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Effects of Nitrogen Addition on Rhizosphere Soil Microbial Community and Yield of Wheat in Loess Plateau

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Abstract: The soil microbial community diversity of wheat rhizosphere was affected by the amount of nitrogen fertilizer. In addition to bacterial community, ammonia-oxidizing archaea, nitrogen-fixing bacteria and denitrifying bacteria also play important roles in nitrogen cycle. At present, the microecological mechanism of its response to nitrogen application is still unclear. In this study, the rhizosphere soil microorganisms of winter wheat were used as the research object. The changes of soil bacteria, ammonia-oxidizing archaea, nitrogen-fixing bacteria and denitrifying bacteria communities under five nitrogen rates of 0 (N0), 90 (N6), 180 (N12), 240 (N16) and 300 (N20) kg N · hm^{-2} were studied by high-throughput sequencing technology. Among them, under N12 treatment, the Alpha diversity index of bacteria, AOA and *nifH* nitrogen-fixing bacteria and the Shannon and Simpson indexes of *nirK* denitrifying bacteria were significantly increased. N12 treatment significantly increased the relative abundance of Proteobacteria, but no significant difference was found at the *nifH* and *nirK* bacterial phyla levels. Under the high nitrogen treatment of N16 and N20, the dominant bacteria of *nirK*-type denitrifying bacteria increased significantly compared with N12 treatment, there was no significant difference in microbial community distribution between N20 and the control group. Therefore, the nitrogen addition under N12 treatment was most conducive to the absorption and utilization of nitrogen fertilizer by soil microorganisms. The effect of nitrogen addition on microbial community was weaker than that of soil properties and wheat yield, and nitrogen addition was significantly correlated with yield, reaching the highest yield at 300 kg·hm^{-2}.

Keywords: nitrogen addition; winter wheat; rhizosphere soil; functional microbial; soil properties; yield

Introduction

Winter wheat is the main food crop in the Loess Plateau, the area of wheat accounts for nearly 50 % of the total area[1]. The Loess Plateau is a typical ecologically fragile area. Restricted by natural geographical conditions, soil erosion, drought and barren have become the main limiting factors for the breakthrough of wheat yield in the Loess Plateau[2]. Nitrogen is one of the essential elements to maintain crop growth and development. As the most widely used fertilizer in agricultural production, nitrogen fertilizer plays an important role in improving soil nutrients and increasing crop yield. Its invention and application are considered as one of the greatest contributions of mankind in the 20th century[3]. Nitrogen is an integral part of proteins, chlorophylls, enzymes, various coenzymes, certain plant hormones, and secondary metabolites[4]. Therefore, there is a significant correlation between nitrogen fertilizer application and crop yield. However, with the increase of grain demand, the excessive application of nitrogen fertilizer in production also has certain impacts on Chinese agricultural development, such as the late maturity of plants, soil eutrophication and global climate change[5,6]. It is a major research direction of current wheat cultivation and production to clarify the reasonable amount of nitrogen fertilizer, reduce fertilizer and nitrogen, improve nitrogen use efficiency, and ensure the stable yield and increase of wheat in the Loess Plateau while ensuring the sustainable and safe development of grain production.

Soil microbial is the most widely distributed organism in soil ecosystem. The improvement of element cycle and physical and chemical properties of soil ecosystem driven by microbial[7] is one of the methods to improve the yield and quality of cultivated crops. Nitrogen is the main factor for maintaining metabolic activity of microorganisms[8]. Similarly, functional microorganisms are also involved in soil nitrogen cycling. Among them, ammonia oxidizing bacteria and denitrifying bacteria, as the most critical rate-limiting substances in nitrification and denitrification processes, are involved in the key links of microbial nitrogen utilization and loss[9,10]. Nitrogen - fixing bacteria are also involved in soil nitrogen transformation[11]. Therefore, the application of nitrogen fertilizer causes the change of soil microbial community, which is one of the favorable ways to improve soil quality.

At present, there are many studies on the effects of nitrogen fertilizer on soil microbial community. For example, reasonable nitrogen fertilizer application improved the abundance and diversity of bacterial community in citrus soil and improve its community structure[12]; Noah Fierer et al.[13] proved that nitrogen application helped to build richer and more stable microbial communities. Lu et al.[14] showed that half application of nitrogen fertilizer could significantly improve the utilization of soil microbial community to multiple carbon sources, thereby increasing their community diversity. In summary, the improvement of microbial community structure and functional diversity is driven by nitrogen fertilizer, which is closely related to the addition of nitrogen fertilizer. However, there is no clear research to confirm it.

In this study, high-throughput sequencing technology was used to study the soil microbial community structure in winter wheat rhizosphere under different nitrogen additions in the Loess Plateau and clarify the response of microbial communities to nitrogen fertilizer application. At the same time, combined with the diversity of nitrogen-related functional microbial ammonia-oxidizing archaea, nitrogen-fixing bacteria and denitrifying bacteria communities, the most reasonable nitrogen

application rate was sought without increasing nitrogen leaching and loss during nitrification and denitrification.

Materials and Methods

Experimental design

The experiment was conducted in Shangyuan Village, Hougong Township, Wenxi County, Shanxi Province (34° 35'N, 110° 15'E), from 2016 to 2020, and sampled in 2020. The region is located in the semi-arid region of the southeast Loess Plateau, with a warm temperate continental monsoon climate. The annual average temperature is 8-14℃, the annual average precipitation is 506mm, the annual average sunshine hours are about 2242.0 h, and the frost-free period is 190d. The soil type is calcareous cinnamon soil.

Liangxing 99 was selected as the tested wheat variety. The tested fertilizer was urea (N 46%). Five nitrogen additions were set as 0 (N0), 90 (N6), 180 (N12), 240 (N16) and 300 (N20) kg $N \cdot hm^{-2}$, respectively. A total of 15 plots were repeated three times, and the plot area was 50 m^2, which was completely randomized into groups. 1m guard rows were set between every two plots. The application rates of phosphorus and potassium in all plots were 150 kg $P_2O_5 \cdot hm^{-2}$ and 150 kg $K_2O \cdot hm^{-2}$. Wheat was sowed on Oct. 10, 2019， and harvested on Jun. 7, 2020.

Soil sampling

Soil samples were collected by sterilized soil drill in wheat mature period (Jun. 7, 2020). Three sampling points were randomly selected and mixed evenly in each plot. The soil samples were stored in a refrigerator and transported back to the laboratory. Some of them were stored in dry air for the determination of physical and chemical properties, and others were stored in a refrigerator at −80 °C for the determination of microbial community structure.

DNA extraction and Illumina-based sequencing

Fast DNA SPIN Extraction Kits kit (MP Biomedicals, USA) was used for soil total DNA extraction according to the steps of DNA extraction kit. The purity and integrity of extracted DNA were detected by agarose gel electrophoresis, and the concentration and purity of extracted DNA were detected by Nanodrop NC-2000.

The samples were sequenced using Illumina MiSeq high-throughput sequencing technology platform by Shanghai Personal Biotechnology Co., Ltd. The total DNA extracted from soil microorganisms was used as template to amplify bacterial and nitrogen functional microorganisms by RCR with primers (Table 1). The PCR amplification system was 5×reaction buffer 5.0μL, 5×GC buffer 5.0μL, dNTP (2.5 mM) 2.0μL, upstream and downstream primers (10uM) 1.0μL, DNA template 1.0μL, Q5 DNA polymerase 0.25μL, and ddH2O 9.75μL. PCR products were purified by Agencourt AMPure Beads (Beckman, USA) and quantified by PicoGreen ds DNA Assay Kit15 kit (Invitrogen, USA).

Table 1. The PCR primers and amplification sequences of bacteria and three functional microorganisms

Category	Target gene	Primer	Sequence (5'-3')
Bacteria	Bacteria V3V4	338F	5'-TGCGAYCCSAARGCBGACTC-3'
		806R	5'-GGACTACHVGGGTWTCTAAT-3'
	Archaea amoA	Arch-amoA26F	5'-GACTACATMTTCTAYACWGAYTGGGC-3'
		Arch-amoA417R	5'-GGKGTCATRTATGGWGGYAAYGTTGG-3'
Functional microbial	*nifH*	PolyF	5'-TGCGAYCCSAARGCBGACTC-3'
		PolyR	5'-ATSGCCATCATYTCRCCGGA-3'
	nirK	*nirk*-F	5'-TCATGGTGCTGCCGCGYGANGG-3'
		nirk-R	5'-GAACTTGCCGGTKGCCCAGAC-3'

Sequences data analyses

Using QIIME software, the UCLUST sequence alignment tool (Edgar, 2010) was called to merge and divide the obtained sequences into OTUs according to 97% sequence similarity, and the highest abundance sequence in each OTU was selected as the representative sequence of the OTU. For each OTU representative sequence, the default parameters are used in QIIME software to obtain the corresponding taxonomic information of each OTU by comparing the OTU representative sequence with the template sequence of the corresponding database.

