Biological nitrogen fixation in rice paddy soils is driven by multiple edaphic factors and available phosphorus is the greatest contributor

Tianlong HU[1, 2], Yanhui ZHANG[1, 2], Hui WANG[1, 2], Haiyang JIN[1, 2], Benjuan LIU[1, 2], Zhibin LIN[1, 2], Jing MA[1, 2], Xiaojie WANG[1, 5], Qi LIU[1, 3], Hongtao LIU[1,2], Zhe CHEN[1, 2], Rong ZHOU[1, 2], Penghui JIN[1, 2], Jianguo ZHU[1], Gang LIU[1], Qicheng BEI[1, 4], Xingwu LIN[1], Zubin XIE[1,*]

[1] *State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, 210008, China*

[2] *University of Chinese Academy of Sciences, No. 19A, Yuquan Road, Beijing, 100049, China*

[3] *Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, No. 159, Longpan Road, Nanjing, 210037, China*

[4] *Department of Soil Ecology, Helmholtz Centre for Environmental Research - UFZ, Halle (Saale), Germany*

[5] *Environmental Engineering College, Nanjing Polytechnic Institute, Nanjing 210048, China*

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ABSTRACT

Biological nitrogen fixation (BNF) is important for sustainable rice cultivation. Various edaphic factors have been individually evaluated for their effects on BNF in paddy soils. However, no single factor could fully explain the different soil outcomes. Paddy BNF is more likely to be simultaneously influenced to various degrees by combinations of several factors; however, the relative importance of the interaction of multiple edaphic factors on the regulation of BNF in rice soils is still unclear. Twenty-seven paddy soil samples with different soil properties were collected from major cropping areas in southwest and northeast China. Rice was transplanted into pots of these soils and grown in a $^{15}N_2$ enriched airtight chamber. Estimation of BNF was based on measurements of ^{15}N enrichment in the different soils and rice plants at the end of a 77-day incubation period. BNF amounts ranged from 0.66 to 12.3 $kg \cdot ha^{-1}$. BNF had a significant positive relationship with available phosphorus (AP) and significant quadratic relationships with available molybdenum (AMo) and total soil nitrogen (TN). AP explained 42% of the observed variation in BNF, TN explained 17%, and AMo explained 13%. The specific interaction between the soil cation exchange capacity (CEC) and available soil N (ASN, as determined by rice N uptake) accounted for 28% of the variation. BNF was reduced when AP was < 14 $mg \cdot kg^{-1}$, AMo < 0.09 $mg \cdot kg^{-1}$, or when TN was > 3.2 $g \cdot kg^{-1}$. These results provide valuable benchmarks that could be used to guide farmers in managing their soils to improve the potential contribution of paddy BNF to soil fertility.

Key Words: available molybdenum, available soil nitrogen, influencing factors, multiplicative effect, rice field

[*]**Corresponding author**. E-mail: zbxie@issas.ac.cn

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INTRODUCTION

Flooding conditions in rice fields create a suitable environment for biological nitrogen fixation (BNF), which greatly contributes to the sustainability of paddy productivity before utilizing N fertilization (Ladha and Reddy, 2003). BNF is more environmentally friendly than N fertilization, but the amount of BNF is limited by edaphic factors (Buresh *et al.*, 1980). Some studies have investigated the effects of different edaphic factors (e.g., pH, **N** availability, molybdenum, phosphorus, and oxygen concentration) on BNF from physiological, genetic, and ecological perspectives (Reed *et al.*, 2011; Smercina *et al.*, 2019). Although some factors have been found to affect paddy BNF, how these edaphic factors work together on BNF and how important they remain elusive (Reed *et al.*, 2011).

BNF can be limited by edaphic factors in two different but inclusive ways: BNF is actively decreased for environmental adaptation or passively decreased because of environmental limitations (Reed *et al.*, 2011). Diazotrophs fix atmospheric N_2 for physiological demand, but at the cost of high energy consumption (Stam *et al.*, 1987). To balance **N** acquisition and the energy cost of N_2 reduction, BNF is highly regulated by a sophisticated regulatory network at the gene and protein levels (Dixon and Kahn, 2004). Most diazotrophs switch off BNF by downregulating the expression of the *nifA* gene and inactivating nitrogenase when the external mineral **N** is sufficient for their growth (Halbleib and Ludden, 2000; Dixon and Kahn, 2004). Similarly, because of the oxygen sensitivity of nitrogenase, its expression level is tightly controlled by oxygen concentration (Hill, 1988). Furthermore, the BNF is also passively reduced owing to external restrictions. Survival of diazotrophs is essential for BNF. Aluminum compounds repress the growth of cyanobacteria and decrease BNF (Jurgensen, 1973; Jančula and Maršálek, 2011; Wang *et al.*, 2019). Diazotrophs tend to live in a neutral environment, whereas an acidic or alkaline environment represses BNF (Meng *et al.*, 2021). Molybdenum is necessary for the synthesis of Mo-dependent nitrogenases. Mo deficiency decreases nitrogenase activity by obstructing its synthesis (Jurgensen, 1973; Ma *et al.*, 2019a). In addition, due to the high demand for ATP, BNF is repressed under the constraints of energy sources and phosphorus (Vitousek *et al.*, 2002; Chiewattanakul *et al.*, 2022). The survival of diazotrophs, *nif* genes transcription, assembly of Fe-Mo cofactors, and ATP consumption are necessary processes for BNF, and different edaphic factors influence these processes. Only when the environmental requirements of these processes are met can the N_2 be reduced to ammonium by nitrogenases. Therefore, we hypothesized that BNF amount is the result of the multiplication of edaphic factors rather than a simple sum (Fig. 1).

Fig. 1

Fig. 1 The framework of how multiple edaphic factors affect **BNF**. The fulfillment of **BNF** needs four necessary processes — the survival of diazotrophs, transcription of *nif* genes, assembly of Fe-Mo cofactor, and ATP consumption. These components work sequentially; failure of any part will limit BNF. So, we inferred that **BNF** is affected by edaphic factors multiplicatively rather than additively and hypothesized that BNF amount is the result of the

multiplication of edaphic factors than a simple sum.

To test this hypothesis, 27 paddy soils, including eight subgroups, were sampled from the southwest to the northeast and incubated with rice plantations in an airtight, transparent, and $^{15}N_2$ enriched growth chamber until the rice was ripe to measure BNF amounts. We attempted to answer four questions: (1) what are the driving edaphic factors of paddy BNF amounts? (2) how do these factors work together on BNF? (3) what is their relative importance? (4) What are their limiting thresholds?

