Variation of Soil Bacterial Communities during Lettuce Continuous Cropping

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Abstract. The variation of bacterial community in lettuce continuous cropping was determined by high throughput sequencing. During the continuous planting of lettuce, the richness and diversity of bacterial communities in the soil increased, and the ACE index and Chao index increased by 40.21 % and 36.91 %, respectively. The proportion of Actinobacteria, Chloroflexi, Firmicutes and Nitrospirae in the soil increased, while the abundance of Acidobacteria, Bacteroidetes, Gemmatimonadetes, Planctomycetes and Proteobacteria gradually declined. And the abundance in the soil accounting for 1 % of the dominant bacterial genera increased to 11, among them, Anaerolinea, Bacillus, Nitrosomonas, and Xanthomonas etc became the dominant bacterium genus in the soil after lettuce continuous cropping. After the lettuce had been planted 8 times, the yield decreased by 21.20 % compared to the first harvest. Lettuce continuous cropping had an effect on bacterial community and lettuce yield to some extent.

1 Introduction

Lettuce (*Lactuca sativa* L.) is an annual herb of the family Asteraceae, which in vitamins, carotenoids, fiber, as well as polyphenols. Lettuce contains a variety of minerals and beneficial to human health, such as Fe, Zn, Ca, P, Mg, Mn, K, etc. Lettuce has been eaten by more and more people as a healthy food in recent years in China^[1-2]. Lettuce can be divided into six types, namely crisphead lettuce, butterhead lettuce, romaine or cos lettuce, leaf or cutting lettuce, steam or stalk lettuce and latin lettuce, according to the shape, size, texture, head formation and stem type of lettuce leaves^[3]. Spain, Italy and France are the main lettuce producing countries in Europe, with production accounting for 35 %, 21 % and 13 % of total lettuce production in Europe, respectively^[4]. China is one of the important country in the output of lettuce in the world^[5]. In 2011-2016, only the proportion of leafy vegetable plantings in Beijing accounted for 44.0 % of total vegetable acreage^[6]. According to survey, in 2013, the area planted with leafy vegetables in Beijing was 30000 hectares, of which lettuce accounted for 14.4 % and the output reached 200 million kilograms^[7-8]. Due to the planting area is finite, the demand of lettuce rises to be able to bring about continuous cropping obstacle, unavoidably. Long-term cultivation of lettuce may be susceptible to disease, such as downy mildew, gray mold and virus disease, as described by Cui^[9]. Because of the continuous cropping, some hazardous substance were accumulated in the soil, which became

the cause of the disease in the following year. Microorganisms are efficient bioindicators of soil biological characteristics, because their ability to respond quickly to environmental changes^[10]. Many studies had shown that changes in the size and activity of the soil microbial community were major contributors to soil degradation caused by agricultural management^[11]. Soil organisms play important roles in maintaining soil health and quality^[12]. The purpose of this study is to analyze the variation of soil bacterial communities during lettuce continuous cropping for eight times.

2 Materials and Methods

2.1 Field experiment

The experimental field is located in the plastic greenhouse of the crop variety test demonstration base (116.14 ° east longitude, 40.19 ° north latitude) in Changping District, Beijing. The test field is 400 m² (50 m long, 8 m wide), soil type is sandy loam. The planting density is 80 plants per bed, and the plant spacing is 0.3 m × 0.3 m. During the experiment, organic fertilizer was applied at 3 000 kg/acre, and the basic physical and chemical indicators of the soil were tested: soil alkali N 153.21 g/kg, soil available P 270 g/kg, soil available K 312.45 g/kg, pH 6.90. The planting lettuce variety is North Sansheng No. 2. The test was conducted from September 2016 to June 2018. The specific sample

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information is shown in Table 2-1. The continuous crop has 12 beds, 6.5 m long and 1.2 m wide, in winter had a leisure period. The sample naming method is N_ planting year_ planting times_ planting (1)/ harvest (2), for example: N_17_5_1 indicates the 5th lettuce in 2017 before planting.