Statistical analysis

The data of physical and chemical properties of samples were processed by Microsoft Excel 2010, and the charts were drawn. Analysis of variance and correlation by SPSS 26.0 software. Cluster analysis and heat map of the top 50 genera and NMDS analysis of weighted UniFrac distance matrix were performed using R software. In addition, the generalized linear model (GLM) and quadratic regression model were used to reflect the response of microbial Alpha diversity and wheat yield to nitrogen addition. Structural equation modeling used Amos 23.0 software and data fitting used maximum likelihood estimation.

Results

Effect of N Addition on diversity index of soil microbial

The high-throughput sequencing of bacteria, AOA, *nifH* and *nirK* in each soil sample were carried out, and a total of 503870, 547229, 1120,571 and 1356357 sequences were obtained. The 97% sequence similarity was used as the OTU division threshold, and the number of OTUs of the four microorganisms was 6905 ~ 9763,4488 ~ 7569,7538 ~ 11196 and 6905 ~ 9763, respectively. The Alpha diversity index was used to reflect the richness and diversity of microbial communities. The larger the Chao1 or ACE index, the higher the community richness. The greater the Shannon or Simpson index, the higher the community diversity. Except for the *nirK* denitrifying bacteria, the Alpha diversity indexes

of the other three microorganisms showed a trend of first increase and then decrease. The effect of N addition on Alpha diversity index of bacteria, ammonia-oxidizing archaea and nitrogen-fixing bacteria was the highest under N12 treatment. Under N20 treatment, the Shannon index and Simpson index of denitrifying bacteria were the highest, and Chao1 and ACE indexes were the same as those of other microorganisms on nitrogen gradient (Fig. 1).

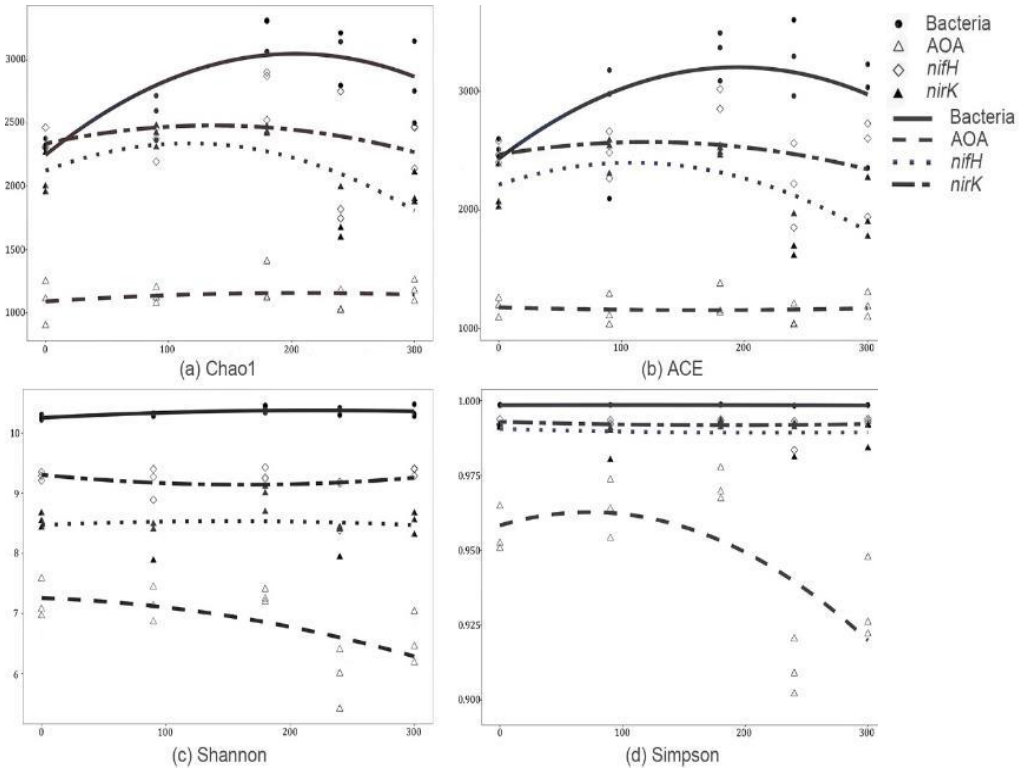


Fig.1 Relationship between microbial Alpha diversity index and nitrogen addition. Horizontal coordinates are nitrogen addition, ordinate coordinates are Chao1 (a), ACE (b), Shannon (c), Simpson (d) index

According to NMDS analysis, N6, N12 and N16 samples were far away from N0, indicating that the species composition of V3~V4 region of bacteria was changed under these three treatments, while the change was not obvious under N20 treatment (Fig. 2a); N12 was far from the control N0 which significantly changed the species composition of ammonia-oxidizing archaea, while N16 and N20 were closer to N0 (Fig. 2b). In the nitrogen-fixing bacteria communities, the effect of N20 and N6 treatment was not obvious compared with N0, and the distance among those three was close, while the species composition of N16 and N12 were significantly differed from the control (Fig. 2c). N0, N6, N16 and N20 treatments had similar community structure of denitrifying bacteria, but they were significantly different from those of N12 treatment (Fig. 2d).

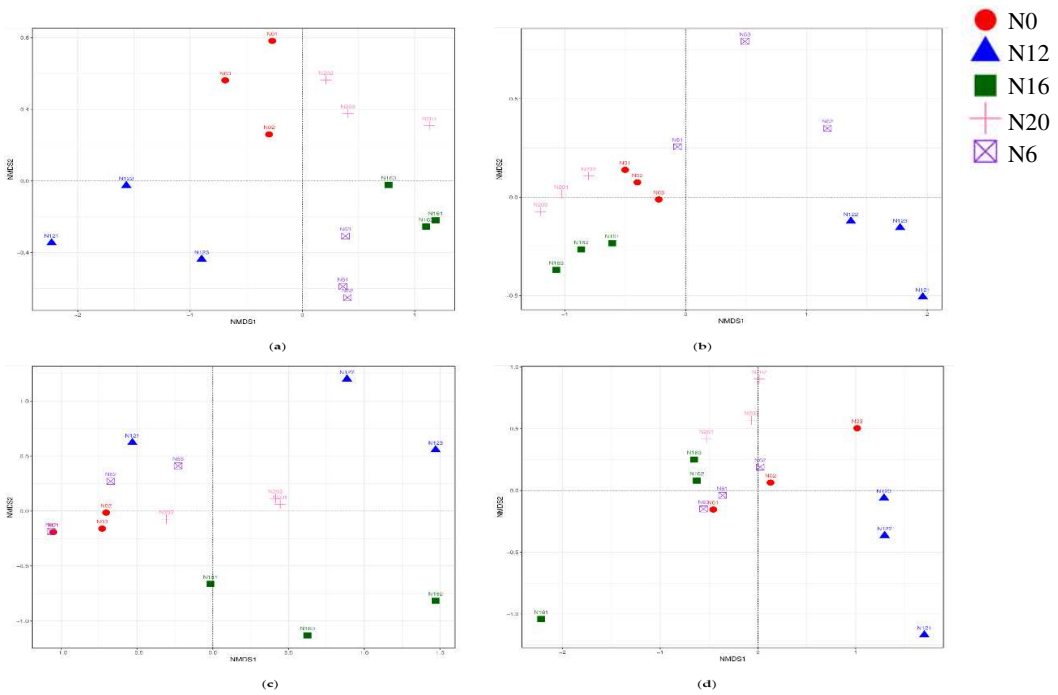


Fig.2 NMDS analysis of four microorganisms, each point represents a sample, the closer the distance between the two points, indicating that the higher the similarity of microbial community structure between the two samples, the smaller the difference. Treatment N0, N6, N12, N16 and N20 were 0, 90, 180, 240 and 300 kg·ha^{-1}, respectively

Soil microbial community structure responses to N fertilization

In the bacterial community composition, the five dominant phyla under different nitrogen additions were Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexiand Gemmatimonadetes. In each treatment, the abundance of the first 10 bacterial categories accounted for 97.3–98.9% of the total sequence. There were significant differences in the relative abundances of Proteobacteria, Actinobacteria, Acidobacteria and Chloroflexi with Bacteroidetes at all nitrogen levels (P<0.05). The relative abundances of Bacteroidetes were significantly increased by N12 and N16 treatments. The relative abundances of Actinobacteria, Acidobacteria and Chloroflexi were significantly higher than those of other treatments under N0 treatment. The relative abundances of Proteobacteria, Nitrospira and Rokubacteria were significantly increased by N12 treatment. Among them, the relative abundance of Actinobacteria was large under N16 treatment, while that of Proteobacteria was large under N0 treatment. (Fig. 3a).

Archaea amoA obtained Thaumarchaeota, unclassified phyla and other phyla under different nitrogen treatments. Among them, the abundance distribution of Thaumarchaeota at phylum classification level reached 99 %. Under N0, N6 and N12 treatments, Thaumarchaeota decreased with the increase of nitrogen additions, while Thaumarchaeota was dominant in the treatment of N16 and N20. (Fig. 3b).

