Long-Term Straw Return Increases Biological Nitrogen Fixation by Increasing Soil Organic Carbon and Decreasing Available Nitrogen in Rice–Rapeseed Rotation

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Research Article

Keywords: Straw return, Soil depth, Potential nitrogen fixation rate, Nitrogen-fixing bacteria, Rice–rapeseed rotation, nifH gen

Posted Date: January 17th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1253413/v1>

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Abstract

Background

Straw return can affect the soil nitrogen (N) balance by changing soil carbon (C) and N components and contents. To understand how straw return affects soil N fixation capacity, relations between biological N fixation and changes in soil C and N contents at different depths need to be examined.

Method

Soil samples were collected at 20-cm intervals from 0 to 100 cm after 5 years of a rice–rapeseed rotation system with straw return (ST) and without (CK). Organic C and inorganic N contents, potential N fixation rate, and abundance and population composition of N-fixing bacteria were analyzed at different soil depths.

Results

Compared with CK, ST increased soil organic C (SOC), particulate organic C (POC), mineral-associated organic C (MOC), and total N (TN) but decreased nitrate-N (NO_3^- -N). Straw return also increased abundance of N-fixing bacteria 2.9 to 18.2-fold at depths from 0 to 40 cm and potential N fixation rate at 0–20 cm by 24.78%, which was equivalent to an annual increase in N fixation of approximately 0.01 to 0.54 $\text{kg}\cdot\text{ha}^{-1}$. Long-term ST significantly affected the composition of N-fixing bacteria at depths from 0 to 40 cm.

Conclusions

Long-term straw return can effectively increase potential N fixation rate in soil and change the structure of the N-fixing bacterial community by increasing SOC and decreasing N availability. The experiment showed that SOC, POC, TN, and NO_3^- -N were the main environmental factors driving changes in abundance, activity, and population composition of soil N-fixing bacteria under ST.

Introduction

Crop straw is an important agricultural resource because it is a rich source of organic carbon (C) and nutrients such as nitrogen (N), phosphorus (P), and potassium (K). In China, output of crop straw reached 1.15 billion tonnes in 2019, accounting for nearly one-third of the global output (Li et al., 2018, China Statistical Yearbook, 2020). Straw return is an important way to maintain and increase soil organic C (SOC) and is currently the most common method of using straw resources in China (Huang et al., 2019). Returning straw to the field can significantly improve soil physical properties (Zhang et al., 2017), promote fixation of SOC and N (Liu et al., 2014, Said-Pullicino et al., 2014), and change abundance, composition, and activity of soil bacterial populations (Guo et al., 2017). Thus, straw return has become one of the main types of human interference with soil C and N cycles in agroecosystems.

In farmland ecosystems, atmospheric N_2 can be converted into inorganic N by lightning or by soil N-fixing bacteria. The soil inorganic N can be directly absorbed by plants, converted into nitrite and nitrate by soil nitrifying bacteria before plant uptake, or converted into N_2O or N_2 by soil denitrifying bacteria (Kuypers et al., 2018). Soil N-fixing bacteria can fix 60 to 100 million tonnes of N annually in farmlands and are one of the main sources of N in agroecosystems (Galloway et al., 2008). Nitrogen-fixing bacteria are an essential functional group in agroecosystems and include free-living, associated, and symbiotic N-fixing bacteria (Harindintwali et al., 2021). Free-living N-fixing bacteria such as *Azotobacter* and *Bacillus* can independently fix N, whereas associated N-fixing bacteria such as *Enterobacter* and *Arthrobacter* often live in the rhizosphere of plants. Symbiotic N-fixing microbes such as *Rhizobium* and *Nostoc* can only fix N in symbiosis with higher plants or other organisms (Ferreira et al., 2019). Ladha et al. (2016) assessed a 50-year global N budget and found that 80% of the N in rice was from biological N fixation. Under corn and wheat, up to 13 $\text{kg}\cdot\text{ha}^{-1}$ N was fixed in soil each year, of which nonsymbiotic N-fixing microbes, i.e., free-living and associated, fixed approximately 50%. Nonsymbiotic N-fixing microbes are important in meeting the high N demand of plants and balancing N loss (Chalk et al., 2017). The *nifH* gene is a genetic marker of N-fixing bacteria because it encodes the conserved subunit of the dinitrogenase iron protein (Lema et al., 2014). The gene also has one of the largest nonribosomal gene data sets for uncultured microorganisms, and thus, it is widely used in studies of soil N-fixing bacteria (Yu et al., 2019).