MATERIALS AND METHODS

Soil collection

In the Second Soil Survey of China, paddy soils were classified into eight subgroups according to the pedogenic classification of Soil Taxonomy of China. They are bleached paddy soils, gleyed paddy soils, percolated paddy soils, de-gleyed paddy soils, submerged paddy soils, salty paddy soils, periodical water-logging paddy soil and others (fluvo-aquic soils, gray fluvo-aquic soils). The following criteria were considered during soil sampling site selection: 1) all subgroups were covered, 2) the site was the main rice production area, and 3) each subgroup was located in different areas. Finally, 27 sites were selected based on the detailed addresses and surrounding conditions described in the Second Soil Survey of China. A total of 27 soil samples were collected in 2015 and 2016 (Fig. 2). In each site, a soil sample was collected in an “S” shape at several points from the plow layer (0-15cm) after the harvest of rice. All paddy soil samples were air-dried and passed through a 2-mm sieve to remove plant residues and stones. The thoroughly homogenized soil samples were then used to analyze the soil physicochemical properties and for field $^{15}N_2$ labeling experiment. Information on the sampling sites and soil properties is shown in Fig. 2 and Table I.

Fig. 2

Fig. 2 Sampling locations for paddy soils.

Table I

Physiochemical properties of 27 soil samples used for $^{15}N_2$ labeling incubation.

Sample ID	pH	OC	TN	TP	TK	AP	AK	AMo	CEC	Bulk Density
			g·kg^{-1}				mg·kg^{-1}		cmol·kg^{-1}	g·cm^{-3}
L1	6.05	12.06	1.05	0.40	18.06	27.67	112.21	0.24	16.14	1.41
L2	7.71	13.58	1.29	0.58	19.38	34.82	274.30	0.19	17.56	1.11
L3	6.21	14.74	1.36	0.79	21.54	67.98	265.98	0.38	19.04	1.16
L4	5.65	12.57	1.13	0.49	19.56	14.19	178.71	0.25	20.53	1.29
L5	7.65	16.41	1.77	0.66	18.96	43.59	374.04	0.34	30.69	1.18
L6	7.77	11.45	0.99	0.45	20.30	21.92	224.42	0.22	14.03	1.21
L7	7.85	16.78	1.86	1.05	18.50	57.38	174.55	0.11	19.47	1.33
L8	6.70	15.98	1.77	0.49	16.98	19.83	162.08	0.18	22.03	1.14
L9	6.86	16.69	1.84	0.50	17.67	18.52	232.74	0.19	25.24	1.24
L10	7.51	25.81	2.92	1.00	19.69	52.80	149.62	0.32	20.60	1.08
L11	6.56	30.58	3.14	0.71	18.71	21.24	103.90	0.18	23.23	1.17
L12	8.00	13.68	1.54	0.93	15.15	29.35	66.50	0.06	9.42	1.25
L13	5.27	19.57	1.86	0.77	13.63	36.12	128.84	0.41	21.91	1.03
L14	5.01	14.82	1.23	0.49	15.06	34.72	174.55	0.09	19.80	1.14
L15	5.60	14.01	1.30	0.31	19.41	2.51	78.96	0.05	22.88	1.31
L16	4.74	16.54	1.56	0.21	20.35	2.80	132.99	0.08	20.46	0.90
L17	8.14	20.63	1.98	0.86	15.87	17.84	54.03	0.09	17.18	1.31
L18	5.29	18.66	2.03	0.36	15.99	15.89	153.77	0.78	19.65	1.23
L19	4.80	15.28	1.85	0.78	24.14	36.29	170.40	0.19	17.18	1.14
L20	5.34	16.52	1.91	0.27	17.20	10.03	78.96	0.12	10.02	1.16
L21	7.45	29.40	2.82	0.86	14.16	24.15	187.02	0.09	18.74	1.14
L22	5.99	28.52	3.29	0.55	16.97	14.49	295.08	0.26	25.66	0.98
L23	5.06	54.61	3.97	0.71	12.17	9.62	191.18	1.51	35.46	0.96
L24	6.22	30.87	3.35	0.70	9.98	14.80	95.59	0.12	16.63	1.15
L25	6.54	31.57	3.15	0.76	13.19	27.38	132.99	0.24	19.77	1.08
L26	7.12	37.43	3.87	1.07	12.18	52.04	33.25	0.07	17.76	0.97
L27	6.99	22.40	2.24	1.82	6.28	71.11	70.65	0.95	26.87	1.25

Soil physical and chemical analysis

The soil physicochemical properties were analyzed following the methods described by Lu (2000). The soil texture (clay, silt, and sand fractions) was determined using the standard pipette method. The soil pH was measured in deionized water at a soil-to-water ratio of 1:2.5 (w/v). The soil cation exchange capacity (CEC) was determined using the ammonium acetate method. Soil organic carbon (OC) concentrations were determined using the dichromate redox titration method. Total soil N concentration was measured using a Vario Max CN Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Soil total P was determined using the molybdenum-blue method following digestion by H_2SO_4-$HClO_4$. Soil total K was determined using a flame photometer following digestion with HF-$HClO_4$. Available P in the soil was extracted using sodium bicarbonate and determined using the molybdenum-blue method. Available K in the soil was extracted by ammonium acetate and determined using a flame photometer. The total Fe and Mo in the soil were measured using an inductively coupled plasma atomic emission spectrophotometer (ICP-AES) after HF-HNO_3-$HClO_4$ digestion. The quantities of available Fe in soils were determined using the ICP-AES following extraction with diethylene triamine penta-acetic acid (DTPA). Available Mo was

extracted with an acid ammonium oxalate solution and then analyzed using inductively coupled plasma mass spectrometry with high performance liquid chromatography (HPLC-ICP-MS).