At the phylum level, the dominant phylum of *nifH*-type nitrogen-fixing bacteria was Proteobacteria, followed by Unidentified and Verrucomicrobia. Under different nitrogen treatments, N0 significantly increased the community abundance of Proteobacteria compared with other treatments, but there was no

significant difference between them. N16 and N20 treatments significantly decreased the community abundance of Verrucomicrobia (Fig. 3c). In the community composition of *nirK*-type denitrifying bacteria, Proteobacteria was the dominant phylum under different nitrogen treatments, followed by Actinobacteria, a total of 8 groups. The abundance of Proteobacteria community in N12 treatment was significantly higher than that in N0 treatment, but there was no significant difference between N12 and other treatments (Fig. 3d)

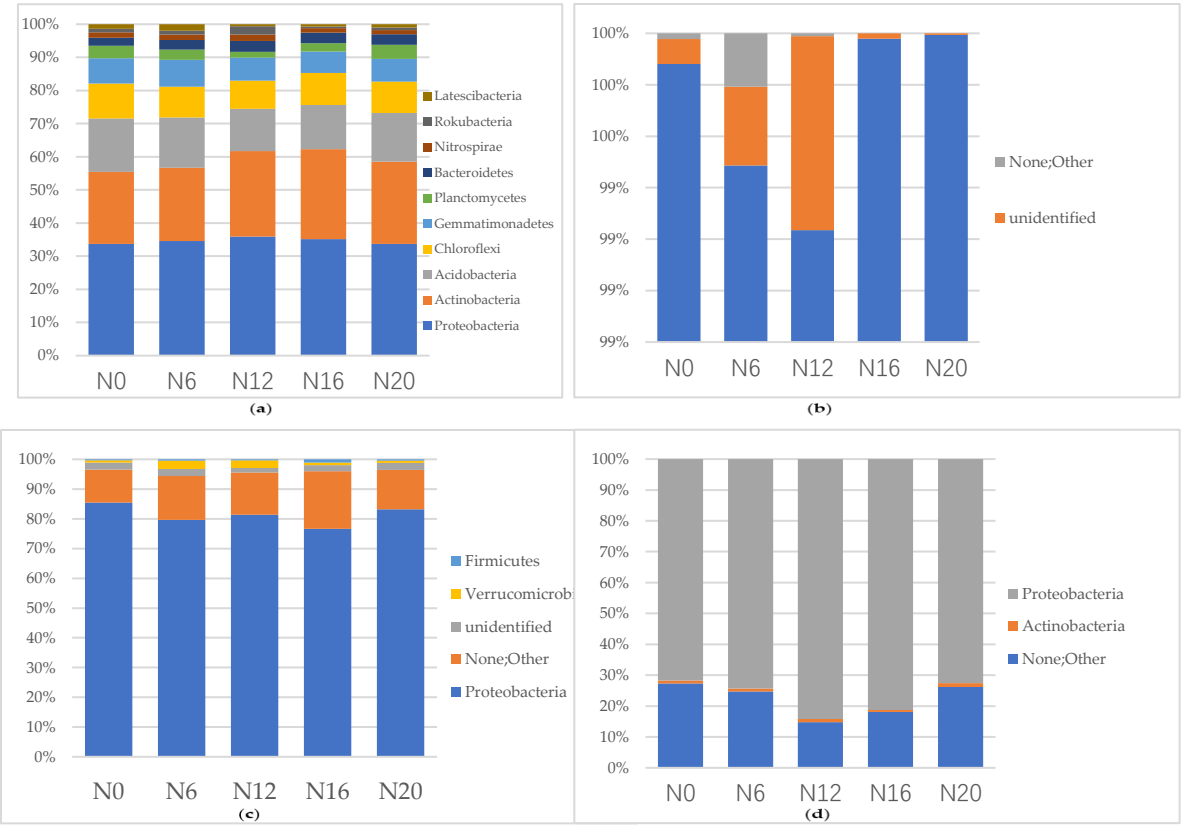


Fig.3 The accumulation column diagram of bacteria and three functional microorganisms reflects the composition relationship of different phyla that is in the overall proportion. Treatment N0, N6, N12, N16 and N20 were 0, 90, 180, 240 and 300 kg·ha^{-1}, respectively

The relative abundance of soil bacterial genera varied greatly under different nitrogen rates. The abundance of *Kribbella*, *Cellulomonas*, *Nocardioides* and *Streptomyces* under N0 was significantly lower than that under other treatments. N12 treatment significantly increased the relative abundance of *MND1*, *Subgroup_10*, *Gaiella*, *Nitrospira* and *Polycyclovorans*. The abundance of *Pseudomonas*, *Mycobacterium*, *Agromyces*, *Arthrobacter* and *Kribbella* under N16 treatment was significantly higher than that under other treatments. According to the results of cluster analysis, the similarity of community structure between N12 and N16 was high (Fig. 4a). At the bacterial genus level, the inter-group difference analysis based on the classification hierarchy tree showed that a total of 16 bacterial genera such as *Pseudomonas*, *Mycobacterium* and *Streptomyces* had significant changes in N16 treatment (Fig. 5a).

Candidatus.Nitrososocosmicus, *Nitrososphaera* and *Candidatus.Nitrosotalea* were detected under all nitrogen treatments. Compared with the control, N12 treatment significantly reduced the relative abundance of *Nitrososphaera*, while *Candidatus.Nitrososocosmicus* significantly increased the relative

abundance. Under N20 treatment, the relative abundance of *Candidatus.Nitrosotalea* was significantly increased; there was no significant difference in bacterial abundance between the other treatments (Fig. 4b). With regard to ammonia-oxidizing archaea, only *Nitrososphaera* under N16 treatment was found to have distinct taxa at genus level (Fig. 5b).

The *nifH*-type nitrogen-fixing bacteria had similar community structure at the genus level under N0 and N20 treatments. The abundance of *Ideonella*, *Azohydromonas*, *Dechloromonas* and *Rubrivivax* was higher under N0 treatment, while the relative abundance of *Klebsiella*, *Yangia* and *Skermanella* were higher under N20 treatment. *Paraburkholderia*, *Rhodobacter* and *Paenibacillus* had higher relative abundance under N16 treatment (Fig. 4c). At the genus level of *nifH*-type nitrogen-fixing bacteria, it was also shown that distinct taxa were mainly found in N16 treatment (Fig. 5c).

The relative abundance of the main genera of soil *nirK*-type denitrifying bacteria was significantly different under different N additions. The relative abundance of *Ensifer, Burkholderia* and *Roseitalea* under N12 treatment was significantly higher than that under other treatments, and the community similarity under N12 and control treatments (N0) was relatively high. (Fig. 4d). Through the analysis of the classification tree, it was found that four *nirK*-type denitrifying bacteria, *Ralstonia*, *Burkholderia*, *Ottowia* and *Massilia*, mainly changed in N12 treatment. In N20 treatment, only *Devosia* showed significant differences between groups at genus level (Fig. 5d).