Biological N fixation is an energy-consuming process, and it is sensitive to changes in soil environmental factors, especially SOC (Fan et al., 2018) and inorganic N (Calderoli et al., 2017). Long-term (20 years) straw return increases SOC and soil N reserves, providing energy for N-fixing bacteria and enhancing N mineralization, thereby increasing the abundance of *nifH* genes 1 to 1.5-fold (Tang et al., 2017; Che et al., 2018; Xia et al., 2021) and N fixation activity in topsoil 59–68% (Tang et al., 2017). However, after five consecutive years, straw return did not significantly affect N fixation rate or *nifH* gene copy number in topsoil of rice, because it increased soil N content and therefore N availability (Liao et al., 2018). Nitrogen-fixing bacteria could preferentially use exogenous N for vegetative reproduction, and therefore, reduced the energy-consuming N fixation (Fan et al., 2018). Thus, straw return can have different effects on soil N fixation, likely related to variation in SOC composition and N content.

Effects of straw return on SOC and N contents are closely related to factors such as amount of straw returned, number of years straw is returned, and depth of the soil layer. In summer maize–winter wheat rotation systems with long-term straw return, SOC, mineral-associated organic C (MOC) (Zhao et al., 2018), and dissolved organic C (DOC) in the 0–20- and 20–40-cm layers (Lu et al., 2021) increased by 15–26%, 13–31%, and 15–20%, respectively. However, He et al. (2019) reported that after 9 years of straw return, SOC increased significantly only in the 0–10-cm soil layer, and the 10–50-cm layer was not affected. In addition, Gai et al. (2019) found that after returning wheat straw to the field for 27 years, total N (TN), dissolved organic N (DON), and microbial biomass N (MBN) increased significantly at 0–20 cm. However, effects of straw return were limited in deeper soil from 40 to 200 cm. Differences in effects of straw return on organic C and N contents in different soil layers likely affect the intensity of biological N fixation in different soil layers. Currently, most studies focus primarily on the effects of straw return on soil N fixation in the 0–20-cm layer (Rahav et al., 2016; Wang et al., 2017; Yang et al., 2018; Wang et al., 2019), and as a result, effects of long-term straw return on N fixation in deep soil layers have not been well documented. Such information is important in order to better understand N transformations in different soil layers with straw return and to effectively guide nutrient management of crops, especially those crops with more developed root systems, such as rapeseed and wheat. In addition, effects of SOC and N on N-fixing bacterial populations have not been quantified.

Therefore, a five-year experiment was conducted to determine the effects of straw return on SOC and N contents at depths from 0 to 100 cm. Potential N fixation rate and *nifH* copy number and composition of N-fixing bacterial populations were determined at depths from 0 to 60 cm. The objective was to reveal relations between C and N in different soil layers and biological N fixation potential. Redundancy and variance partition analyses were used to quantify contributions of soil C and N factors to variation in N-fixing bacterial populations.

Materials And Methods

Experimental design

The experiment was conducted in Huangbilong Village (29.02°N, 119.43°E), Jiangtang Township, Wucheng District, Jinhua City, Zhejiang Province, China, from June 2015 to May 2020. The area has a subtropical monsoon climate, with an annual average temperature of 17.3°C to 18.2°C and annual precipitation of 1,109.0 to 1,305.2 mm. A rice–rapeseed rotation system was used. The growing season of rice was from June to October, whereas that of rapeseed was from November to May of the following year. The soil type was loam soil. Before the experiment, topsoil (0–20cm) chemical properties were the following: pH 6.55, organic matter 32.1 g·kg⁻¹, TN 1.24 g·kg⁻¹, available N (AN) 193.39 mg·kg⁻¹, available P 5.98 mg·kg⁻¹, and available K 72.5 mg·kg⁻¹.

Plots in the experiment were 2.7 m × 7.5 m. There were two treatments: CK (i.e., without straw return) and straw return (ST). There were three plots per treatment, for a total of six plots. Treatments were randomly distributed among the plots. In ST, average rice straw returned to the field was 8,100 kg·ha⁻¹, whereas rapeseed straw input was approximately 6,150 kg·ha⁻¹. The depth of returned straw was 20 cm. In the rice season, N fertilizer amount was 270 kg·ha⁻¹, with 40% applied as basal fertilizer, 30% as tiller fertilizer, and 30% as ear fertilizer. Potassium fertilizer (K₂O) amount was 108 kg·ha⁻¹, of which 50% was tiller fertilizer and 50% was ear fertilizer. Phosphate fertilizer (P₂O₅) was applied at 67.5 kg·ha⁻¹ as base fertilizer. In the rapeseed season, N fertilizer was applied one time at 51.75 kg·ha⁻¹ as base fertilizer. Potassium fertilizer amount was 112.5 kg·ha⁻¹, with 40% applied as basal fertilizer and 60% as topdressing. Other field management measures were consistent with local practices.