Field $^{15}N_2$ labeling experiment

Field $^{15}N_2$ labeling experiments were conducted in Xiaoji Town, Jiangdu City, Jiangsu Province, China (32°35′N, 119°42′E) from July 27 to October 11, 2018. Three chambers (length × width × height = 100 × 100 × 120 cm) (ITIGCN Co., Ltd., Nanjing, China) were installed in a flooded rice field. The temperature and CO_2 concentration in the chambers were automatically controlled according to ambient air temperature (ambient temperature ± 2 °C) and CO_2 concentration (400 ± 20 ppm). Excessive O_2 generated by the rice photosynthesis in the chambers was removed using an oxygen absorber composed of iron powder, salt, and water (RUI-KOCH Desiccant Co., Ltd., Shenzhen, China). The detailed design and control system of the chamber were described by Bei *et al.* (2013) and Zhang *et al.* (2021). Three chambers were installed, and 27 pots (length × width × height = 9 × 9 × 20 cm) were placed randomly in each growth chamber. Air-dried soil was filled into each pot to a depth of 15 cm, according to their bulk density (Table I). The soils were submerged with 1–2 cm of water above the soil surface for two weeks before seedling transplantation. One seedling of two-month-old rice (*Oryza sativa* L., Wuyunjing 23) was transplanted into each pot from an adjacent rice field. Then, 116.37 mg KH_2PO_4 and 32.55 mg KCl (equivalent to 70 kg of P_2O_5 ha^{-1} and 70 kg of K_2O ha^{-1}, which is the commonly applied amount in local rice production) were added to each pot before transplantation, while N fertilizer was not applied. After transplanting for 10 days, approximately 40 L of air in the $^{15}N_2$-labeling chamber was replaced with enriched $^{15}N_2$ (approximately 95 atoms% ^{15}N), as described by Bei *et al.* (2013). The ^{15}N enrichment of N_2 within the labeling chamber was constantly monitored during the 77-day labeling period (Fig. S1). The soil and plant samples in the growth chambers were sampled after 77 d of labeling. The rice plants were separated into two parts: aboveground and underground. The soil in each pot was sectioned from the top at two intervals (0–1 cm and 1–15 cm). A subsample of soil and plant samples was then dried and ground using a Retsch MM 400 mixer mill (Retsch, Haan, Germany), and analyzed for total nitrogen (TN) content and ^{15}N-enrichment using a Thermo Finnigan Delta plus Advantages Mass Spectrometer coupled with an elemental analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Calculations and statistical analysis

The amount of BNF was calculated as follows:

$$^{15}N_{excess\ sample}(\%) = {}^{15}N_{chamber\ sample}(\%) - {}^{15}N_{ambient\ sample}(\%)\ ;$$

$$^{15}N_{excess\ gas}(\%) = {}^{15}N_{chamber\ gas}(\%) - {}^{15}N_{ambient\ gas}(\%)\ ;$$

$$\%N_{fixed} = \frac{^{15}N_{excess\ sample}(\%)}{^{15}N_{excess\ gas}(\%)} \times 100;$$

$$BNF(mg/pot) = \sum_{i=1}^{n} \{N_{i\ sample} \times \%N_{fixed}\};$$

$$BNF(kg/ha) = \frac{BNF(mg/pot)}{0.09 \times 0.09} \times 0.01,$$

where $\%N_{fixed}$ is the percentage of N dirived from BNF, $N_{i\ sample}$ is the amount of N in rice plants (aboveground and underground) and soil (0–1cm and 1–15cm) in the $^{15}N_2$-labeled chamber.

We used rice N uptake per unit of soil to estimate available soil nitrogen (ASN). ASN was calculated as:

$$ASN = \frac{N_a \times M_a + N_u \times M_u}{V \times BD}$$

where V is the volume of soil in each pot; BD represents the bulk density of soil; N and M represent the N concentration and biomass, respectively; and the subscripts a and u represent the aboveground and underground parts of rice, respectively. Information on the ASN and rice biomass is shown in Table S1.

The normality of log-transformed BNF and rice biomass was tested using the Shapiro-Wilk method and visualized using a normal Q-Q plot (Royston, 1982). Pairwise correlation of soil properties was analyzed by "corr.test" function with no adjustment for multiple tests and visualized by "corrplot" function (Wei and Simko, 2017; Revelle, 2018). The regression analysis of BNF and soil properties was conducted by the "lm" function and visualized by the "ggplot2" package (Wickham, 2016; R Core Team, 2022). The significance level of *P value* was set at 0.05. The parameters of our model were estimated by minimizing the residual sum of squares using a Newton-type algorithm (Schnabel *et al.*, 1985). The relative importance of edaphic factors was calculated by the "relaimpo" package (Grömping, 2007). The limiting thresholds of edaphic factors were determined by minimizing the *p-values* of t-test of ln(BNF) between soils above and below thresholds. All data and R code supporting the results are available from the figshare: https://doi.org/10.6084/m9.figshare.20453658.v3

RESULTS

The BNF amounts measured by $^{15}N_2$ labeling incubation for 77 days ranged from 0.66 to 12.36 kg·ha^{-1} (Fig. 3a). The mean and median of BNF amounts were 3.64 and 2.97 kg·ha^{-1} respectively. In contrast to the normal distribution of rice biomass, BNF followed a lognormal distribution (Fig. 3b, c).

Fig. 3

Fig. 3 BNF amounts measured by $^{15}N_2$ labeling incubation for 77 days until the rice was ripe (a), and normal Q-Q plots for log-transformed BNF (b) and biomass of rice (c). *P* means the probability that the distribution is normal.

Considering the lognormal distribution of BNF amounts, BNF amounts were transformed using the logarithmic method before regression analysis. The BNF had a significant positive linear relationship with pH, available phosphorus (AP), and bulk density (Fig. 4a, c, e). Significant quadratic relationships were observed between the BNF amounts and total nitrogen (TN), available Mo (AMo), and organic carbon (OC) (Fig. 4b, d, f). BNF amounts had a significant positive correlation with the ratio of CEC to available soil nitrogen (ASN) (Fig. 4g), but no significant relationship was observed between BNF amounts and CEC, and between BNF amounts and ASN.

Fig. 4

Fig. 4 Statistical analysis of regression between BNF amounts with pH (a), total nitrogen (b), available phosphorus (c), available molybdenum (d), bulk density (e), organic carbon (f), and the ratio of CEC to available soil nitrogen (g).