According to the experimental design, the sampling range is determined, the surface floating soil is removed, the soil with a depth of 0-20 cm is excavated, and the visible impurities are removed. After the sample is collected, it is loaded into the sampling bag and numbered. Each sample was sampled by 5-point sampling method. After mixing and removing impurities, 5-10 g was taken, stored in a sterile centrifuge tube, placed in an incubator, and transport-ed to the laboratory in 1 hand store at -80 °C .

2.2 Determination of lettuce yield

When the lettuce was harvested, fresh plants were gathered, and 9 plants were randomly taken from each bed and repeated 3 times to determine the fresh weight of the plants.

Table 1. Description of soil samples

Samples	Collection date	Depth (cm)	State of crop growth	Cultivation time
CK	2016.09.09	0-20	—	—
N_16_1_1	2016.09.09	0-20	Before cultivation	1st
N_16_1_2	2016.10.20	0-20	Harvest	1st
N_17_2_1	2017.03.10	0-20	Before cultivation	2nd
N_17_2_2	2017.04.27	0-20	Harvest	2nd
N_17_3_1	2017.05.23	0-20	Before cultivation	3rd
N_17_3_2	2017.06.20	0-20	Harvest	3rd
N_17_4_1	2017.07.02	0-20	Before cultivation	4ht
N_17_4_2	2017.07.26	0-20	Harvest	4th
N_17_5_1	2017.09.19	0-20	Before cultivation	5th
N_17_5_2	2017.10.20	0-20	Harvest	5th
N_17_6_1	2017.11.05	0-20	Before cultivation	6th
N_17_6_2	2017.12.13	0-20	Harvest	6th
N_18_7_1	2018.03.20	0-20	Before cultivation	7th
N_18_7_2	2018.05.03	0-20	Harvest	7th
N_18_8_1	2018.05.18	0-20	Before cultivation	8th
N_18_8_2	2018.06.21	0-20	Harvest	8th

2.3 Total DNA extraction and amplification of 16S rRNA

Microbial DNA was extracted from 1.0g soil samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's protocols. The quality of the extracted DNA was determined using agarose gel electrophoresis (0.8%), and the DNA was quantified using a UV spectroph-otometer. The extracted DNA was stored at -80°C prior to analysis. The V3-V4 region of the bacteria 16S ribosomal RNA gene were amplified by PCR 95 ° C for 3 min, followed by 25 cycles at 95 ° C for 30 s, 50 ° C for 30 s, and 72 ° C for 45 s and a final extension at 72 ° C for 10 min using primers 338F(5'-barcode-ACTCCTACGGGAGGCAGCAG)-3'a

nd 806R(5'-GGACTACHVGGGTWTCTAAT -3')[13], according to previously published protocols, where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate 20 µL mixture containing 4 µL of 5×FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase, and 10 ng of template DNA.

2.4 Illumina MiSeq sequencing

Amplicons were extracted from 2 % agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced (2×250) on an Illumina MiSeq platform according to the standard protocols.

2.5 Processing of sequencing data

Raw fastq files were demultiplexed, quality-filtered using QIIME (version 1.17) with the following criteria: (i) The 300 bp reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50 bp. (ii) exact barcode matching, 2 nucleotide mismatch in primer matching, reads containing ambiguous characters were removed. (iii) only sequences that overlap longer than 10 bp were assembled according to their overlap sequence. Reads which could not be assembled were discarded. Operational Units (OTUs) were clustered with 97 % similarity cutoff using UPARSE(ver-sion 7.1 <http://drive5.com/uparse/>) and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier (<http://rdp.cme.msu.edu/>) against the silva (SSU115) 16S rRNA database using confidence threshold of 70 %^[14]

3 Results

As shown in Fig.1, the yield of lettuce in the previous four plantings was 5.14 kg/m², 5.29 kg/m², 5.01 kg/m² and 4.96 kg/m², respectively, and the total remained at about 5.10 kg/m². With the increased of continuous cropping times, the yield of lettuce decreased significantly during the 5th harvest, which was 13.96 % lower than that of the 1st harvest. Then, the yield decreased by 16.24 %, 16.04 % and 21.20 % respectively, in 6th, 7th and 8th planting times. This indicates that as the time of continuous crops increases, it will have an impact on the yield of lettuce, and the yield will decrease significantly after 5th lettuce continuous croppings.