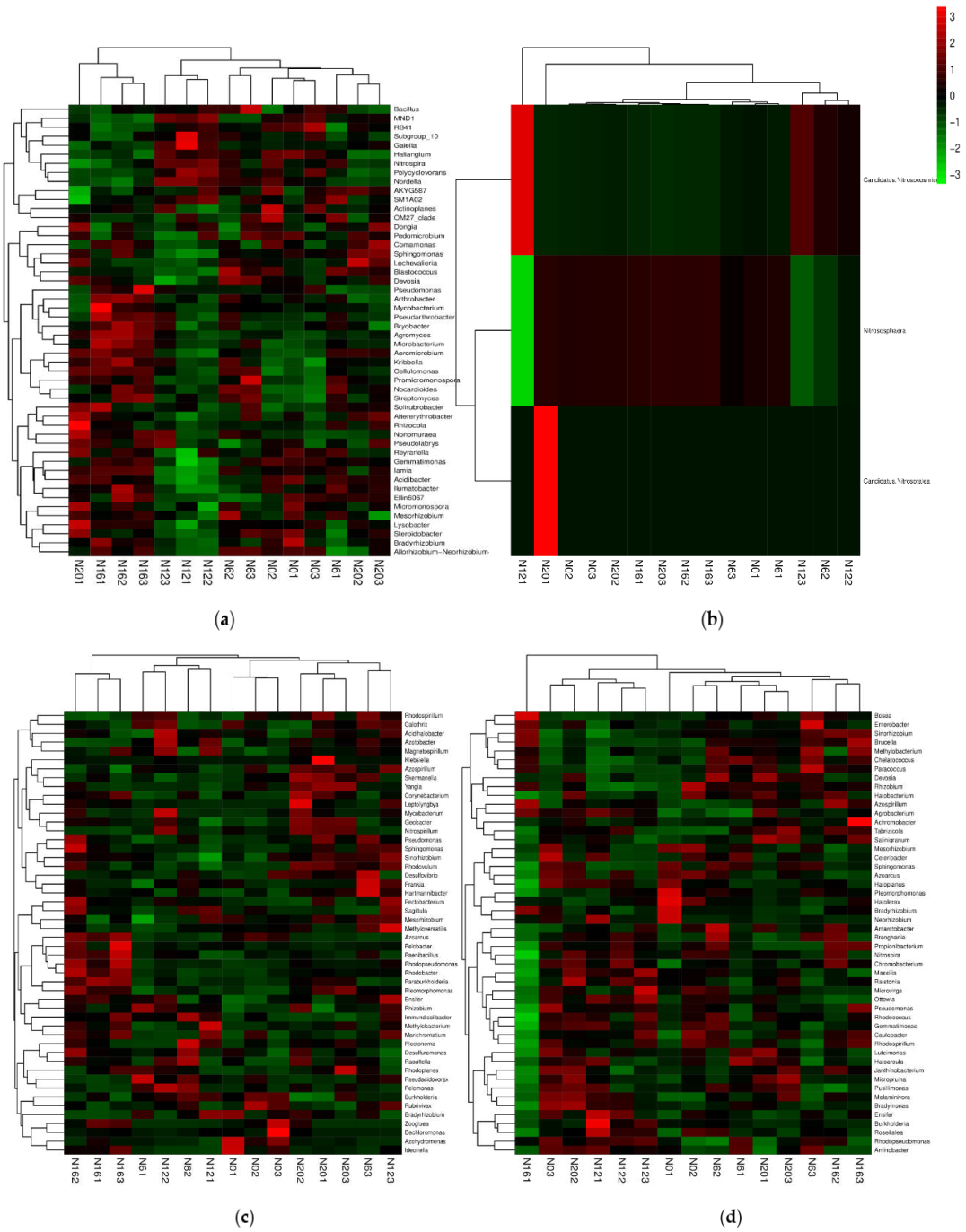
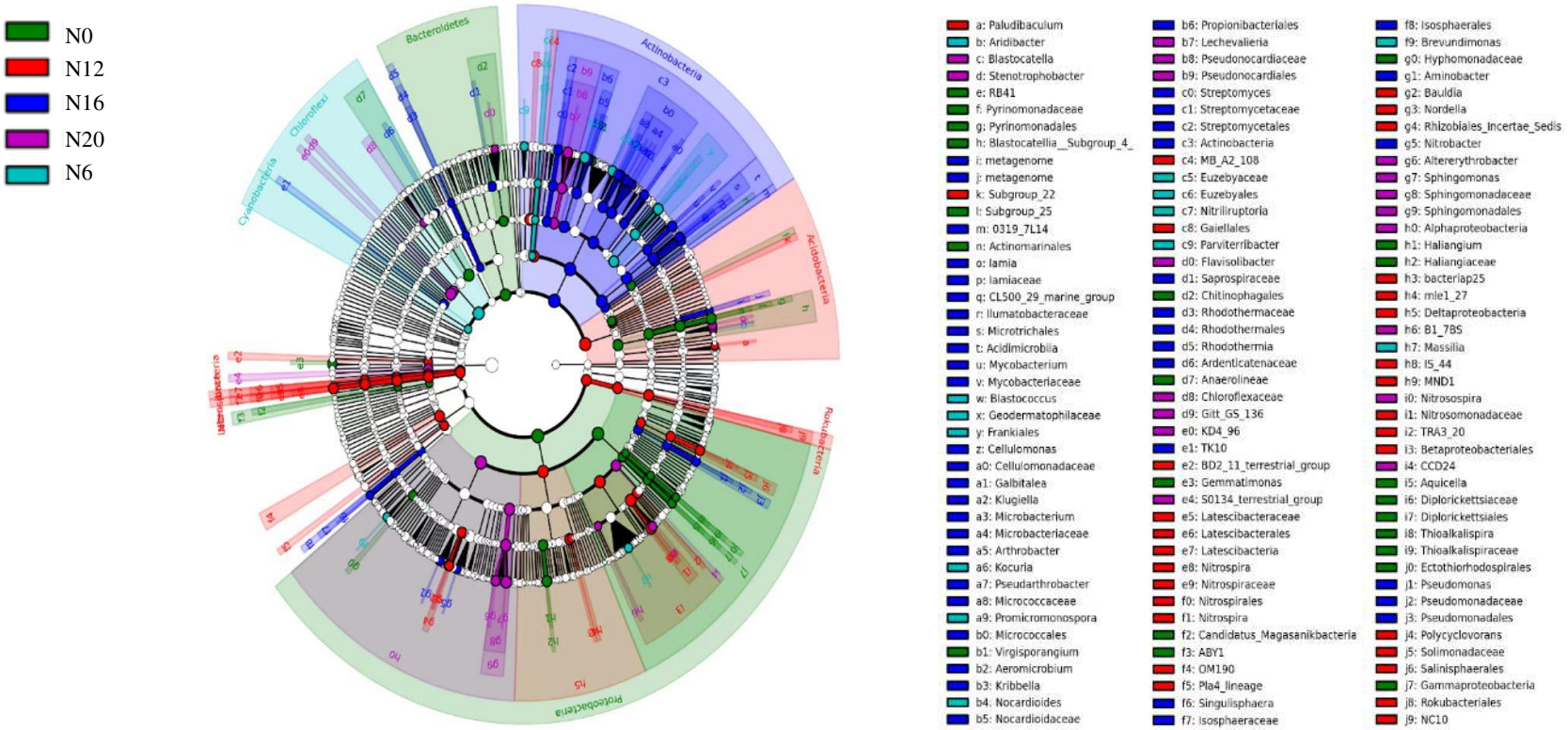


Fig.4 Bacteria (a), ammonia-oxidizing archaea (b), nitrogen-fixing bacteria (c) and denitrifying bacteria (d) of the genus level community composition heat map, the left clustering tree shows the similarity between the various treatments. Red represents the genus with high abundance in the corresponding samples, and green represents the genus with low abundance. N0, N6, N12, N16 and N20 were 0, 90, 180, 240 and 300 kg·ha^{-1}, respectivel


N0
N12
N16
N20
N6
Bacteroidetes
Actinobacteria
Chloroflexi
Cyanobacteria
Acidobacteria
Rokubacteria
Proteobacteria
a: Paludibaculum
b: Aridibacter
c: Blastocatella
d: Stenotrophobacter
e: RB41
f: Pyrinomonadaceae
g: Pyrinomonadales
h: Blastocatellia__Subgroup_4_
i: metagenome
j: metagenome
k: Subgroup_22
l: Subgroup_25
m: 0319_7L14
n: Actinomarinales
o: Iamia
p: Iamiaceae
q: CL500_29_marine_group
r: Ilumatobacteraceae
s: Microtrichales
t: Acidimicrobiia
u: Mycobacterium
v: Mycobacteriaceae
w: Blastococcus
x: Geodermatophilaceae
y: Frankiales
z: Cellulomonas
a0: Cellulomonadaceae
a1: Galbitalea
a2: Klugiella
a3: Microbacterium
a4: Microbacteriaceae
a5: Arthrobacter
a6: Kocuria
a7: Pseudarthrobacter
a8: Micrococcaceae
a9: Promicromonospora
b0: Micrococcales
b1: Virgisporangium
b2: Aeromicrobium
b3: Kribbella
b4: Nocardioides
b5: Nocardioidaceae
b6: Propionibacteriales
b7: Lechevalieria
b8: Pseudonocardiaceae
b9: Pseudonocardiales
c0: Streptomyces
c1: Streptomycetaceae
c2: Streptomycetales
c3: Actinobacteria
c4: MB_A2_108
c5: Euzebyaceae
c6: Euzebyales
c7: Nitriliruptoria
c8: Gaiellales
c9: Parviterribacter
d0: Flavisolibacter
d1: Saprospiraceae
d2: Chitinophagales
d3: Rhodothermaceae
d4: Rhodothermales
d5: Rhodothermia
d6: Ardenticatenaceae
d7: Anaerolineae
d8: Chloroflexaceae
d9: Gitt_GS_136
e0: KD4_96
e1: TK10
e2: BD2_11_terrestrial_group
e3: Gemmatimonas
e4: S0134_terrestrial_group
e5: Latescibacteraceae
e6: Latescibacterales
e7: Latescibacteria
e8: Nitrospira
e9: Nitrospiraceae
f0: Nitrospirales
f1: Nitrospira
f2: Candidatus_Magasanikbacteria
f3: ABY1
f4: OM190
f5: Pla4_lineage
f6: Singulisphaera
f7: Isosphaeraceae
f8: Isosphaerales
f9: Brevundimonas
g0: Hyphomonadaceae
g1: Aminobacter
g2: Bauldia
g3: Nordella
g4: Rhizobiales_Incertae_Sedis
g5: Nitrobacter
g6: Altererythrobacter
g7: Sphingomonas
g8: Sphingomonadaceae
g9: Sphingomonadales
h0: Alphaproteobacteria
h1: Haliangium
h2: Haliangiaceae
h3: bacteriap25
h4: mle1_27
h5: Deltaproteobacteria
h6: B1_7BS
h7: Massilia
h8: IS_44
h9: MND1
i0: Nitrosospira
i1: Nitrosomonadaceae
i2: TRA3_20
i3: Betaproteobacteriales
i4: CCD24
i5: Aquicella
i6: Diplorickettsiaceae
i7: Diplorickettsiales
i8: Thioalkalispira
i9: Thioalkalispiraceae
j0: Ectothiorhodospirales
j1: Pseudomonas
j2: Pseudomonadaceae
j3: Pseudomonadales
j4: Polycyclovorans
j5: Solimonadaceae
j6: Salinisphaerales
j7: Gammaproteobacteria
j8: Rokubacteriales
j9: NC10

(a)