BNF amounts were driven multiplicatively by AP, AMo, TN, ASN and CEC. $BNF = (1.64 \cdot AP + 61.06) \cdot \frac{1}{\exp(6.04 \cdot ASN - 0.05 \cdot CEC + 9.79) + 1} \cdot (-19.56 \cdot AMo^2 + 20.43 \cdot AMo + 3.80) \cdot (-23.64 \cdot TN^2 + 98.74 \cdot TN + 23.67)$, which could explain 86% of BNF variation, and the residues follow a normal distribution (Fig. 5a). Among the driving factors, AP contributed 42%, CEC and ASN contributed 28%, TN contributed 17%, and AMo contributed 13% to the BNF (Fig. 6a). Removing any one of them would make the R^2 lower than 0.80 (Figure 6a). Adding factors (pH, OC, TP, TK, TFe, TMo, AK, AFe, and Clay) did not increase the R^2 (Fig. 6b). Adding factors (bulk density, water holding capacity, silt, and sand) slightly increased R^2, but the increase was less than or equal to two percentage points (Fig. 6b).

Fig. 5

Fig. 5 Linear regression analysis of predicted BNF and measured BNF (a), and Normal Q-Q plots for residues (b). R^2 means the explained proportion, and *P* means the probability that the distribution of residues is normal.

Fig. 6

Fig. 6 Effect of removing factors (AMo, TN, ASN, CEC, AP) (a) and adding factors (pH, OC, TP, TK, TFe, TMo, AK, AFe, and Clay, bulk density, water holding capacity, silt, and sand) (b) on R^2. "All" means the variables that had been selected, namely AP, CEC, ASN, TN, and AMo. "-Varible" means deleting a variable from the model, "+Variable" means adding a variable to the model. The abbreviations are as follows: TN = total nitrogen, OC = organic carbon, TP = total phosphorus, TK = total potassium, TMo = total molybdenum, AP = available phosphorus, AK = available potassium, AFe = available iron, AMo = available molybdenum, CEC = cation exchange capacity, BD = bulk density, Clay = the percentage of clay content, Silt = the percentage of silt content, Sand = the percentage of clay content, WHC = water holding capacity, ASN = available soil N.

The limiting thresholds of AP, AMo and TN were 14 $mg \cdot kg^{-1}$, 0.09 $mg \cdot kg^{-1}$ and 3.2 $g \cdot kg^{-1}$, respectively. When AP was lower than 14 $mg \cdot kg^{-1}$ or AMo lower than 0.09 $mg \cdot kg^{-1}$, increasing AP or AMo significantly increased BNF amounts (Fig. 7a, b). When TN was higher than 3.2 $g \cdot kg^{-1}$, BNF amount was significantly limited (Fig. 7c).

Fig. 7

Fig. 7 Thresholds of AMo (a), AP (b) and TN (c) to limit BNF. AMo, available molybdenum; AP, available phosphorus; TN, total nitrogen.

DISCUSSION

The BNF amounts estimated at the end of a 77-day incubation ranged from 0.66 to 12.36 $kg \cdot N \cdot ha^{-1}$ (9–160 $g \cdot N \cdot ha^{-1} \cdot day^{-1}$, Fig. 3a). The mean and median of BNF amounts was 3.64 and 2.97 $kg \cdot N \cdot ha^{-1}$ (47 $g \cdot N \cdot ha^{-1} \cdot day^{-1}$ and 39 $g \cdot N \cdot ha^{-1} \cdot day^{-1}$) respectively. These BNF amounts were lower than those in previous studies using the same $^{15}N_2$ incubation measurement technology, 45 $kg \cdot N \cdot ha^{-1}$ (643 $g \cdot N \cdot ha^{-1} \cdot day^{-1}$) (Bei *et al.*, 2013), and 22 to 39 $kg \cdot N \cdot ha^{-1}$ (297 to 527 $g \cdot N \cdot ha^{-1} \cdot day^{-1}$) depending on the rice cultivar grown (Ma *et al.*, 2019b), and similar to 2.2 to 20.1 $kg \cdot N \cdot ha^{-1}$ (46–419 $g \cdot N \cdot ha^{-1} \cdot day^{-1}$, mean 186 $g \cdot N \cdot ha^{-1} \cdot day^{-1}$) across four different soil types (Wang *et al.*, 2019). The lower BNF amounts in this study might be due to the smaller pot size. The pot used in this study was 9×9 cm^2 which is smaller than Bei *et al.* (2013), Ma *et al.* (2019b), and Wang *et al.* (2019). The smaller pot size decreased BNF by reducing root respiration and exudates through hindering rice growth (Bei *et al.*, 2013; Wang *et al.*, 2020). Furthermore, the smaller pot size caused a higher planting density of rice, which can block sunlight and repress the growth of phototrophic diazotrophs, which are the main contributors to BNF in paddy soils (Wang *et al.*, 2020). Considering the important role of rice planting in paddy BNF, keeping pot-grown rice plants resembling field-grown plants is important for estimating the BNF in the field.

While considering the effects of edaphic factors on BNF, previous studies have broadened our knowledge about the effects of single factors (such as **Mo**, **P**, and **N** availability) on BNF (Reed *et al.*, 2011). Our results showed that BNF amounts were determined by edaphic factors AP, CEC, ASN, AMo and TN in the form of multiplication, namely BNF amounts = f_1(available P) × f_2(ASN, CEC) × f_3(AMo) × f_4(TN), where f_1 was a linear function, f_2 was a logistic function, and f_3 and f_4 were quadratic functions. These factors could explain 86% of the variation of BNF amounts. The multiplicative effects of edaphic factors resulted in the lognormal distribution of BNF amounts rather than a normal distribution. Log-normal distribution of BNF in the ocean has also been observed (Tang *et al.*, 2019). These results highlight the multiplicative effects of edaphic factors on BNF amounts, suggesting that multiple improving approaches for different restrictions would be more efficient in enhancing BNF than a single approach, and the benefit might be multiplicative.

Although phosphorus was fertilized in our study, AP (measured before the experiment) still showed significant positive effects on BNF amounts and was the primary edaphic factor driving paddy BNF amounts (Fig. 4c and Fig. 6a). AP has also been reported as a driver in the ocean, where photosynthetic **N** fixers dominate the BNF (Tang *et al.*, 2020). In contrast to heterotrophic diazotrophs, which are often limited by the availability of carbon compounds (Buresh *et al.*, 1980), photosynthetic **N** fixers, the major contributor to paddy BNF (Bei *et al.*, 2013; Wang *et al.*, 2020), are often limited by phosphorus (Buresh *et al.*, 1980). Applied phosphorus can stimulate **N** fixation in rice soils (Tang *et al.*, 2017). We found that when AP was lower than 14 $mg \cdot kg^{-1}$, the BNF amount was significantly limited (Fig. 7b). Phosphorus fertilization is an efficient way to improve BNF, especially in paddy soils with AP lower than 14 $mg \cdot kg^{-1}$.