The rarefaction curve reflects the depth of sampling and can be used to assess whether or not the number of sequences is sufficient to cover all taxa. The bacteriarichness rarefaction curve showed that, as the number of sequences increases, the increase

Fig.1. Lettuce yield during continuous cropping

in the number of OTUs in each sample tends to flatten and eventually reaches saturation. The amount of coverage obtained during the sample sequencing was calculated at a similarity level of 97 %. The results showed that the amount of coverage in all of the sample libraries was adequate. The rate was approximately 99 %, indicating that the sampling was sufficient and the confidence in the structure of the bacteria community that was obtained in the real environment was high, which indicates that our analysis truly reflects the bacteria community present in the soil samples(Fig. 2).

3.1 Soil bacterial diversity and community structure during continuous cropping

The bacterial 16S rRNA diversity index in continuous cropping soil samples was analyzed (Table 2), and the bacterial diversity index fluctuated with the increased of continuous cropping times. The bacterial diversity index of continuous cropping soil samples showed an increasing trend except shannon index, which decreased by 0.46 %. OTU increased from 2010 to 2623, which increased by 30.50 %. ACE index, Chao index and Simpson index increased by 40.21 %, 36.91 % and 26.55 %. This indicated that continuous cropping could increase the diversity of bacteria in soil.As shown in Fig. 3, before continuous cropping of lettuce, the dominant bacterial phyla with more

Fig.2. Rarefaction curves indicating the OTUs observed at a genetic distance of 3 % in all soil samples.

than 5 % abundance in the soil was Proteobacteria, Acidobacteria, Bacteroidetes, Chloroflexi, Gemmatimonadetes and Actinobacteria, accounting for 41.36 %,21.23 %, 8.01 %, 7.75 %, 6.57 % and 5.72 %

respectively in the soil, accounting for 90.65 % in total. Firmicutes, Nitrospirae and Planctomycetes, which account for 1-5 % of abundance, account for

Table 2 Index of bacterial richness and diversity inlettuce continuous cropping soil samples

Samples	OTU	ACE	Chao	Simpson	Shannon	Coverage
CK	2010	2196.3776	2238.4194	0.0029	6.5981	0.9934
N_16_1_1	2330	2461.4484	2519.1346	0.0046	6.5293	0.9940
N_16_1_2	2267	2439.0364	2490.7783	0.0048	6.5307	0.9931
N_17_2_1	2372	2502.4326	2544.6884	0.0033	6.7137	0.9942
N_17_2_2	2440	2545.4091	2561.0041	0.0035	6.7126	0.9947
N_17_3_1	2399	2501.7245	2536.5756	0.0026	6.8166	0.9947
N_17_3_2	2469	2538.7585	2542.3797	0.0026	6.8606	0.9958
N_17_5_1	2793	3219.1124	3239.8060	0.0039	6.6393	0.9875
N_17_5_2	2727	3117.2521	3119.2598	0.0037	6.6618	0.9883
N_18_8_1	2465	3026.7693	3086.6196	0.0057	6.3946	0.9868
N_18_8_2	2623	3079.4499	3064.7124	0.0037	6.5678	0.9875