Soil sample collection

After harvest in May 2020, the five-point sampling method was used to collect soil cores (4-cm diameter) from each plot at five different depths (0–20, 20–40, 40–60, 60–80, and 80–100-cm). After removing debris and plant residues, soil samples from the same soil layer at the five sample points in each plot were mixed evenly. One portion of the samples was stored at 4°C in a refrigerator and used to

measure DOC, microbial biomass C (MBC), MBN, ammonia N ($\text{NH}_4^+\text{-N}$), nitrate N ($\text{NO}_3^-\text{-N}$), and potential N fixation rate (PNFR). A second portion was stored at -20°C until DNA extraction. The remaining portion was air-dried and screened through a 2-mm mesh to determine SOC, TN, AN, and particulate organic carbon (POC). Mineral-associated organic C was determined by subtracting POC from total SOC.

Soil chemical properties

Dissolved organic C was measured by the deionized water extraction method (Haynes and Francis, 1993). Microbial biomass C and MBN were extracted by the chloroform fumigation–extraction method (Vance et al., 1987). Particulate organic C was measured by sodium hexametaphosphate dispersion (Cambardella and Elliott, 1992). Before measurement of SOC and TN, soil was air-dried and ground to pass through a 0.15-mm sieve. An automatic element analyzer (Vario Isotope Cube, Elementar, Jena, Germany) was used to measure SOC and TN (Nelson and Sommers, 2005). Soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ were extracted with $2\text{ mol}\cdot\text{L}^{-1}$ KCl and measured on an AA3 continuous flow analyzer (Auto Analyzer 3, Bra+Luebbe, Hamburg, Germany) (Liu, 1996). Available N content was determined by the alkaline solution diffusion method after samples were air-dried, ground, and sieved (Page et al., 1982).

Measurement of potential nitrogen fixation rate

According to the steps in Hsu and Buckley (2009), ^{15}N labeling was used to determine PNFR. First, 5.0 g of fresh soil sample was put in a 100-mL serum bottle, and soil water content was adjusted to 60% of field water holding capacity. The bottle was vacuumed and then filled with 20% O_2 (v/v) and 80% $^{15}\text{N}_2$ (v/v). This process was repeated three times for each sample. Unlabeled N_2 was used in the control. Samples were incubated for 7 days at room temperature in a dark room. After incubation, samples were freeze-dried and screened through a 0.15-mm sieve. Total N content and ^{15}N abundance were determined by an elemental analyzer–isotopic ratio mass spectrometer technique. Based on the difference in ^{15}N content between labeled and unlabeled samples, the PNFR ($\mu\text{g}(\text{N})\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) was calculated.

Abundance and population composition of soil nitrogen-fixing bacteria

DNA was extracted from soil samples, 1.0–1.5 g, with an E.Z.N.A.®soil DNA kit (Omega, Norcross, GA, USA). A Nanodrop 2000UV-VIS spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to determine DNA concentration and purity, which was then stored at -80°C . Sample DNA was measured using a MiSeq sequencing platform at Shanghai Majorbio Bio-pharm Technology (Shanghai, China), and the primer sequences were *nifH*F (5'-AAAGGYGGWATCGGYAARTCCACCAC-3') and *nifH*R (5'-TTGTTSGCSGCRATCATSGCCATCAT-3') (Rösch et al., 2002).

Raw sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME) website (http://qiime.org/scripts/assign_taxonomy.html) and the UPARSE platform (version 7.0.1090; <http://drive5.com/uparse/>). Standard primer sets and bar codes were excluded, and sequences with quality scores less than 20 were removed. Sequences shorter than 50 bp or those containing unresolved nucleotides were also removed. Extracting nonrepetitive sequences from the optimized sequences was convenient in reducing redundant computation in the intermediate process of analysis, and single sequences without duplication were removed. According to 97% similarity, nonrepetitive sequences (excluding single sequences) were clustered into operational taxonomic units (OTUs), with chimeras removed in the clustering process to obtain representative sequences of OTUs. A representative sequence was selected for each OTU under default parameters, and each representative sequence was assigned taxonomy using the Ribosomal Database Project (RDP) Classifier (Ou et al., 2019). Taxonomic information was obtained, and the community composition of each sample was determined at phylum and genus levels. The OTUs in each replicate were analyzed for richness and diversity. The sobs, Chao, Simpson, and Shannon indices were calculated to assess the alpha diversity of N-fixing bacteria, with sobs and Chao indices reflecting community richness and Shannon and Simpson indices estimating community diversity (Zheng et al., 2018).

