Watanabe I (1986) Dynamics of Available Nitrogen in Paddy Soils. Soil Science Plant Nutrition 32:561–577. doi:10.1080/00380768.1986.10557538
10. Irisarri P, Gonnet S, Monza J (2001) Cyanobacteria in Uruguayan rice fields: diversity, nitrogen fixing ability and tolerance to herbicides and combined nitrogen. J Biotechnol 91:95–103
11. Knauth S, Hurek T, Brar D, Reinhold-Hurek B (2005) Influence of different Oryza cultivars on expression of nifH gene pools in roots of rice. Environ Microbiol 7:1725–1733. doi:10.1111/j.1462-2920.2005.00841.x
12. Kox MAR, Lüke C, Fritz C, Elzen E, Alen T, Camp H, Lamers L, Jetten MSM, Ettwig KF (2016) Effects of nitrogen fertilization on diazotrophic activity of microorganisms associated with *Sphagnum magellanicum*. Plant Soil 406:83–100. doi:10.1007/s11104-016-2851-z
13. Ladha JK, Reddy PM (2003) Nitrogen fixation in rice systems: state of knowledge and future prospects. Plant Soil 252:151–167
14. Ladha JK, Tirol-Padre A, Reddy CK, Cassman KG, Verma S, Powlson DS, Kessel C, Richter DB, Chakraborty D, Pathak H (2016) Global nitrogen budgets in cereals: A 50-year assessment for maize, rice, and wheat production systems. Sci Rep 6:19355. doi:10.1038/srep19355
15. Lu Y, Rosencrantz D, Liesack W, Conrad R (2006) Structure and activity of bacterial community inhabiting rice roots and the rhizosphere. Environ Microbiol 8:1351–1360. doi:10.1111/j.1462-2920.2006.01028.x
16. Ma J, Bei QC, Wang XJ, Lan P, Liu G, Lin XW, Liu Q, Lin ZB, Liu BJ, Zhang YH, Jin HY, Hu TL, Zhu JG, Xie ZB (2019a) Impacts of Mo application on biological nitrogen fixation and diazotrophic communities in a flooded rice-soil system. Sci Total Environ 649:686–694. doi:10.1016/j.scitotenv.2018.08.318
17. Ma J, Bei QC, Wang XJ, Liu G, Cadisch G, Lin XW, Zhu JG, Sun XL, Xie ZB (2019b) Paddy System with a Hybrid Rice Enhances Cyanobacteria Nostoc and Increases N2 Fixation. Pedosphere 29:374–387. doi:10.1016/s1002-0160(19)60809-x
18. Norman JS, Friesen ML (2017) Complex N acquisition by soil diazotrophs: how the ability to release exoenzymes affects N fixation by terrestrial free-living diazotrophs. ISME J 11:315–326. doi:10.1038/ismej.2016.127
19. Ohyama T, Kumazawa K (1981) A simple method for the preparation, purification and storage of15N2gas for biological nitrogen fixation studies. Soil Science Plant Nutrition 27:263–265. doi:10.1080/00380768.1981.10431278
20. Patra AK, Le Roux X, Abbadie L, Clays-Josserand A, Poly F, Loiseau P, Louault F (2007) Effect of Microbial Activity and Nitrogen Mineralization on Free-living Nitrogen Fixation in Permanent Grassland Soils. J Agron Crop Sci 193:153–156. doi:10.1111/j.1439-037X.2006.00247.x
21. Poly F, Ranjard L, Nazaret S, Gourbiere F, Monrozier LJ (2001) Comparison of nifH gene pools in soils and soil microenvironments with contrasting properties. Appl Environ Microbiol 67:2255–2262.

doi:10.1128/AEM.67.5.2255-2262.2001

22. Rao VR, Rao JLN (1984) Nitrogen fixation (C2H2 reduction) in soil samples from rhizosphere of rice grown under alternate flooded and nonflooded conditions. Plant Soil 81:111–118
23. Reed SC, Cleveland CC, Townsend AR (2011) Functional Ecology of Free-Living Nitrogen Fixation: A Contemporary Perspective. Annu Rev Ecol Evol Syst 42:489–512. doi:10.1146/annurev-ecolsys-102710-145034
24. Roger PA, Watanabe I (1996) Technologies for utilizing biological nitrogen fixation in wetland rice: potentialities, current usage, and limiting factors. Fertilizer Research 9:39–77
25. Rosenblueth M, Ormeño-Orrillo E, López-López A, Rogel MA, Reyes-Hernández BJ, Martinez-Romero JC, Reddy PM, Martinez-Romero EM (2018) Nitrogen Fixation in Cereals. Front Microbiol 9:1794. doi:10.3389/fmicb.2018.01794
26. Santiago-Ventura, Bravo M, Daez C, Ventura V, Watanabe I, App AA (1986) Effects of N-fertilizers, straw, and dry fallow on the nitrogen balance of a flooded soil planted with rice. Plant Soil 93:405–411
27. Scherer S, Ernst A, Chen TW, Böger P (1984) Rewetting of drought-resistant blue-green algae: Time course of water uptake and reappearance of respiration, photosynthesis, and nitrogen fixation. Oecologia 62:418–423
28. Smercina DN, Evans SE, Friesen ML, Tiemann LK (2019) To Fix or Not To Fix: Controls on Free-Living Nitrogen Fixation in the Rhizosphere. Appl Environ Microbiol 85(6):e02546–e02518. doi:10.1128/AEM.02546-18
29. Tan ZY, Hurek T, Reinhold-Hurek B (2003) Effect of N-fertilization, plant genotype and environmental conditions on nifH gene pools in roots of rice. Environ Microbiol 5:1009–1015
30. Valiente EF, Ucha A, Quesada A, Leganes F, Carreres R (2000) Contribution of N2 fixing cyanobacteria to rice production: availability of nitrogen from 15N-labelled cyanobacteria and ammonium sulphate to rice. Plant Soil 221:107–112
31. Wang XJ, Liu BJ, Ma J, Zhang YH, Hu TL, Zhang H, Feng YC, Pan HL, Xu ZW, Liu G, Lin XW, Zhu JG, Bei QC, Xie ZB (2019) Soil aluminum oxides determine biological nitrogen fixation and diazotrophic communities across major types of paddy soils in China. Soil Biol Biochem 131:81–89. doi:10.1016/j.soilbio.2018.12.028
32. Wang XJ, Bei QC, Yang W, Zhang H, Hao JL, Qian Li, Feng YC, Xie ZB (2020) Unveiling of active diazotrophs in a flooded rice soil by combination of NanoSIMS and 15N2-DNA-stable isotope probing. Biol Fertil Soils 56:1189–1199. doi:10.1007/s00374-020-01497-2
33. Yamamuro S (1986) Behavior of Nitrogen in Paddy Soils. Japan Agriculture Reserch Quarterly 20:100–107
34. Yoshida T, Ancajas RR (1971) Nitrogen fixation by bacteria in the root zone of rice. Soil Sci Soc Amer Proc 35:156–158
35. Zeng F, Ali S, Zhang H, Ouyang Y, Qiu B, Wu F, Zhang G (2011) The influence of pH and organic matter content in paddy soil on heavy metal availability and their uptake by rice plants. Environ

Pollut 159:84–91. doi:10.1016/j.envpol.2010.09.019

36. Zhang X, Davidson EA, Mauzerall DL, Searchinger TD, Dumas P, Shen Y (2015) Managing nitrogen for sustainable development. Nature 528(7580):51–59