2.86 %, 1.54 % and 1.70 %, respectively, in soils. As the growth of the continuous cropping time, dominant bacterial in the soil phyla proportion also gradually changed, Actinobacteria, Chloroflexi, Firmi-cutes and Nitrospirae the proportion in the soil was increasing after lettuce planting for 8 times, account-ed for 13.88 %, 19.48 %, 14.16 % and 2.58 % res-pectively, compared with before the continuous cropping were increased by 8.16 %, 11.73 %, 11.31 % and 1.04%. However, Acidobacteria, Bacteroidetes, Gemmatimonadetes, Planctomycetes and Proteobacteria, abundance accounting in the soil decreased, the relative abundance in soil was 18.66 %, 2.72 %, 2.01 %, 1.52 % and 22.13 % respectively, which decreased by 2.57 %, 5.29 %, 4.56 %, 0.18 % and 19.23 %. It can be seen that continuous cropping has a certain impact on the composition of soil bact-erial community.

Fig. 3. Relative abundance of the bacterial phyla presents in the continuous cropping soil samples.

There are 8 dominant bacteria genus belonging to the soil with a relative abundance of more than 1 % at the

beginning of planting, as shown in Fig.4, *Adhaeribacter*, *Bacillus*, *Bryobacter*, *H16*, *Lysobacter*, *Pontibacter*, *RB41* and *Sphingomonas*, the proportion was 1.06 %, 1.84 %, 1.31 %, 1.89 %, 6.22 %, 1.10 %, 2.10 % and 7.29 %, respectively. After 8 times lettuce cropping, soil dominant genus were *Bacillus*, *H16*, *Nitrospira*, *norank_c_Acidobacteria*, *norank_c_KD4-96*, *norank_f_Anaerolineaceae*, *norank_f_Gemmatimonadaceae*, *norank_f_Nitrosomonadaceae*, *norank_o_AKYG1722*, *norank_o_JG30-KF-CM45* and *norank_o_Xanthomonadales*, accounting abundance in the soil were 7.32 %, 1.21 %, 2.58 %, 15.52 %, 2.18 %, 6.70 %, 1.19 %, 1.19 %, 1.09 %, 4.04 %, 1.37 %, the type and number of advantages compared with the previous species planted have undergone significant. The change of dominant bacteria from 8 to 11, of which only *Bacillus* and *H16* is the dominant genus in the soil through the lettuce continuous cropping. The results showed that the continuous cropping of lettuce can increase the diversity of bacterial communities in the soil, and the composition of bacterial communities in the soil can be changed with the increased of continuous cropping time.

Fig. 4. Relative abundance of the bacterial genus presents in the continuous cropping soil samples

At the genus level, clusters of the genus of the sample and the sample are clustered. According to the different OTU numbers in each sample after clustering, a Heatmap is generated corresponding to the abundance of the contained sequence (Fig.5), and the change of the color gradient can reflect the difference in bacterial community structure of each sample at the level of the genus. The abundance of the genus is affected by the conditions of lettuce continuous cropping, *norank_o_Anaerolineaceae*, *norank_c_Acidobacteria*, *Bacillus*, *norank_c_KD4-96*, *norank_o_JG30-KF-CM45* and *Nitrospira*.

4 Discussion

From the results of high-throughput sequencing, the coverage of each sample in continuous soil was over 99 %, which proved that the sequencing results can represent the real situation of microorganisms in the soil. With the continuous cropping of lettuce, the diversity and richness of bacterial communities in the soil increased gradually, and the ACE index and Chao index increased by 40.21 % and 36.91 %, respectively. The higher is the diversity index, the higher the diversity of microbial communities in the soil, which is mainly composed of the diversity and richness of the community^[15]. Zhao et al. found that the species richness of bacterial communities in soil increased after 1 year of continuous crop-ping, which was consistent with our experiment, but after strawberry continuous cropping for 8 years, the species richness of soil bacterial communities is reduced, which is different from our experiment result. It may be due to the short planting period of lettuce, and the diversity of bacteria has not decreased significantly^[16].