Wang *et al.* (2019) conducted a similar experiment to determine the driving factors of paddy BNF and found that paddy BNF was mainly determined by Al oxides but not AP. Aluminum compounds can be used as algaecides to suppress cyanobacteria growth (Jančula

and Maršálek, 2011). As shown in Fig. 1, if aluminum oxides kill diazotrophic cyanobacteria, the positive effects of available phosphorus cannot be brought about. In addition, the AP ranged from 24 to 41 $mg \cdot kg^{-1}$ in Wang *et al.* (2019), while 2–71 $mg \cdot kg^{-1}$ in this study. AP in Wang *et al.* (2019) had less variation than that in this study and was higher than the limiting threshold of 14 $mg \cdot kg^{-1}$ detected in this study. Therefore, the effect of AP cannot be displayed in Wang *et al.* (2019). Wang *et al.* (2019) suggested that pH affects BNF indirectly by affecting the activity of aluminum. The solubility of Al increases with a decrease in pH, in acidic soils ($pH \leq 5.5$), the mineral forms of Al can dissolve and release Al ions into the soil solution (Zhou *et al.*, 2011). Acidic soils with a pH <5.5 account for 50% of all samples in Wang *et al.* (2019), and 22% in this study. The importance of Al oxides and pH was diluted in this study. We also found a positive relationship between pH and BNF (Fig. 4), and a positive correlation between pH and AP (Fig. S2). Therefore, in this study, pH indirectly affected the BNF by affecting the AP instead of Al oxides in Wang *et al.* (2019). Although the detected driving factors differ between this study and that of Wang *et al.* (2019), the potential microbial mechanisms might be similar. Aluminum oxides inhabit paddy BNF by repressing diazotrophic cyanobacteria (Wang *et al.*, 2019), and the AP can improve paddy BNF by promoting diazotrophic cyanobacteria.

Available soil N (ASN) was a secondarily important edaphic factor driving paddy BNF (Fig. 6a). The low rate of N fertilizer (5.64 $mg \cdot N \cdot kg^{-1}$ soil) does not depress BNF, while a high rate of N fertilizer (99.72 $mg \cdot N \cdot kg^{-1} \cdot$soil) eliminates BNF (Santiago-Ventura *et al.*, 1986). The repressed rate of N fertilizer on BNF is 86.15% at 250 $kg \cdot ha^{-1}$ and 83.21% at 125 $kg \cdot ha^{-1}$ (Zhang *et al.*, 2021). These results indicate that the effect of ASN on BNF is non-linear. Logistic function transforms continuous values to the range 0–1 and has an S shape. Therefore, logistic function was used to model the response of BNF to ASN in this study. No significant relationship was observed between BNF amount and ASN, but BNF amount had a significant positive relationship with the ratio of CEC to ASN (Fig. 4g). ASN was estimated by rice N uptake in this study, so ASN can represent the ability of the soil to supply N to rice. However, there might be some differences between N availability to rice and to diazotrophs. Ammonium can be adsorbed in the negatively charged sites of soils (Brady *et al.*, 2008). The adsorbed ammonium might still be available to rice, but not to diazotrophs. The number of negatively charged sites that can adsorb positively charged ions is measured by CEC (Brady *et al.*, 2008). When CEC is higher in the soil, a greater amount of free ammonium may be fixed (Nieder *et al.*, 2011). CEC can represent the soil's ability to adsorb ammonium N. Therefore, the ratio of CEC to ASN represents the tradeoff between absorbing and supplying ammonium to diazotrophs. The BNF amount was positively correlated with the ratio of CEC to ASN instead of ASN or CEC separately. This result implies that a higher CEC may reduce the negative effect of ammonium N on BNF. Increasing soil CEC, such as using biochar (Domingues *et al.*, 2020), might efficiently increase BNF under N fertilization conditions, especially in highly weathered and sandy soils.

BNF amounts had a significant quadratic relationship with soil total **N** (Fig. 4b). This quadratic relationship might be attributed to the balance between diazotrophs growth and the preference for obtaining **N** from the soil instead of atmosphere with the increase of soil total N. The soil total N is positively correlated with the number of diazotrophs (Han *et al.*, 2019). When soil total N is too low, there may be not enough diazotrophs to fix atmospheric N, resulting in a positive correlation between soil total N and BNF. In addition to BNF, diazotrophs can obtain N by secreting exoenzymes to decompose soil organic **N** into biologically available **N** (Norman and Friesen, 2017). The benefit of producing exoenzymes would be greater for diazotrophs in soils with higher total N concentrations. Therefore, when soil total N is too high, BNF might be limited by the preference of diazotrophs for obtaining **N** from the soil but not the atmosphere. The optimal soil total N concentration for BNF in paddy fields was 2.1 $g \cdot kg^{-1}$ (Fig. 4b). Excessive soil total N decreased the BNF amount. In particular, when TN was higher than 3.2 $g \cdot kg^{-1}$, BNF amounts were significantly limited (Fig. 7c).

BNF amount had a significant quadratic relationship with available Mo, and 0.5mg·kg^{-1} available Mo was the optimal concentration for BNF in paddy fields (Fig. 4d). Mo limitation have been widely reported across ecosystems (Barron *et al.*, 2009; Rousk *et al.*, 2017; Ma *et al.*, 2019a). The deficient threshold of available Mo is 0.09 mg·kg^{-1} in soils for paddy BNF (Fig. 7a), which is lower than 0.15 mg·kg^{-1} in soils for legume BNF (Zou *et al.*, 2008). This may be because the Mo demand of the asymbiotic BNF was lower than that of the symbiotic BNF. Mo application can increase BNF (Ma *et al.*, 2019a), but when available Mo exceeds a certain value, there might be a toxic effect on diazotrophs (Fig. 4d). Compared to 1kg of Mo ha^{-1}, the application of 10 kg of Mo ha^{-1} resulted in lower nodule weights per plant (Jabbar *et al.*, 2014). The Mo application to seeds with *Bradyrhizobium* inoculation, reduced *Bradyrhizobium* survival, nodulation, and fixation efficiency (Albino *et al.*, 2000). Mo application has been recommended as an approach to improve BNF in paddy soils (Ma *et al.*, 2019a); however, the application rate of Mo needs to be strictly controlled within an appropriate range to avoid toxic effects on BNF.

