

# Dynamics of soil properties and fungal community structure in continuous-cropped alfalfa fields in Northeast China

Qin Yao<sup>Equal first author, 1</sup>, Yanxia Xu<sup>Equal first author, 2</sup>, Xuefeng Liu<sup>Corresp., 2</sup>, Junjie Liu<sup>1</sup>, Xinyu Huang<sup>2</sup>, Weiguang Yang<sup>2</sup>, Zhao Yang<sup>2</sup>, Lan Lan<sup>2</sup>, Jingming Zhou<sup>2</sup>, Guanghua Wang<sup>Corresp. 1</sup>

<sup>1</sup> Key Laboratory of Mollisols Agroecology, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin, China

<sup>2</sup> Heilongjiang Province Institute of Animal Science, Qiqihar, China

Corresponding Authors: Xuefeng Liu, Guanghua Wang  
Email address: lxf.888888@163.com, wanggh@iga.ac.cn

To compensate for the seasonal imbalance between livestock and forage yield in the cold region of Northeast China, alfalfa (*Medicago sativa* L.) continuous cropping has been widely employed in animal husbandry. However, the effects of continuous cropping of alfalfa on soil properties, including physical, chemical and biological properties, are poorly understood. In this study, we investigated the soil properties and fungal community composition of alfalfa fields under continuous cropping for different time periods (i.e., 1, 2, 6, 9, 12, 13 and 35 years). The results showed that soil moisture, total C, total N, NO<sub>3</sub><sup>-</sup>-N and available K content decreased at less than 10 years of continuous cropping and then increased at more than 10 years of continuous cropping, but soil total P and available P content showed the opposite tendency. The soil fungal community composition determined using Illumina Miseq sequencing showed that continuous cropping increased the fungal alpha diversity and changed the fungal community structure. The relative abundances of *Guehomyces* and *Chaetomium* decreased, but the relative abundances of *Phaeomyocentrospora* and *Paecilomyces* increased with continuous cropping time. In addition, continuous cropping of alfalfa increased the relative abundances of some plant pathogens, such as *Haematonectria haematococca* and *Cyphellophora* sp. Soil total P and available P content were important soil factors affecting the soil fungal community diversity, fungal community structure and the relative abundances of specific fungi in this alfalfa continuous cropping system.

**Title:** Dynamics of soil properties and fungal community structure in continuous-cropped alfalfa fields in Northeast China

**Author names:** Qin Yao<sup>1,†</sup>, Yanxia Xu<sup>2,†</sup>, Xuefeng Liu<sup>2\*</sup>, Junjie Liu<sup>1</sup>, Xinyu Huang<sup>2</sup>, Weiguang Yang<sup>2</sup>, Zhao Yang<sup>2</sup>, Lan Lan<sup>2</sup>, Jingming Zhou<sup>2</sup>, Guanghua Wang<sup>1\*</sup>

**Affiliation:**

1. Key Laboratory of Mollisols Agroecology, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin 150081, China

2. Heilongjiang Province Institute of Animal Science, Qiqihar 161005, China

† These authors contributed equally to this work

**\* Corresponding author:**

Guanghua Wang

No.138 Haping Road, Harbin, Heilongjiang, 150081, China

E-mail address: [wanggh@iga.ac.cn](mailto:wanggh@iga.ac.cn)

Xuefeng Liu

No.2 Heyi Road, Qiqihar, Heilongjiang, 161005, China

E-mail address: [lx.f.888888@163.com](mailto:lx.f.888888@163.com)

22

23 **Abstract:** To compensate for the seasonal imbalance between livestock and forage yield in the  
 24 cold region of Northeast China, alfalfa (*Medicago sativa* L.) continuous cropping has been  
 25 widely employed in animal husbandry. However, the effects of continuous cropping of alfalfa on  
 26 soil properties, including physical, chemical and biological properties, are poorly understood. In  
 27 this study, we investigated the soil properties and fungal community composition of alfalfa fields  
 28 under continuous cropping for different time periods (i.e., 1, 2, 6, 9, 12, 13 and 35 years). The  
 29 results showed that soil moisture, total C, total N, NO<sub>3</sub><sup>-</sup>-N and available K content decreased at  
 30 less than 10 years of continuous cropping and then increased at more than 10 years of continuous  
 31 cropping, but soil total P and available P content showed the opposite tendency. The soil fungal  
 32 community composition determined using Illumina Miseq sequencing showed that continuous  
 33 cropping increased the fungal alpha diversity and changed the fungal community structure. The  
 34 relative abundances of *Guehomyces* and *Chaetomium* decreased, but the relative abundances of  
 35 *Phaeomyocentrospora* and *Paecilomyces* increased with continuous cropping time. In addition,  
 36 continuous cropping of alfalfa increased the relative abundances of some plant pathogens, such  
 37 as *Haematonectria haematococca* and *Cyphellophora* sp. Soil total P and available P content  
 38 were important soil factors affecting the soil fungal community diversity, fungal community  
 39 structure and the relative abundances of specific fungi in this alfalfa continuous cropping system.