Fluorescence quantitative PCR was used to determine the copy number of *nifH* genes in sample DNA, and the primers were the same as those used for sequencing. The PCR procedure was as follows: pre-denaturation at 95°C for 3 min; then, 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s; and a repair extension at 72°C for 8 min. Temperature range of the melt curve was 65°C to 95°C ; the temperature was increased in increments of 0.5°C and held for 5 s to collect data.

Statistical analyses

Statistical analyses were conducted using SPSS 20.0 software for Windows (SPSS Inc., Chicago, IL, USA). Levene's test (Lin et al., 2018) confirmed that all data met the assumptions of normality and homogeneity of variance required for ANOVA. One-way ANOVA and least

significant difference (LSD) were used to assess significance of effects of ST and soil depth on soil C and N contents, PNFR, *nifH* gene copy number, and diversity of N-fixing bacterial populations. Pearson correlations were used to test relations between different SOC and N components and PNFR, *nifH* gene copy number, and N-fixing bacteria in R with the “corrplot” package (<https://corrplot.r-forge.r-project.org>).

Differences in N-fixing bacterial populations under different treatments were analyzed using principal coordinates analysis (PCoA) based on a Bray–Curtis distance matrix in R with the “vegan” package (Oksanen et al., 2019). Variance inflation factors were computed to confirm collinearities among environmental properties. Usually, environmental properties that have variance inflation factors (VIFs) greater than 10 are of little value. Environmental properties were selected by the functions `envfit` (`permu = 999`) and `vif.cca` in R with the “vegan” package and then were removed until all VIF values were less than 10 (Ji et al., 2020) (Table S1). Redundancy analysis was performed to analyze the relations between N-fixing bacterial populations and selected soil properties (DOC, POC, TN, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$) and assess the effects of long-term straw return on composition and structure of N-fixing bacterial populations throughout the soil profile (Oksanen et al., 2019). The P and R^2 values indicated the significance and importance of the effect of a soil variable on N-fixing bacterial populations. In addition, variance partitioning analysis (VPA) was also performed in order to quantify the contributions of soil organic C (SOC, POC, MOC, DOC, MBC) and N (TN, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$) components to variation in composition of N-fixing bacterial populations (Ji et al., 2020). Results were plotted using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA), GraphPad Prism 8.0 (GraphPad Software, California, USA), and R (v 4.1.0).

Results And Analysis

Effects of straw return on soil organic carbon and nitrogen components at different depths

Straw returned changed SOC and N contents at different depths. Compared with CK, ST significantly increased SOC at depths from 0 to 80 cm, POC at depths from 0 to 40 cm, and MOC at depths from 20 to 60 cm, with increases of 13.19–243.48%, 36.39–142.75%, and 89.34–272.68%, respectively. However, compared with CK, ST decreased DOC at depths from 0 to 60 cm and MBC at depths from 0 to 80 cm, with decreases of 40.29–75.88% and 35.64–75.43%, respectively. At the 80–100-cm depth, there were no significant differences in contents of different SOC components between the two groups (Fig. 1).

Compared with CK, ST significantly increased TN content at depths from 0 to 80 cm, with increases of 14.26–90.34%. Compared with CK, ST significantly decreased MBN at depths from 0 to 80 cm and $\text{NO}_3^-\text{-N}$ and AN at depths from 0 to 60 cm, with decreases of 41.83–72.98%, 12.92–61.87%, and 12.99–67.80%, respectively. Compared with CK, ST significantly decreased $\text{NH}_4^+\text{-N}$ 11.61–24.80% at depths from 0 to 40 cm, but significantly increased it 44.65% at 40–60 cm.

Effects of straw return on activity and abundance of nitrogen-fixing bacteria at different soil depths

Potential N fixation rate, to a certain extent, characterizes activity of N-fixing bacteria in soil. The PNFR decreased as soil depth increased, ranging from $0.14 \mu\text{g (N)} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ to $3.08 \mu\text{g (N)} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (Fig. 2A). Compared with CK, PNFR increased significantly by 24.78% at 0–20-cm in ST, although there were no significant differences between treatments at depths from 20 to 60 cm (Fig. 2a). Correlations between PNFR and C and N contents were different in different soil layers (Fig. 3). At 0–20-cm, PNFR was positively correlated with SOC and TN and negatively correlated with MBC, $\text{NH}_4^+\text{-N}$, and $\text{NO}_3^-\text{-N}$. At 20–60-cm depths, PNFR was only negatively correlated with $\text{NH}_4^+\text{-N}$ (Fig. 3).