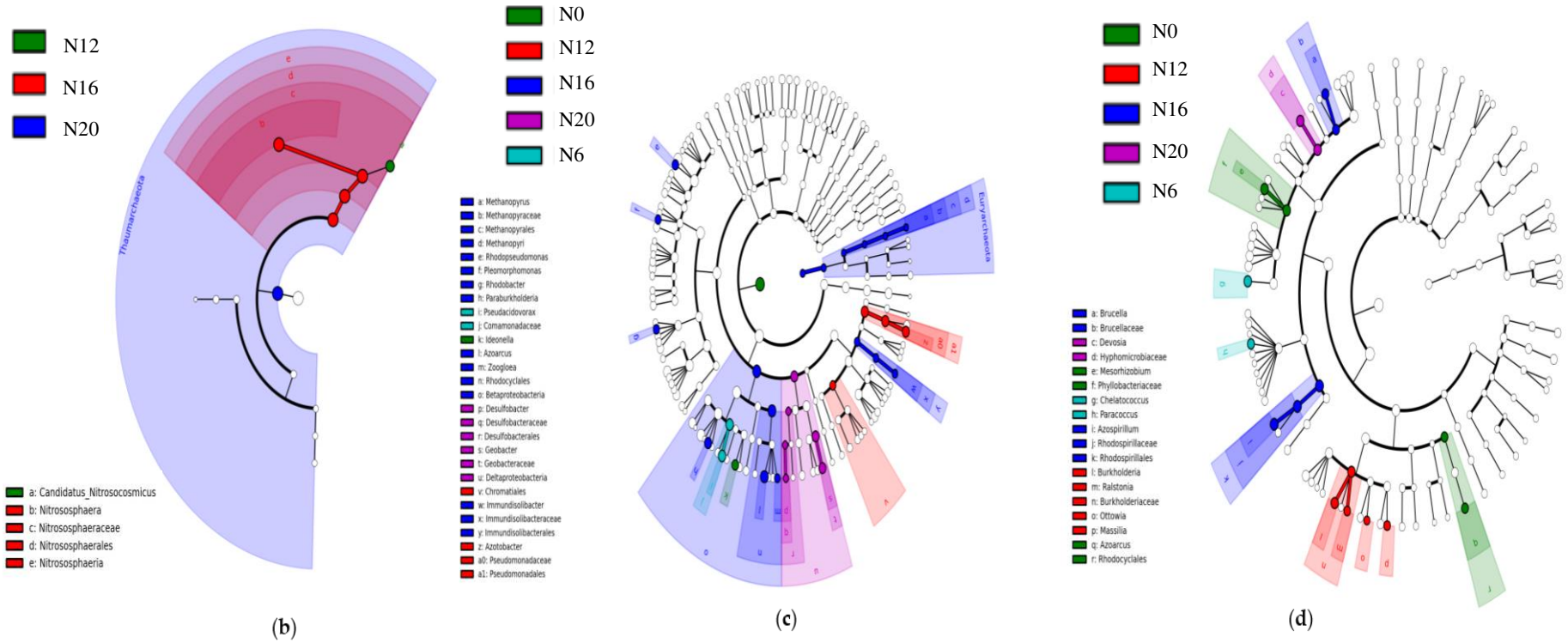


Fig. 5 The hierarchical tree of bacteria (a), ammonia-oxidizing archaea (b), nitrogen-fixing bacteria (c) and denitrifying bacteria (d) showed the hierarchical relationship of all taxonomic units from phylum to genus (arranged from inner circle to outer circle) in the community. The node size corresponds to the average relative abundance of the taxonomic unit. The yellow node represents the taxonomic unit that does not reflect the significant difference between groups, while other colors (such as green and red) show that these taxonomic units reflect the significant difference between groups, and the abundance is higher in the group samples represented by the color. N0, N6, N12, N16 and N20 were 0, 90, 180, 240 and 300 kg·ha^{-1}, respectively

Response of soil physical-chemical-biological properties to different nitrogen additions

By analyzing soil physiochemical properties of different treatments (Table 2), nitrogen application significantly reduced soil pH, ranging from 7.92 to 8.00. Soil total nitrogen and total carbon showed a trend of increasing first and then decreasing with the increase of nitrogen fertilizer additions. Under N16 treatment, soil total carbon content reached 22.63 g · kg^{-1}, which was significantly higher than N0 and N20. The soil bulk density decreased with the increase of nitrogen fertilizer, and the activities of urease, phosphatase and sucrase of N12 and N16 were higher than other nitrogen additions.

Table 2 The soil physical-chemical-biological properties

Treatments	pH	TN ($g \cdot kg^{-1}$)	TC ($g \cdot kg^{-1}$)	BD	Urease ($mg \cdot g^{-1} \cdot 24h^{-1}$)	Phosphatase ($mg \cdot g^{-1} \cdot 24h^{-1}$)	Invertase ($mg \cdot g^{-1} \cdot 24h^{-1}$)
N0	8.00 a	1.36 a	20.53 bc	1.54 a	0.59 c	1.74 ab	13.47 c
N6	7.98 a	1.36 a	21.53 ab	1.49 b	0.57 c	1.59 b	16.84 b
N12	7.98 a	1.40 a	21.72 ab	1.40 c	0.87 a	1.94 a	18.24 ab
N16	7.94 b	1.46 a	22.63 a	1.39 c	0.86 a	1.79 ab	19.42 a
N20	7.92 b	1.35 a	19.57 c	1.39 c	0.72 b	1.71 ab	12.77 c

* TN refers to soil total nitrogen, TC refers to soil total carbon, BD refers to soil bulk density. Different letters in the same column indicate significant differences at $P < 0.05$

Wheat yield and its components

The application of nitrogen fertilizer had a significant effect on wheat yield, which showed a gradual upward trend with the increase of nitrogen additions, and reached the highest yield of 8610 $kg \cdot hm^{-2}$ when the nitrogen addition was 300 $kg \cdot hm^{-2}$. (Fig. 6) In order to analyze the changes of yield components in the process of wheat yield growth, the linear regression analysis of spike number, grain number per spike and 1000-grain weight with yield was carried out. The results showed that with the increase of yield level, spike number, grain number per spike and 1000-grain weight showed an increasing trend. Among them, the correlation between spike number and yield reached extremely significant level (P<0.01), while the correlation between 1000-grain weight and yield was not significant. (Fig. 7)

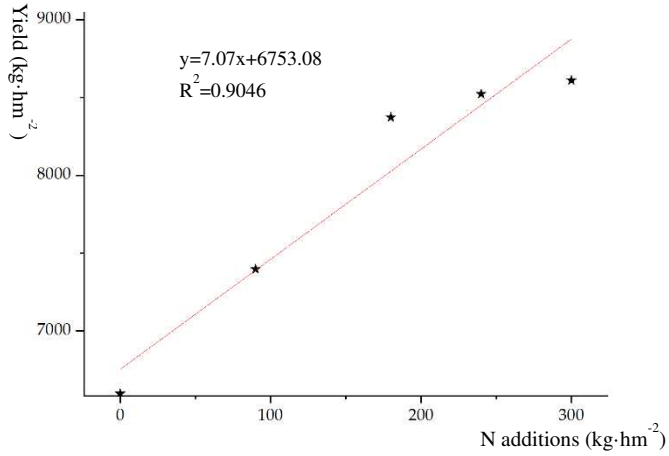


Fig.6 Linear analysis of wheat yield and N addition, R^2 is the determination coefficient to measure the fitting degree of regression equation