CONCLUSIONS

Our study provides a systematic framework for how BNF amount responds to multiple edaphic factors in paddy soils. The paddy BNF amount was driven multiplicatively by AP, ASN, CEC, TN, and AMo. AP contributed 42%, ASN and CEC 28%, TN 17%, and AMo 13% to the BNF amount. BNF amount was significantly limited when AP was lower than 14 mg·kg^{-1}, or AMo was lower than 0.09·mg kg^{-1}, TN higher than 3.2 g·kg^{-1}. To balance rice production and environmental protection, China must reduce its reliance on synthetic **N** and increase the input of BNF (Ladha *et al.*, 2022). This study provides valuable benchmarks that can be used to guide farmers in managing their soils to improve the potential contribution of paddy BNF to soil fertility.

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SUPPLEMENTAL MATERIAL

Supplementary materials for this article can be found in the online version. Supplementary materials include two parts: Supplementary Tables and Figures, and Supplementary Materials and Methods. The operation of the $^{15}N_2$-labeling growth chamber during the entire experimental period is shown in Fig. S1. The pairwise correlation of the soil properties is shown in Fig. S2. Available soil N and rice biomass are listed in Table S1. A more detailed description of how paddy BNF was measured is presented in the Supplementary Materials and Methods.

CONTRIBUTION OF AUTHORS

Xie Zubin conceived the idea and designed the experiment. Hu Tianlong, Zhang Yanhui, Jin Haiyang, Lin Zhibin, Liu Qi, Ma Jing, Wang Xiaojie and Lin Xingwu collected and treated soil samples. Zhang Yanhui and Hu Tianlong conducted the

experiment for BNF measurement with the assistance of Wang Hui and Liu Hongtao. Gang Liu and Jianguo Zhu gave technical guidance for setting up the control system of $^{15}N_2$-gowth-chamber. Hu Tianlong conducted the data analysis and wrote the first draft. Chen Zhe, Zhou Rong and Jin Penghui helped perform the analysis with constructive discussions. Xie Zubin finalized the manuscript. All authors commented on and revised the paper.

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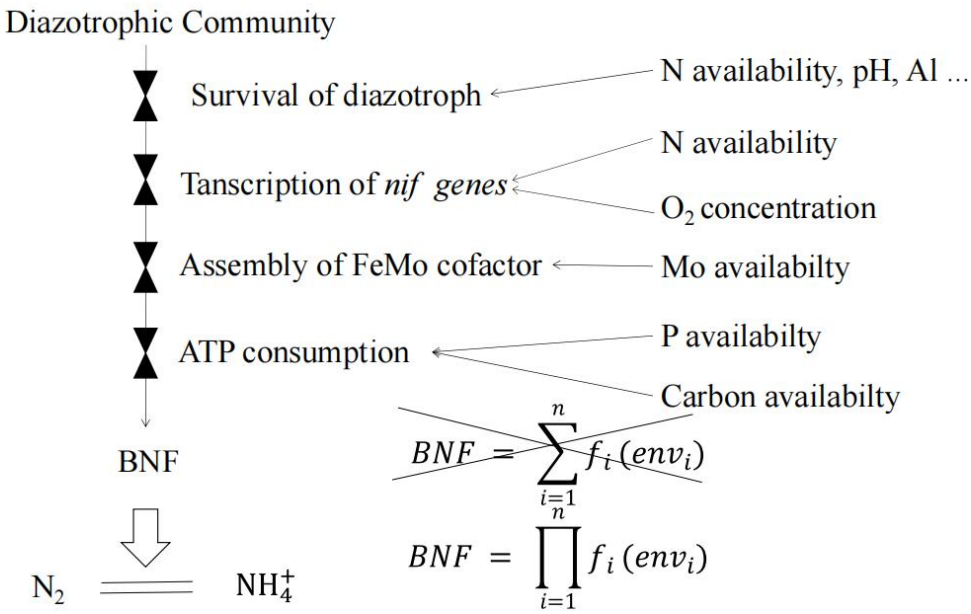
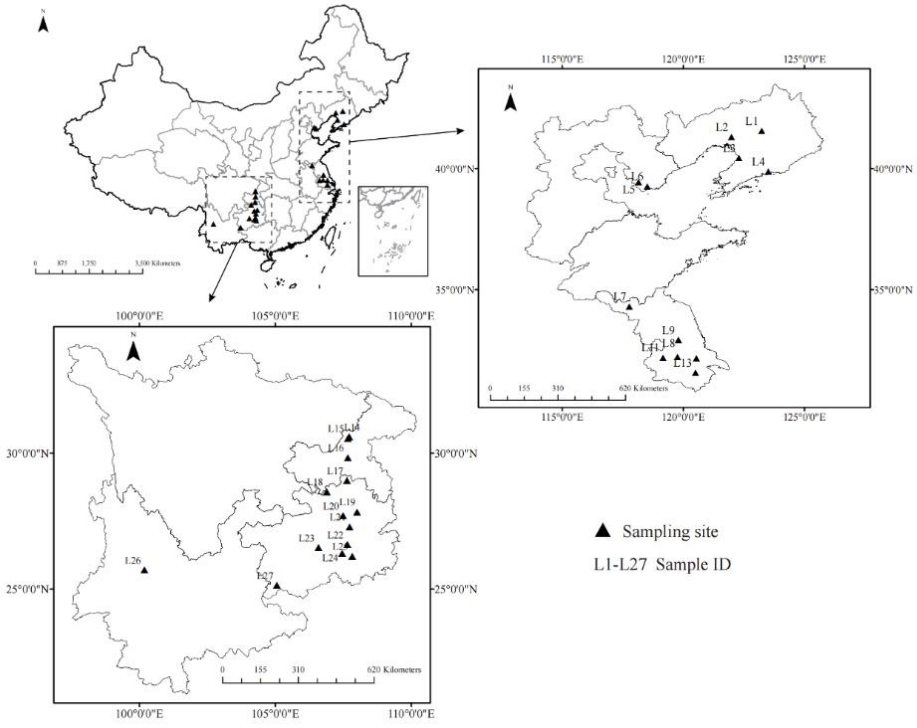