Similar to changes in PNFR, the abundance of *nifH* N-fixing bacteria also decreased as soil depth increased (Fig. 2b). Compared with CK, copy numbers of the *nifH* gene in 0–20- and 20–40-cm soil layers increased by 2.9 and 18.2-fold, respectively. The *nifH* gene copy numbers in different soil layers were significantly positively correlated with SOC, MOC, and TN and significantly negatively correlated with DOC, MBC, MBN, $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, and AN (Fig. 3).

Effects of straw return on population composition of *nifH* nitrogen-fixing bacteria at different soil depths

Compared with CK, ST significantly increased the sobs index of N-fixing bacteria in 0–20- and 20–40-cm soil layers (Table 1), with increases of 11.13% and 28.55%, respectively. However, the sobs index of N-fixing bacteria in ST decreased in the 40–60-cm soil layer compared within that in CK (Table 1). In addition, compared with CK, ST significantly increased Chao and Shannon indices of N-fixing bacteria in the 20–40-cm soil layer by 20.77% and 19.47%, respectively.

Table 1
Effects of long-term straw return (ST) and no straw return (CK) on α diversity and phylum-level composition of populations of *nifH* nitrogen-fixing bacteria at three soil depths from 0 to 60 cm.

Diversity and phylum components		Treatment					
		CK			ST		
		0–20-cm	20–40-cm	40–60-cm	0–20-cm	20–40-cm	40–60-cm
Diversity index	Sobs	730±17d	706±15 d	953±11 a	812±21 c	908±27 b	717±7 d
	Chao index	1151±56 b	1148±112 b	1210±108 b	1237±41 ab	1386±88 a	1118±71 b
	Shannon index	5.10±0.11 a	4.34±0.27 b	5.36±0.48 a	5.12±0.10 a	5.19±0.12 a	4.90±0.14 a
	Simpson index	0.018±0.002 c	0.120±0.011a	0.019±0.008c	0.022±0.002c	0.040±0.006b	0.033±0.008bc
Relative abundance of phylum (%)	Proteobacteria	70.19±1.97ab	75.00±9.40ab	72.83±4.37ab	67.92±3.26b	77.66±1.35a	73.99±3.03ab
	Unclassified bacteria	17.76±3.71b	14.99±2.69b	22.40±3.72a	17.08±2.12b	16.20±0.48b	14.84±1.14b
	Unclassified	11.92±3.51a	7.31±1.18bc	4.10±0.77c	14.78±1.28a	5.81±1.81c	10.72±4.26ab
Note: Values are the mean ± SE(n=3). Different lowercase letters in the same column indicate significant differences between groups ($P < 0.05$). <i>unclassified_k_norank_d_Bacteria</i> was unclassified nitrogen-fixing bacteria phylum; <i>unclassified_d_Unclassified</i> was other unclassified phylum besides unclassified bacteria phylum							

Proteobacteria was the main phylum of *nifH* N-fixing bacteria in soil layers in the upper 60 cm (relative abundance 67.92–77.66%; Table 1). There was no significant difference between CK and ST. In addition, compared with CK, relative abundance of *unclassified_k_norank_d_Bacteria* (unclassified N-fixing bacterial phylum) decreased by 33.77% in ST; but relative abundance of *unclassified* increased by 161.25% at 40–60 cm.

Thirty-nine genera of *nifH* N-fixing bacteria were identified, and eight genera had relative abundances greater than 1%: *unclassified_p_Proteobacteria*, *unclassified_k_norank_d_Bacteria*, *unclassified_c_Deltaproteobacteria*, *unclassified_d_unclassified*, *Geobacter*, *unclassified_c_Alphaproteobacteria*, *Anaeromyxobacter*, and *unclassified_o_Rhizobiales* (Fig. 4a). Compared with CK, ST significantly increased the relative abundance of *unclassified_c_Deltaproteobacteria* at 0–20 cm by 44.3% (Fig. 4b), but that of *Geobacter* and *Anaeromyxobacter* decreased significantly by 17.03% and 21.05%, respectively. In addition, compared with CK, ST also increased the relative abundance of *Geobacter* and *unclassified_o_Rhizobiales* at 20–40-cm and that of *unclassified_c_Alphaproteobacteria* at 40–60-cm.