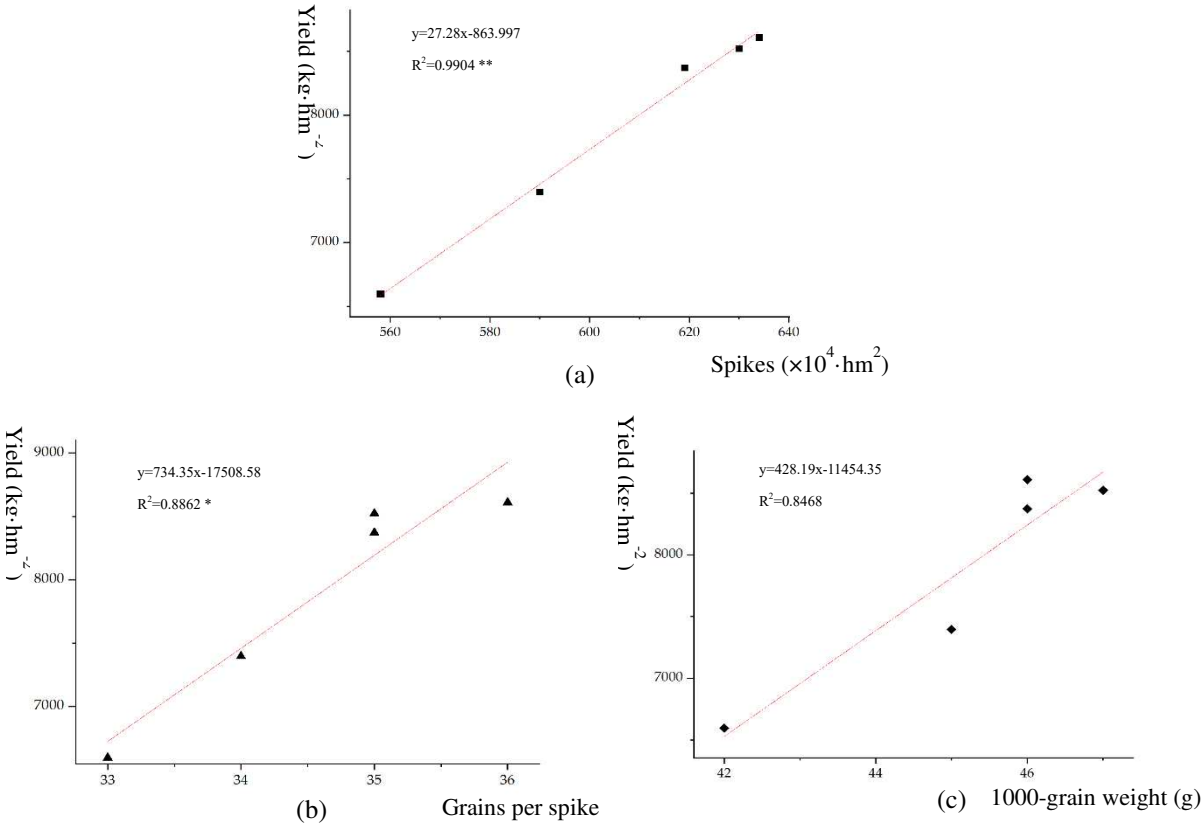


Fig. 7 Linear analysis of wheat yield and its components, where (a), (b), (c) denote the linear fitting of wheat spike number, grain number per spike and 1000-grain weight with yield, respectively, R^2 is the determination coefficient to measure the fitting degree of regression equation. * expressed at $P < 0.05$ level, ** expressed at $P < 0.01$ level

Response of soil properties, microbial community and yield to nitrogen addition

SEM model was constructed to evaluate the direct or indirect effects of nitrogen addition on soil properties, microbial diversity and wheat yield. (Fig. 8) The model showed that nitrogen addition had significant direct effects on wheat yield and soil properties ($P \leq 0.001$), and the λ values were 0.72 and 0.70, respectively. The effect on microbial diversity was not significant, $\lambda = 0.27$. Nitrogen addition increased spike number ($\lambda = 1$, $R^2 = 0.99$) and Urease ($\lambda = 0.95$, $R^2 = 0.90$) to improve yield and soil properties. The indirect effect of microbial diversity on crop yield through nitrogen addition was not significant ($P > 0.05$). Similarly, the indirect effect of soil traits on microbial diversity through nitrogen addition was not significant ($P > 0.05$). The effect of bacteria ($\lambda = 0.96$, $R^2 = 0.93$) was greater than that of *nifH* ($\lambda = 0.42$, $R^2 = 0.19$), followed by *nirK* ($\lambda = 0.3$, $R^2 = 0.09$) and AOA ($\lambda = -0.18$, $R^2 = 0.03$).

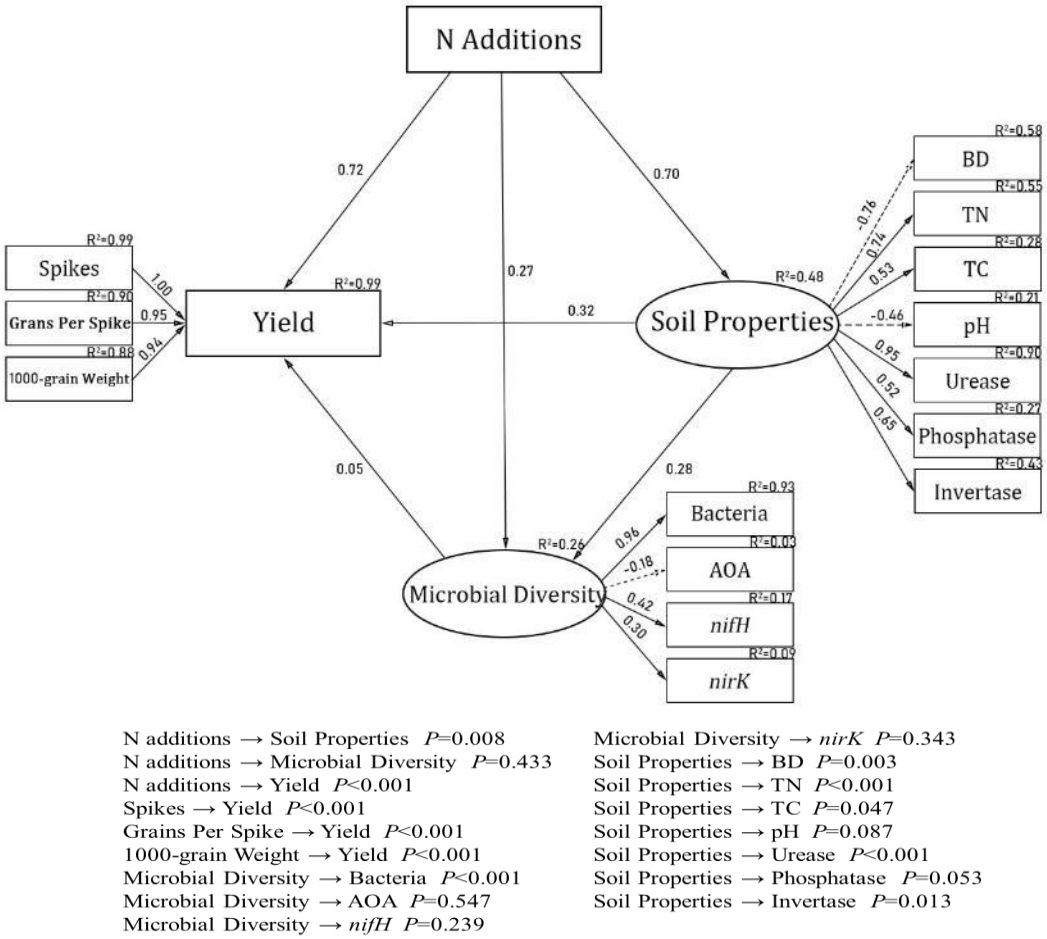


Fig.8 Structural equation model(SEM) of effects of nitrogen addition on soil properties, microbial diversity and wheat yield. The microbial diversity was reflected by the Shannon diversity index of bacteria, AOA, *nifH* nitrogen-fixing bacteria and *nirK* denitrifying bacteria. The number (λ) above the arrow represents the path coefficient, the solid line is positive, and the dashed line is negative. R^2 represents the variance ratio of the latent variable and the observed variable, and the significance is implied by $P < 0.05$

Discussion

Soil microbial community structure under different nitrogen additions

Soil microbial community determines soil fertility to a certain extent[15]. In the soil ecosystem, microorganisms mainly act as decomposers to decompose organic substances such as plant residues to complete the nutrients circulation and the energy flow of the food web. Kathleen et al.[16] showed that the competition between microorganisms and plants for the same resources led to a certain nutrient limitation effect. The reason for the above two results may be related to the differences in dominant bacteria in different microbial communities. For example, the products of organic matter decomposition will inhibit the growth of bacterial Actinomycetes, Therefore, studies by Mei et al.[17] on 40 years of organic fertilizer input showed that fertilizer application significantly reduced the relative abundance of actinomycetes. These results showed that the abundance of Actinomycete phylum in N0 treatment was significantly higher than that in other treatments, which revealed a similar effect.

Ammonia oxidizing archaea was the main driving force of ammonia oxidation process. Xu et al.[18] found that AOA community was significantly associated with soil potential nitrification (PNA), NH_4-N and NO_3-N. Liu et al.[19] also confirmed that AOA was sensitive to nitrogen fertilizer input and showed a downward trend, which was consistent with the trend under N0, N6 and N12 treatments in this study. In this experiment, Thaumarchaeota was the dominant phylum of ammonia-oxidizing archaea, and its abundance decreased first and then increased with the increase of nitrogen fertilizer addition, so Thaumarchaeota may have good tolerance to high nitrogen environment. Both *nifH* and showed changes in community structure stability under high nitrogen treatment. Studies have shown that pH is the most important factor affecting the *nifH* community[20]. In this study, with the increase of nitrogen fertilizer application, soil pH decreased gradually, and the number of dominant genera of *nifH* increased first and then decreased. *nirK* is involved in the denitrification process in soil[21], according to the abundance changes of dominant phylum and genera with significant differences, the relative abundance decreased significantly under high nitrogen treatment. This process aggravates the loss of nitrogen in farmland.