539
540 Fig. 1
541



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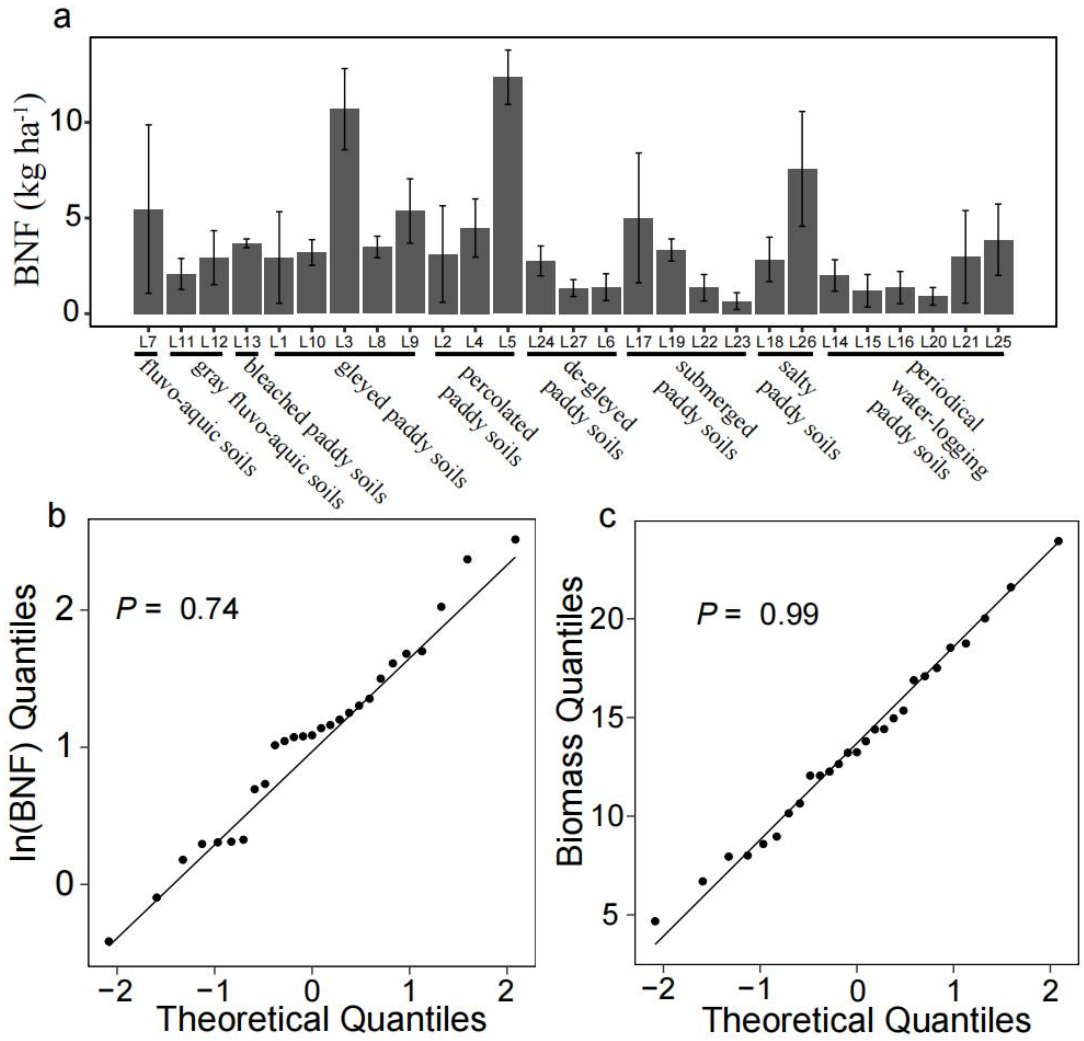
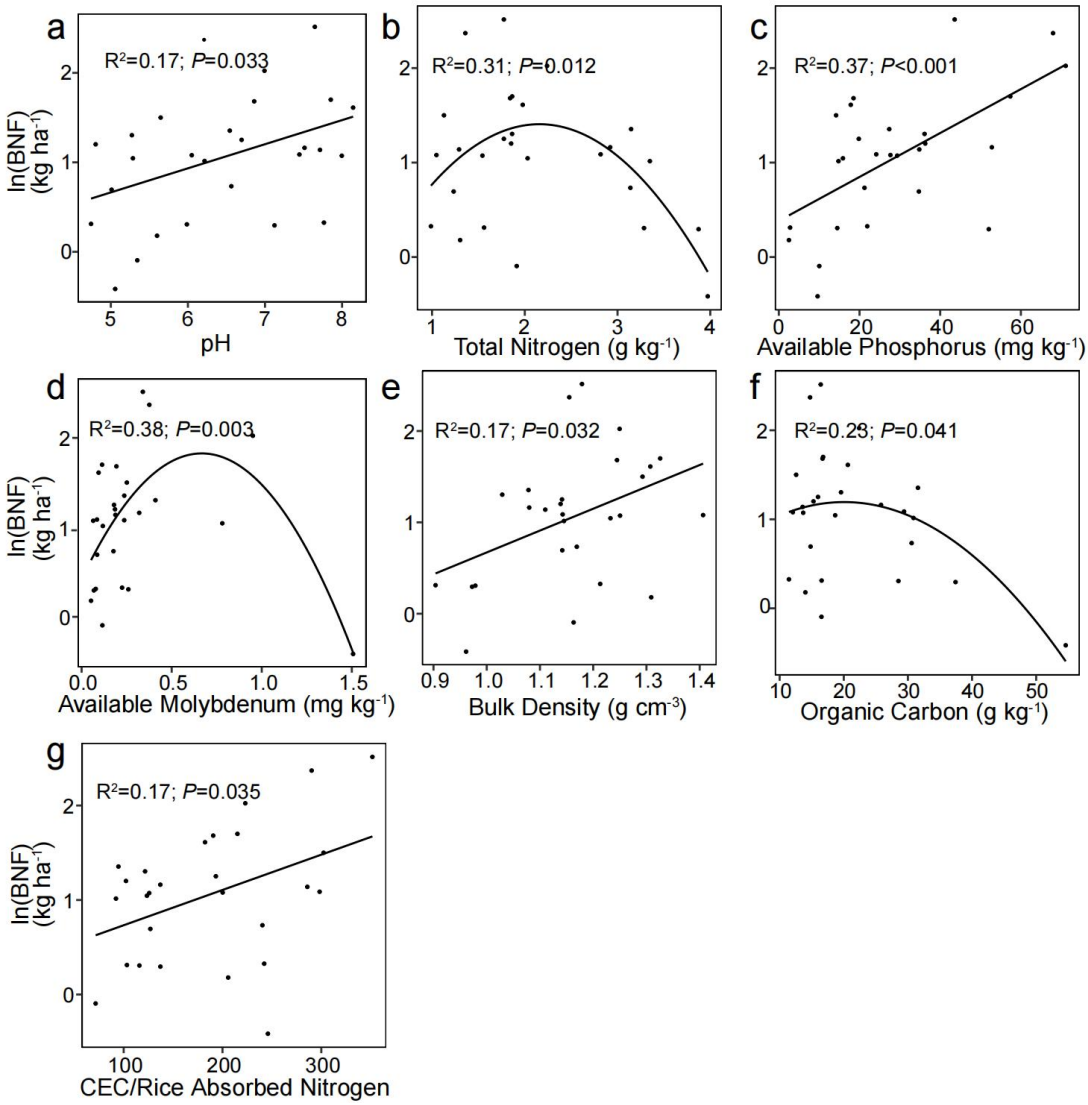
543 Fig. 2