In the PCoA, N-fixing bacteria in different soil layers were primarily clustered into two groups: (1) 0–60-cm in ST and 40–60-cm in CK, and (2) 0–20- and 20–40-cm in CK (Fig. 5a). The results indicated that the effect of ST on population composition of N-fixing bacteria was mainly concentrated at depths from 0 to 40 cm. In the RDA (Fig. 5b), environmental factors explained 35.69% of the total variation in population composition of *nifH* N-fixing bacteria, with RD1 and RD2 explaining 31.25% and 4.44%, respectively. Among the factors, POC ($R^2 = 0.413$, $P < 0.05$), TN ($R^2 = 0.531$, $P < 0.01$), and NO_3^- -N ($R^2 = 0.365$, $P < 0.05$) significantly affected the composition of N-fixing bacterial populations. In addition, VPA was used to determine the relative contributions of organic C (DOC, MBC, POC, MOC, and SOC) and N (NH_4^+ -N, NO_3^- -N, and TN) components to the composition of N-fixing bacterial populations (Fig. 5c). The variables explained 92.16% of the variation in N-fixing bacterial populations, whereas 7.8% of the variation could not be explained. Changes in soil N and organic C contents after returning straw to the field explained 31.92% and 12.3%, respectively, of the variation in composition of N-fixing bacterial populations.

Correlations between soil factors in different soil layers and dominant genera of bacteria were inconsistent (Fig. 6). At 0–20 cm, *Geobacter* and *Anaeromyxobacter* were significantly negatively correlated with SOC and significantly positively correlated with DOC, MBC, MBN, and NO_3^- -N, while *unclassified_c_Deltaproteobacteria* was significantly positively correlated with SOC and significantly

negatively correlated with DOC, MBC, MBN, and NO_3^- -N. At 20–40 cm, *Geobacter* and *unclassified_o__Rhizobiales* were significantly positively correlated with SOC, MOC, POC, and TN and significantly negatively correlated with DOC, MBC, AN, MBN, and NO_3^- -N. There were no significant correlations between the other bacterial genera and soil factors.

Discussion

Effects of straw return on activity and abundance of nitrogen-fixing bacteria at different soil depths

In this study, PNFR decreased with increasing soil depth, with the rate decreasing from 3.08 to 0.14 $\mu\text{g(N)}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$. Liao et al. (2018) used $^{15}\text{N}_2$ tracing and estimated PNFRs were between 14.6 and 20.4 $\mu\text{g(N)}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ in paddy soil after 30 years of straw return. Therefore, in comparison, the rates in the present study were relatively low. The lower rates might be because nitrogenase in N-fixing bacteria was sensitive to oxygen, and the catalytic reaction usually occurs under anaerobic conditions. Thus, the aerobic environment on dry land is not favorable for biological N fixation by N-fixing bacteria (Keuter et al., 2014). Compared with CK, ST significantly increased PNFR at 0–20 cm but did not affect it at depths from 20 to 60 cm. These results are consistent with those of Wang et al. (2019). However, in contrast to the 0–20-cm layer in this study, they found soil biological N fixation mainly occurred in the 0–5-cm soil layer. The difference might be because long-term straw return effectively increased organic C and N components in the 5–20-cm soil layer, which provided C and N sources for N-fixing bacteria, thereby ensuring a good environment and energy supply to support them. Furthermore, N availability in soil can greatly affect activity of N-fixing bacteria, with low N generally stimulating biological N fixation (Wang et al., 2017, Wang et al., 2019). Crop straw contains relatively low N and high C contents, and soil microorganisms absorb available N in soil to facilitate growth. Therefore, the significant decreases in soil NO_3^- -N and NH_4^+ -N after straw return might also explain the increase in activity of N-fixing bacteria in the 0–20-cm soil layer. The decreases might also explain the negative correlations between PNFR and NO_3^- -N and NH_4^+ -N at 0–20 cm and the positive correlations between PNFR and SOC and TN.

Similar to the effect on PNFR, ST effectively increased the abundance of *nifH* N-fixing bacteria at depths from 0 to 60 cm. In general, most N-fixing bacteria in soil are heterotrophic or facultative microorganisms. In ST, large amounts of organic C and energy are input to soil, which can effectively promote the growth of N-fixing bacteria (Valiente et al., 1997; Che et al., 2018). However, high NO_3^- -N and NH_4^+ -N contents inhibit the growth of soil N-fixing bacteria (Wang et al., 2015). Those observations are consistent with the findings of this study, because the *nifH* copy number was positively correlated with SOC, MOC, and TN and negatively correlated with NO_3^- -N and NH_4^+ -N.