Soil microbial community diversity

Fertilization is one of the main factors affecting microbial community diversity[22], The difference in nitrogen fertilizer application rate will significantly affect the diversity of nitrogen cycle-related microbial communities in soil. In this study, with the increase of nitrogen addition, the bacterial richness increased overall, but the diversity index showed a trend of first increase and then decrease, which was different from the study of Castellano-Hinojosa[23] which showed the decrease of rhizosphere diversity of tomato and bean by nitrogen application rate. At present, the adverse effects of inorganic nitrogen fertilizer application on soil microbial diversity have been confirmed[24], but the specific results are still different depending on the amount of nitrogen fertilizer applied, Reasonable addition of nitrogen fertilizer can improve the diversity and activity of soil microbial community, and under the condition of reducing nitrogen fertilizer, nitrogen-related functional microbial community is affected to some extent[25]. The results of this study showed that the response of the diversity of AOA and *nifH* nitrogen-fixing bacteria to nitrogen gradient was roughly consistent with that of bacteria. Denitrification process involved by *nirK* Denitrifying Bacteria is the cause of soil nitrogen loss[26], With the increase of nitrogen fertilizer application amount, the diversity of denitrifying bacteria community had an obvious increasing trend. Therefore, the addition of excessive nitrogen fertilizer would enhance the denitrification process of soil.

Response of Nitrogen Related Functional Microorganisms to Nitrogen addition

Microorganisms involved in nitrogen cycling are closely related to AOA, *nifH* and *nirK* denitrifying bacterial communities[27]. At the phylum and genus level, the abundance of dominant bacteria in ammonia-oxidizing bacteria communities with high nitrogen fertilizer input was higher than that with low nitrogen fertilizer input, and there were more species with significant differences in N16 treatment. This result was consistent with the research result of Huang et al.[28], the effect of earthworm on ammonia oxidation, that is, the ammonia oxidation process is closely related to the abundance of related bacteria in soil.

Kumar[29] showed that the abundance and diversity of nitrogen-fixing bacteria community can be used as an important reference index to reduce nitrogen fertilizer input. Some studies have shown that soil organic carbon and available potassium are the main factors affecting the change of nitrogen-fixing bacteria community structure[30]. In this study, under N16 treatment, the number and abundance of significantly different species of *nifH*-type nitrogen-fixing bacteria were better than those under other treatments, and the community structure was significantly changed. Therefore, it was inferred that the process of soil nitrogen fixation was promoted by multiple factors.

Denitrification is the process of reducing nitrates and nitrites into nitrogen and returning it to the air[31], JI et al.[32] on the effect of nitrogen fertilizer application on denitrification process concluded that high nitrogen fertilizer input often has high N_2O emissions, this process may be related to the change of soil denitrifying bacteria community structure. N16 and N20 treatments significantly increased the abundance of denitrifying bacteria community, while the community diversity index and community composition under N12 treatment were higher than those under other treatments. Therefore, excessive nitrogen application mainly enhanced denitrification process by increasing the number of dominant denitrifying bacteria and reducing community diversity.

In summary, the soil microbial community structure was significantly affected by nitrogen addition in this study. Except for denitrifying bacteria, the other three microbial communities performed best under N12 treatment, but not well under high nitrogen input. This is mainly because with the increase of nitrogen input, nutrient accumulation reduces the diversity of their communities. Under N20 high nitrogen treatment, the leaching loss of nitrogen increased, and the enhancement of denitrification was not conducive to the stability of microbial community.

Effect of nitrogen fertilizer on wheat yield

Nitrogen fertilizer is a necessary element for crop growth and development, and reasonable nitrogen fertilizer input is the condition for wheat to obtain high yield[33]. At present, wheat production is facing continuous deficit, high nitrogen fertilizer input leads to serious low productivity of wheat[34]. The fertilizer requirement of wheat is regulated by many factors, such as wheat type, precipitation, tillage and so on[35,36]. The more rainfall, the lower the average annual temperature, the greater the nitrogen requirement of wheat[37]. In this study, the nitrogen application rate of 300 $kg \cdot hm^{-2}$ wheat reached the highest yield in all treatments. Previous studies showed that the application of nitrogen fertilizer significantly increased the spike number of wheat, while the 1000-grain weight showed a downward trend[38,39]. The results are consistent with our research. The increase of wheat spike number caused by nitrogen application is an important reason for high yield of wheat. In the future, under the premise of controlling nitrogen fertilizer input and improving nitrogen use efficiency, as far as possible to improve 1000-grain weight of wheat will become the focus of our research.

In this study, with the increase of fertilization level, wheat yield increased gradually. However, under the two treatments of N16 and N20, the yield increase was significantly lower than that of N6 and N12. Therefore, combining the two factors of microbial community and crop yield, the optimum

fertilization level is 180 kg·hm^{-2} to ensure the high yield of wheat under the premise of stable microbial community.

Conclusions

Nitrogen application significantly changed the microbial community structure in soil, and the richness of bacteria, archaea ammonia oxidation, *nifH*-type nitrogen-fixing bacteria and *nirK*-type nitrogen-fixing bacteria were the highest under N12 treatment, indicating that the community abundance was relatively high under this treatment. The diversity of *nirK* denitrifying bacteria under N20 treatment were highest.

N12 treatment significantly increased the relative abundance of Proteobacteria, Bacteroidetes, Nitrospira and Rokubacteria, but no significant difference was found at the *nifH* and *nirK* bacterial phyla levels.

N12 treatment significantly improved the community diversity and structural stability of ammonia oxidizing bacteria and *nifH* nitrogen-fixing bacteria, which was beneficial to the recycling of soil nitrogen. Under high nitrogen treatment of N16 and N20, the dominant bacteria of *nirK*-type denitrifying bacteria increased significantly compared with N12 treatment.

The effect of nitrogen addition on soil properties and wheat yield was greater than that on microbial diversity. In order to maintain the stability of microbial community structure under the premise of high yield of wheat, N12 treatment performed best.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request. The sequencing data generated in this study can be obtained in the NCBI SRA database, and their numbers are PRJNA883614 (bacteria), PRJNA883617 (ammonia-oxidizing archaea), PRJNA883622 (nitrogen-fixing bacteria), and PRJNA883626 (denitrifying bacteria).

References

1 Zhang, D. B. *et al.* Trade-offs between winter wheat production and soil water consumption via leguminous green manures in the Loess Plateau of China. *Field Crops Research* **272**, 108278, doi:https://doi.org/10.1016/J.FCR.2021.108278 (2021).

2 Xu, Y. X. *et al.* Effect of terrace construction on soil moisture in rain-fed farming area of Loess Plateau. *Journal of Hydrology: Regional Studies* **37**, 100889, doi:https://doi.org/10.1016/J.EJRH.2021.100889 (2021).

3 Effah, Z. *et al.* Regulation of Nitrogen Metabolism, Photosynthetic Activity, and Yield Attributes of Spring Wheat by Nitrogen Fertilizer in the Semi-arid Loess Plateau Region. *Journal of Plant Growth Regulation*, doi:https://doi.org/10.1007/S00344-022-10617-1 (2022).

4 Khampuang, K., Rerkasem, B., Lordkaew, S. & Prom-u-thai, C. Nitrogen fertilizer increases

grain zinc along with yield in high yield rice varieties initially low in grain zinc concentration. *Plant and Soil* **467**, 239-252, doi:https://doi.org/10.1007/S11104-021-05090-W (2021).

5 Li, B. B. *et al.* Long-term excess nitrogen fertilizer increases sensitivity of soil microbial community to seasonal change revealed by ecological network and metagenome analyses. *Soil Biology and Biochemistry* **160**, 108349, doi:https://doi.org/10.1016/J.SOILBIO.2021.108349 (2021).

6 Nasiroleslami, E., Mozafari, H., Sadeghi-Shoae, M., Habibi, D. & Sani, B. Changes in yield, protein, minerals, and fatty acid profile of wheat (Triticum aestivum L.) under fertilizer management involving application of nitrogen, humic acid, and seaweed extract. *Journal of Soil Science and Plant Nutrition* **21**, 1-10, doi:https://doi.org/10.1007/S42729-021-00552-7 (2021).

7 Siczek, A., Frąc, M. & Gryta, A. Forecrop Effects on Abundance and Diversity of Soil Microorganisms during the Growth of the Subsequent Crop. *Agronomy* **10**, 1971, doi:https://doi.org/10.3390/AGRONOMY10121971 (2020).

8 Traoré, O. Y. A., Kiba, D. I., Bünemann, E. K. & Oberson, A. Nitrogen and phosphorus uptake from isotope - labeled fertilizers by sorghum and soil microorganisms. *Agrosystems, Geosciences & Environment* **3**, e20111, doi:https://doi.org/10.1002/AGG2.20111 (2020).

9 Abbas, T. *et al.* Anammox co-fungi accompanying denitrifying bacteria are the thieves of the nitrogen cycle in paddy-wheat crop rotated soils. *Environment International* **130**, 104913, doi:https://doi.org/10.1016/j.envint.2019.104913 (2019).

10 Nakamura, S., Saliou, P. S., MinakoTakahashi, Ando, Y. & Subbarao, G. V. The Contribution of Root Turnover on Biological Nitrification Inhibition and Its Impact on the Ammonia-Oxidizing Archaea under Brachiaria Cultivations. *Agronomy* **10**, 1003, doi:https://doi.org/10.3390/agronomy10071003 (2020).

11 Zhang, H. M., Li, X. L. & Yang, F. Effects of warming and nitrogen fertilization on soil microbial nitrogen physiology groups in alpine meadow. *Acta Agrestia Sinica* **28**, 606-612 doi:https://doi.org/10.11733/j.issn.1007-0435.2020.03.003 (in Chinese) (2020).