a
BNF (kg ha-1)
0
5
10
L7 L11 L12 L13 L1 L10 L3 L8 L9 L2 L4 L5 L24 L27 L6 L17 L19 L22 L23 L18 L26 L14 L15 L16 L20 L21 L25
fluvo-aquic soils
gray fluvo-aquic soils
bleached paddy soils
gleyed paddy soils
percolated paddy soils
de-gleyed paddy soils
submerged paddy soils
salty paddy soils
periodical water-logging paddy soils
b
P = 0.74
ln(BNF) Quantiles
Theoretical Quantiles
c
P = 0.99
Biomass Quantiles
Theoretical Quantiles

544

545 Fig. 3



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547 Fig. 4

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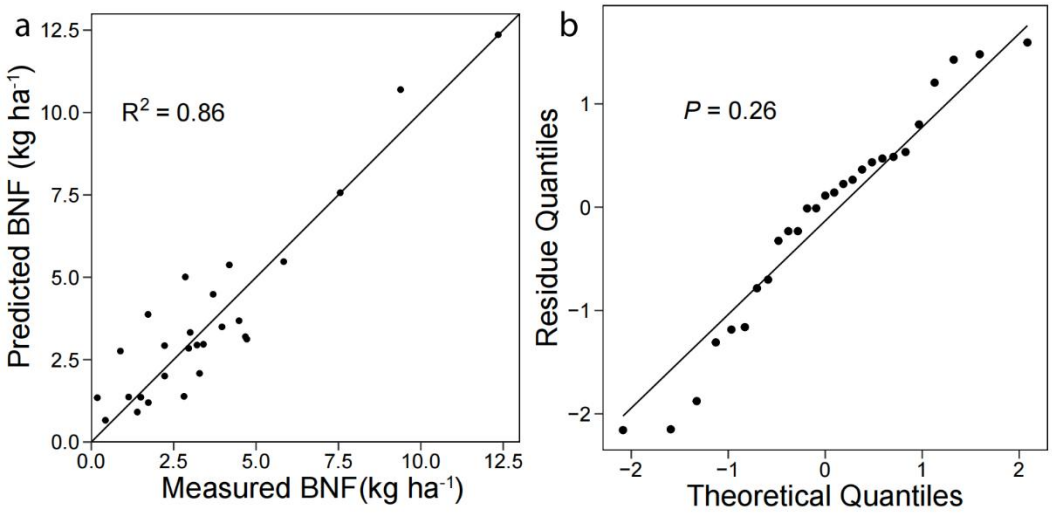

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550 Fig. 5

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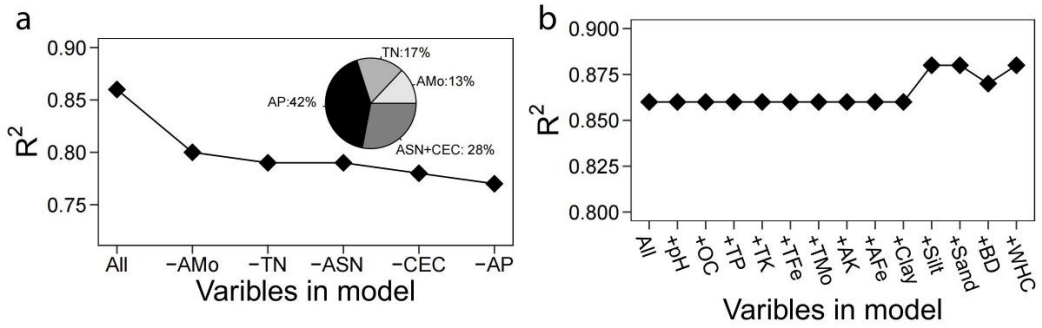

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553 Fig. 6

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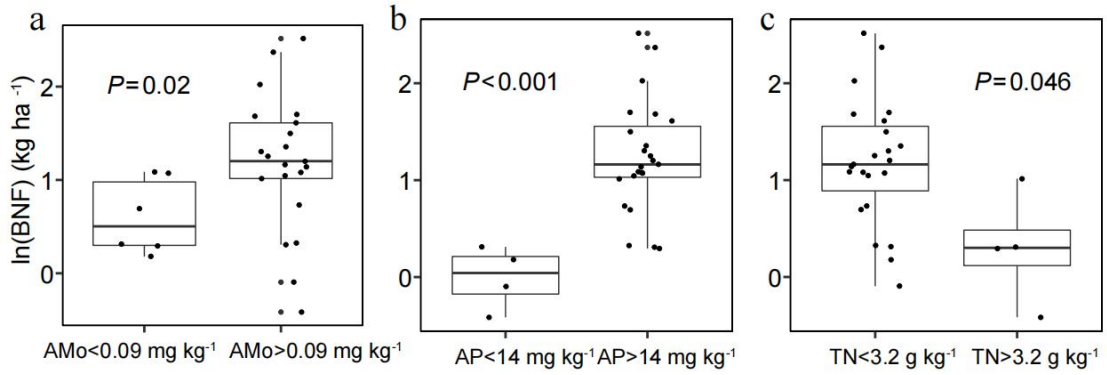

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556 Fig. 7

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