The continuous cropping of lettuce also has an effect on the composition of the bacterial community in the soil. During the continuous cropping of lettuce, the species of dominant bacteria in the soil did not change were Proteobacteria, Acidobacteria, Bacteroides, Chloroflexi, Gemmatimonadetes, Actinobacteria, Firmicutes, Nitrospirae and Planctomycetes, but their relative abundance changes in the soil, including Actinobacteria, Chloroflexi, Firmicutes and Nitrospira in the soil the proportion is increasing, and Acidobacteria, Bacteroides, Gemmatimonadetes, Planctomycetes and Proteobacteria, gradually decreased in the soil. This is consistent with the results of Ouyang et al. who found that the abundance of Firmicutes, Actinomycetes and Chloroflexi increased in the soil of banana continuous cropping for 5 years, and became the main bacterial population in the soil^[17]. Zhao et al. studies had shown that the abundance of Proteobacteria and Bacteroides increasing can promote the transformation of organic matter in soil, which may have had an impact on crop yields, while in this experiment both decline in the process of lettuce continuous cropping and the lettuce yield fell by 21.20% after 8th lettuce continuous cropping^[18].

After 8 times continuous crops of lettuce, the abundance of Anaerolineaceae in the soil increased, becoming the dominant genus in the soil. Anaerolineaceae is a representative group of the Chloroflexi which is a kind of facultative anaerobic organism and can not produce oxygen in photosynthesis and can not nitrogen fixation, but it can decompose organic matter and degrade in anaerobic environment^[19-20]. The abundance of Anaerolineaceae increase during continuous cropping may affect the absorption and utilization of nitrogen by plants. In addition, Bacill-

Fig. 5 Heatmap of the bacterial genus present of the continuous cropping soil samples

-us, Nitrosomonas, and Xanthomonas are the dominant genus in the soil after 5 years of continuous cultivation of *Artemisia annua* L, and these three genera also become the dominant genus in the soil after continuous lettuce production[21]. Studies had found that *Sphingomonas* can absorb Cd in soil and degrade a variety of aromatic chemical contaminants[22]. The abundance of *Sphingomonas* in the continuous soil of lettuce also decreased from 7.29 % to 0.77 %, which may affect the degradation of Cd and aromatic chemical pollutants in the soil, resulting in accumulation in the soil, endangering the growth of lettuce plants.

From the heatmap and community structure, the diversity and abundance of bacteria do not change obviously, which is the same with most field conclusion. Through the observation of potato continuous cropping for 7 years, the abundance of *Fusarium* in the soil increased, and the soil-borne diseases became more serious, which in turn affected crop growth[23]. Continuous cropping had inhibitory effects on bacteria and nitrogen fixation bacteria, but promote the growth and reproduction of fungi. The soil is converted from bacterial to fungal, from high fertility to low fertility[24-25]. After the continuous cropping, the nematode communities of the dominant genus such as the bacterial nematode and the fungus nematode were found to be significantly reduced, while the nematodes with the dominant species of plant parasitic nematodes began to destroy the host plants, which made the soil unfavorable for crop growth, which led to reduced yield[26]. The results showed that the main influencing factors of crop continuous cropping are fungal and nematode diseases, while the number of bacteria will gradually decrease, and

fungal diseases will be serious year by year, which is also consistent with the trend of smaller changes in the abundance of bacteria in heatmap in this experiment. The results showed that variation of bacterial community in soil of short continuous cropping was not the main factor affecting lettuce yield.

5 Conclusion

In this experiment, the diversity and richness of bacterial community in the soil of lettuce continuous cropping increased, but their relative abundance changed in the soil. Their relative abundance altered in the soil, including Actinobacteria, Chloroflexi, Firmicutes and Nitrospirae, while the proportion increased. Other abundance: Acidobacteria, Bacteroides, Gemmatimonadetes, Planctomycetes and Proteobacteria was gradually decreased in the soil. In addition, *Bacillus*, *Nitrosomonas* and *Xanthomonas* became the dominant genus in the soil after continuous lettuce production. From the heatmap and community structure, the diversity and abundance of bacteria did not alter obviously. The change of bacterial community in short continuous cropping soil was not the main factor affecting lettuce yield.

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