12 Wan, Y., Li, W. J., Wang, J. & Shi, X. J. Bacterial diversity and community in response to long-term nitrogen fertilization gradient in citrus orchard soils. *Diversity* **13**, 282, doi:https://doi.org/10.3390/D13070282 (2021).

13 Fierer, N. *et al.* Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. *The ISME journal* **6**, 1007-1017, doi:https://doi.org/10.1038/ismej.2011.159 (2012).

14 Lu, H. *et al.* Effects of half-reduced nitrogen fertilization combined with organic fertilizer on functional diversity of soil microbial communities in oat field. *Chinese Journal of Ecology* **38**, 3660-3666 doi:https://doi.org/10.13292/j.1000-4890.201912.029 (in Chinese) (2019).

15 Yuan, R. W., Liu, L., Zhang, R. & Fan, S. Y. The Interaction mechanism between plant rhizosphere secretion and soil microbe: a review. *Chinese Agricultural Science* **36**, 26-35, doi:https://doi.org/10.11924/j.issn.1000-6850. (in Chinese) (2020).

16 Lemanski, K., Armbruster, M. & Bonkowski, M. Linking soil microbial nutrient limitation to fertilizer regime and sugar beet yield. *Plant and Soil* **441**, 253-259, doi:https://doi.org/10.1007/s11104-019-04114-w (2019).

17 Mei, N. *et al.* Effects of 40 years applications of inorganic and organic fertilization on soil bacterial community in a maize agroecosystem in northeast China. *European Journal of Agronomy* **130**, doi:https://doi.org/10.1016/j.eja.2021.126332 (2021).

18 Xu, A. X. *et al.* Changes in Ammonia-Oxidizing Archaea and Bacterial Communities and Soil Nitrogen Dynamics in Response to Long-Term Nitrogen Fertilization. *International Journal of Environmental Research and Public Health* **19**, 2732, doi:https://doi.org/10.3390/IJERPH19052732 (2022).

19 Liu, L. Z., Ma, S. H., Li, X. L., An, T. T. & Wang, J. K. Effects of long-term fertilization on soil ammonia-oxidizing microorganisms. *Chinese Journal of Applied Ecology* **31**, 1459-1466 doi:https://doi.org/10.13287/j.1001-9332.202005.024 (in Chinese) (2020).

20 Zhang, C. Z. *et al.* Long-term nitrogen fertilization shaped the nifH, nirK and nosZ gene community patterns in red paddy soil in south China. *Canadian Journal of Microbiology* **67**, 310-322, doi:https://doi.org/10.1139/cjm-2020-0300 (2020).

21 Wang, N. *et al.* Roles of bulk and rhizosphere denitrifying bacteria in denitrification from paddy soils under straw return condition. *Journal of Soils and Sediments* **21**, 2179–2191, doi:https://doi.org/10.1007/S11368-021-02942-X (2021).

22 Chen, Y. L. *et al.* Diversity of rice rhizosphere microorganisms under different fertilization modes of slow-release fertilizer *Scientific Reports* **12**, 2694, doi:https://doi.org/10.1038/S41598-022-06155-1 (2022).

23 Castellano-Hinojosa, A., Strauss, S. L., González-López, J. & Bedmar, E. J. Changes in the diversity and predicted functional composition of the bulk and rhizosphere soil bacterial microbiomes of tomato and common bean after inorganic N-fertilization. *Rhizosphere* **18**, 100362, doi:https://doi.org/10.1016/j.rhisph.2021.100362 (2021).

24 Zhang, M., Liu, Y. L., Wei, Q. Q. & Gou, J. L. Biochar enhances the retention capacity of nitrogen fertilizer and affects the diversity of nitrifying functional microbial communities in karst soil of southwest China. *Ecotoxicology and Environmental Safety* **226**, 112819, doi:https://doi.org/10.1016/J.ECOENV.2021.112819 (2021).

25 Liu, G. Y. *et al.* Adding NBPT to urea increases N use efficiency of maize and decreases the abundance of N-cycling soil microbes under reduced fertilizer-N rate on the North China Plain. *PloS one* **15**, e0240925, doi:https://doi.org/10.1371/journal.pone.0240925 (2020).

26 Dai, J. L. & Miao, Y. J. Nitrogen recycling function and micro-organism of different saline-alkali agroecosystems around Yellow river estuary delta. *Journal of Safety and Environment* **19**, 1041-1048 doi:https://doi.org/10.13637/j.issn.1009-6094.2019.03.043 (in Chinese) (2019).

27 Toshihide, H., Masanori, F., Nobuhiko, S., Hisaya, K. & Manabu, F. Influence of understory vegetation on soil bacterial communities and nitrogen cycling gene abundance in cool-temperate and sub-alpine forests along an elevational gradient. *Pedobiologia - Journal of Soil Ecology* **87-88**, 150746, doi:https://doi.org/10.1016/J.PEDOBI.2021.150746 (2021).

28 Huang, K., Xia, H., Cui, G. Y. & Li, F. S. Effects of earthworms on nitrification and ammonia oxidizers in vermicomposting systems for recycling of fruit and vegetable wastes. *Science of the Total Environment* **578**, 337-345, doi:https://doi.org/10.1016/j.scitotenv.2016.10.172 (2017).

29 Kumar, P., Brar, S. K., Pandove, G. & Aulakh, C. S. Bioformulation of Azotobacter spp. and

Streptomyces badius on the productivity, economics and energetics of wheat (Triticum aestivum L.). *Energy* **232**, doi:https://doi.org/10.1016/j.energy.2021.120868 (2021).

30 Wang, Z. K., Xu, Z. H., Chen, Z. Y. & Fragrance. Synergistic effects of organic fertilizer coupled with phosphate-solubilizing and nitrogen-fixing bacteria on nutrient characteristics of yellow-brown soil under carbon deficiency. *Chinese Journal of Applied Ecology* **31**, 3413-3423 doi:https://doi.org/10.13287/j.1001-9332.202010.023 (in Chinese) (2020).

31 Mergel, A., Schmitz, O., Mallmann, T. & Bothe, H. Relative abundance of denitrifying and dinitrogen-fixing bacteria in layers of a forest soil. *FEMS Microbiology Ecology* **36**, 33-42, doi:https://doi.org/10.1016/S0168-6496(01)00113-1 (2001).

32 Ji, M. M. *et al.* Enhanced N2O emission rate in field soil undergoing conventional intensive fertilization is attributed to the shifts of denitrifying guilds *Pedosphere* **31**, 145-156, doi:https://doi.org/10.1016/S1002-0160(20)60050-9 (2021).

33 Tomaz, A. *et al.* Yield, technological quality and water footprints of wheat under Mediterranean climate conditions: A field experiment to evaluate the effects of irrigation and nitrogen fertilization strategies. *Agricultural Water Management* **258**, 107214, doi:https://doi.org/10.1016/J.AGWAT.2021.107214 (2021).

34 Dume, B. Y. *et al.* Integrated use of compost and nitrogen fertilizer and its effects on yield and yield components of wheat: a pot experiment. *bioRxiv*, doi:https://doi.org/10.1101/2020.10.07.329524 (2020).

35 Kandel, T. P., Gowda, P. H., Northup, B. K., Rocateli, A. C. & Moral, M. T. Impacts of tillage systems, nitrogen fertilizer rates and a legume green manure on light interception and yield of winter wheat. *Cogent Food & Agriculture* **5**, doi:https://doi.org/10.1080/23311932.2019.1580176 (2019).

36 Tyutyunov, S., Solntsev, P., Stupakov, A., Kulikova, M. & Khalaf, A. D. H. Agroecological justification of winter wheat fertilization systems in the south-west of the Central Black-soil region. *E3S Web of Conferences* **175**, 07005, doi:https://doi.org/10.1051/e3sconf/202017507005 (2020).

37 Ma, D. K. *et al.* A Meta-analysis of the effects of nitrogen application rates on yield and water use efficiency of winter wheat in dryland of Loess Plateau. *Scientia Agricultura Sinica* **53**, 486-499 doi:https://doi.org/10.3864/j.issn.0578-1752.2020.03.003 (in Chinese) (2020).

38 Lu, J. S. *et al.* Response of yield, yield components and water-nitrogen use efficiency of winter wheat to different drip fertigation regimes in Northwest China. *Agricultural Water Management* **255**, 107034, doi:https://doi.org/10.1016/J.AGWAT.2021.107034 (2021).

39 Wang, X. Y., Zhao, S. D., Zheng, X. F., Wang, Z. H. & He, G. Effects of straw returning and nitrogen application rate on grain yield and nitrogen utilization of inter wheat. *Scientia Agricultura Sinica* **54**, 5043-5053 doi:https://doi.org/10.3864/j.issn.0578-1752.2021.23.010 (in Chinese) (2021).

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Author Contributions

All authors contributed to the study conception and design. Y.F.: software, formal analysis, writing—original draft preparation, visualization. Q.L.: investigation, writing—review and editing. R.H.: software. Y.W.: validation. L.G.: validation, writing—review and editing. Z.Y.: methodology, resources. T.H.: validation, writing—review and editing, project administration. Z.G.: Conceptualization, resources. Y.Q.: Conceptualization, writing—review and editing, supervision, funding acquisition. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.