Zhao et al. (2018) and Yang et al. (2021) showed that expression of functional genes and nitrogenase activity of N-fixing bacteria were inhibited by high concentrations of NO_3^- -N (Zuberer, 2021). In this study, ST led to significant decreases in nitrate N at depths from 0 to 60 cm. The decrease could be explained by an increase in denitrification (Kakuda et al., 2000), which would also increase activity and abundance of N-fixing bacteria in a negative feedback mechanism of the N cycle (Yang et al., 2021). In the rice and rapeseed rotation system, based on average soil bulk density of 1.2 $\text{g}\cdot\text{cm}^{-3}$, annual N fixation at different depths from 0 to 60 cm was approximately 2.70 (0–20 cm), 0.58 (20–40 cm), and 0.13 $\text{kg}\cdot\text{ha}^{-1}$ (40–60 cm) in ST, which was 0.01 to 0.54 $\text{kg}\cdot\text{ha}^{-1}$ higher per year than that in CK. Compared with the amount of N applied, the amount of N fixation in the whole year was negligible, but it remains important to quantify all N inputs in order to further understand changes in the soil N cycle with long-term straw return. Although the increase was similar to the loss of nitrate N (0.36–0.68 $\text{kg}\cdot\text{ha}^{-1}$) in ST, changes in different forms of N can affect crop growth. In dryland crops, absorption of nitrate N is the main source of N. Improvement in soil N fixation capacity in ST might not significantly change the reduction in nitrate caused by increased denitrification. Therefore, straw return may reduce the N supply for dryland crops. Consequently, changes in different forms of soil N should be fully considered when returning straw in order to facilitate efficient and reasonable management of N nutrition.

Effects of straw return on population composition of *nifH* nitrogen-fixing bacteria

In this study, 5 years of ST effectively increased diversity of N-fixing bacteria at depths from 0 to 40 cm, which is consistent with the results of Yang et al. (2018). The increase in diversity likely occurred primarily because most bacteria that can express the *nifH* gene are heterotrophic or facultative, and therefore, returned straw provided sufficient monosaccharides and polysaccharides for those bacteria (Rahav et al., 2016). The increase in diversity of N-fixing microbial communities after straw return may lead to diversity in microbial processes and changes in soil characteristics, which are very important to soil health and plant productivity (Saleem et al., 2019). Therefore, in ST, the increase in diversity of N-fixing microorganisms changed soil nutrients and organic C components, which provided

bacteria, nutrients and C sources for biological nitrogen fixation under different environmental conditions, was conducive to maintaining soil biological N fixation after the change of environmental conditions, and helping to sustain rice–rapeseed rotation production systems.

Straw return not only changed the diversity of N-fixing bacteria in soil but also affected population composition and structure. According to VPA, soil N (31.92%) explained a greater portion of changes in population composition of N-fixing bacteria than that of SOC (12.3%), suggesting that composition of N-fixing bacterial populations in ST largely depended on the level of soil N (Ning et al., 2015). The RDA further validated that changes in POC, TN, and NO_3^- -N after long-term ST were the main factors affecting population composition of N-fixing bacteria. These results are consistent with those of recent reports. Su et al. (2020) showed that POC generated by microorganisms in the process of assimilating straw C was the C source for N-fixing bacteria (e.g., *Bradyrhizobium*, *Burkholderia-Paraburkholderia*, and *Paraphaeosphaeria*). Yang et al. (2018) also showed that NO_3^- -N content with straw return and inorganic fertilizer application was a significant factor affecting abundance and population composition of N-fixing bacteria.

In the present study, differences in the composition of N-fixing bacterial populations in ST primarily occurred at depths from 0 to 40 cm. Straw return significantly increased the relative abundance of *unclassified_c_Deltaproteobacteria* at 0–20 cm, whereas it reduced that of *Geobacter* and *Anaeromyxobacter*. *Deltaproteobacteria* is an aerobic bacterium, whereas *Geobacter* (Wang et al., 2017) and *Anaeromyxobacter* (Aklujkar et al., 2011) are facultative anaerobic gram-negative bacteria (He and Sanford, 2003). Return of straw improves aeration of topsoil and thus, to a certain extent, increases the relative abundance of aerobic N-fixing bacteria such as *Deltaproteobacteria* (Severin et al., 2015) but decreases that of anaerobic bacteria such as *Geobacter* and *Anaeromyxobacter*. In addition, *Geobacter* is an oligotrophic bacterium that is closely associated with N fixation activity (Hug et al., 2015), and its growth may be inhibited in nutrient-sufficient environments (Fan et al., 2018). Relative abundance of *Geobacter* at 0–20 cm was significantly negatively correlated with contents of SOC and TN in ST, whereas that at 20–40 cm was significantly positively correlated with SOC, POC, MOC, and TN. These results suggested that C and N contents at 20–40 cm inhibited growth and metabolism of this bacterium. The increase in organic C and N contents at 20–40 cm under ST could promote the growth of *Geobacter* and therefore also increased relative abundance of *unclassified_o_Rhizobiales*. Based on the above results and the RDA results, soil aeration, SOC, POC, TN, and NO_3^- -N were identified as the main factors driving changes in population composition of *nifH* N-fixing bacteria and soil N-fixing capacity in ST.

In this research, N fixation was only examined following the rapeseed crop in the rice–rapeseed system, and N fixation following the rice crop remains unclear. Qin et al. (2016) showed that abundance and function of microorganisms involved in soil N cycling changed when paddy fields were converted to vegetables. Therefore, the influence of long-term straw return on soil N-fixing microbial community structure in rapeseed or rice crop may directly or indirectly affect the next crop. Thus, future research should investigate the effects of long-term straw return on PNFR and N-fixing microbial communities in rice crops as well as clarify changes in N-fixing microbes between rice and rapeseed crops.

Conclusions

Long-term ST significantly affected abundance, activity, and population composition of N-fixing bacteria by changing soil chemical properties at different depths, with effects reaching 20 cm or deeper. Straw return increased abundance, activity, and diversity of N-fixing bacteria by increasing SOC, POC, and TN and by providing sufficient sources of C. Because of high C and low N contents of straw, soil available N content decreased, especially NO_3^- -N which might be accelerated by denitrification after straw return. Soil NO_3^- -N was also an important factor affecting populations of N-fixing bacteria. To summarize, straw return induced changes in soil organic C and N contents that regulated N-fixing bacterial populations, which had an important role in maintaining the balance between soil C and N. Therefore, for crop nutrient management, it was necessary to full consider the change of different forms of nitrogen levels in soil and the supply of nitrogen nutrition caused by straw returning.

Declarations

Author contributions

Huishan Fan and Yao Su conceived and designed the experiments; Huishan Fan and Shengqiang Jia conducted the experiments; Huishan Fan and Yao Su did the analysis and wrote the main manuscript text; Ma Yu, Xijing Chen and Alin Shen edited the manuscript. All authors contributed to discussion about the results and the manuscript.

Funding

This work was financially supported by the China Natural Science Foundation Youth Fund Project (Grant NO. 41807034), the National Modern Agricultural Technology System of Wheat (Grant NO.CARS-3), and Agricultural Science and Technology project in Ping hu (Grant NO.PH2020004).

Declaration of Competing Interest

The authors have no competing interests to declare that are relevant to the content of this article.

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Figures

Figure 1

Effects of long-term straw return (ST) and no straw return (CK) on organic carbon and nitrogen components at different depths in soil from 0 to 100 cm. Error bars indicate the SE of means (n=3), and different lowercase letters above columns mean significant differences between treatment (p < 0.05).

Figure 2

Comparison of effects of straw return (ST) and no ST (CK) on potential nitrogen fixation rate (PNFR, μg N kg⁻¹ d⁻¹) and copy number of the *nifH* gene (copy g⁻¹ dry soil) at three soil depths from 0 to 60 cm. Error bars indicate the SE of means (n=3), and different lowercase letters above columns mean significant differences between treatment (p < 0.05).

Figure 3

Pearson correlation matrix between soil chemical properties and *nifH* gene copy number and potential nitrogen fixation rate (PNFR) at three soil depths from 0 to 60 cm. Data are means of 3 replicates. Blue and red circles denote positive and negative correlations, respectively. Large to small circles and dark to light color indicate high to low correlations.

Figure 4

Comparison of effects of straw return (ST) and no ST (CK) on (A) relative abundance (%) of dominant genera and (B) significantly different bacterial genera composing *nifH* nitrogen-fixing bacterial populations at three soil depths from 0 to 60 cm. Bars in blue and red represent the treatment with and without straw inputs, respectively. P value <0.05 indicates significant differences between CK and ST treatment.

Figure 5

Comparisons of populations of soil nitrogen-fixing bacteria in straw return (ST) and without (CK) treatments at three soil depths from 0 to 60 cm. (a) Principal coordinates analysis (PCoA) of differences in population composition of soil N-fixing bacteria. (b) Redundancy analysis of population composition of nitrogen-fixing bacteria and soil chemical factors. (c) Variance partition analysis of effects of organic carbon and nitrogen on population composition of nitrogen-fixing bacteria.

Figure 6

Pearson correlation matrix between soil chemical properties and dominant genera of nitrogen-fixing bacteria at 0-20 cm depths(A) and 20-40 cm depths(B). Data are means of 3 replicates. Blue and red circles denote positive and negative correlations, respectively.

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