40

# 41 1. Introduction

Alfalfa (*Medicago sativa* L.), as an important perennial herbaceous forage legume, is widely grown in many countries (Raiesi, 2007; Su, 2007; Li & Huang, 2008; Bagavathiannan, Gulden & Van Acker, 2011) with a great contribution to the development of agriculture and animal husbandry (Han, Jia & Wang, 2005). In China, alfalfa is cultivated in more than  $4 \times 10^6$  hectares every year and is mainly planted in the arid and semiarid regions of northern China (Zhang et al., 2016). Northeast China is an ecotone system between agriculture and animal husbandry, and winter in this region is longer than in other parts of China (Chen et al., 2013). Thus, the animal fodder in this area nearly exclusively relies on pasture in summer and on silage in winter (Su, 2007). Alfalfa could eliminate the seasonal imbalance between livestock and forage yield in Northeast China due to its great yield potential, high nutritional value and wide adaptation (Chen et al., 2013). Therefore, to meet forage demand in the winter season and then enhance the productivity of livestock, a large area of alfalfa was planted continuously in Northeast China (Dong et al., 2003).

Previous studies showed that the length of the alfalfa growth phase was related to grass yield. Alfalfa productivity increased within 8 years after establishment in the dryland region of northwestern China and then decreased when alfalfa continuously grew for  $> 8$  years (Li & Huang, 2008). In addition, a study in the semiarid Loess Plateau of China recommended that the optimal length of the alfalfa growth phase is 9 years (Jiang et al., 2007). These studies also indicated that the soil quality of alfalfa fields changed with increasing age and is reflected in alfalfa productivity (Jiang et al., 2007; Ren et al., 2011). Soil quality, including physical, chemical and biological properties, can directly or indirectly influence soil productivity and

environmental security (Doran & Parkin, 1994). Dong et al. (2016) found that the amounts of soil organic carbon, total nitrogen, total phosphorus and available phosphorus were significantly increased when new alfalfa land was reclaimed from native sandy steppe. A pot experiment also indicated that planting alfalfa significantly increased the contents of soil organic matter, total nitrogen, available nitrogen, available phosphorus and available potassium (Luo et al., 2018). However, a long-term survey showed that the contents of soil organic carbon, total nitrogen, available phosphorus and soil nitrate nitrogen decreased within 10 years of continuous cropping of alfalfa but then increased after alfalfa grew for more than 10 years (Jiang et al., 2007).

Soil microorganisms are important components of soil ecosystems, play a critical role in material cycling (Lupwayi et al., 2004; Bastida et al., 2017), and quickly respond to changes in soil physicochemical properties (Jiang et al., 2007; Xiao et al., 2017). However, studies related to soil microbial characteristics in alfalfa fields have been limited (Beauregard et al., 2010; Zhong et al., 2012; Luo et al., 2018). Jiang et al. (2007) investigated the soil microbial properties under alfalfa continuous cropping fields in the Loess Plateau of China and found that soil microbial biomass and soil basal respiration decreased steadily from 3 years of alfalfa continuous cropping to 9 years but increased from 15 years to 25 years. Luo et al. (2018) assessed the influence of alfalfa revegetation on the soil microbial community in an Entisol of East China and found that alfalfa revegetation significantly increased soil microbial diversity (e.g., OTU richness and Shannon index) and affected the soil microbial community structures through changes in soil physicochemical properties. In addition to the continuous growth of alfalfa, continuous cropping of soybean caused the gradual transformation of soil from ‘bacterial type’ to ‘fungal type’, as

continuous cropping enhances fungal growth while inhibits bacterial proliferation (Jie, Liu & Cai, 2013). In particular, the abundance of pathogenic fungi, which could influence plant growth and crop yield, was increased with continuous cropping (Guo et al., 2011; Bai et al., 2015).

Therefore, considering the change in soil microorganisms (especially fungi or pathogenic fungi), to support the sustainable development of animal husbandry in Northeast China, it is necessary to reveal the relationships between soil quality and long-term continuous cropping of alfalfa. In this study, soil samples were collected from continuous-cropped alfalfa fields of different cropping times in Northeast China, and the soil properties and fungal communities were investigated. The objectives of this study were 1) to assess the dynamic changes in soil properties and fungal community structures with continuous cropping time and 2) to estimate the comprehensive relationships among soil properties, soil fungal communities and continuous cropping time.

## 2. Materials and Methods

### 2.1 Study site and experimental design

The research fields were set up in an experimental field of the Heilongjiang Province Institute of Animal Science, which is located in the Fularji district (47°15'N, 123°41'E), Qiqihar, Heilongjiang Province, China. The average annual temperature is 3 °C, and the average annual precipitation is 450 mm in this area. The soil is aeolian sandy soil, the pH value of the soil is 7.4 and the salinity is 0.24%.

Fields with alfalfa continuous cropping for 1, 2, 6, 9, 12, 13 and 35 years were selected for this study, which were encoded ACC1y, ACC2y, ACC6y, ACC9y, ACC12y, ACC13y and ACC35y, respectively. All the treatments were randomly arranged in a large experimental field, and each treatment covered more than 900 m<sup>2</sup> in area. At the beginning of the established experiment, alfalfa (*Medicago sativa* L.cv. Longmu801) was seeded at a density of 3,000,000 seeds ha<sup>-1</sup>. Chemical compound fertilizer (N 16%, P<sub>2</sub>O<sub>5</sub> 16%, K<sub>2</sub>O 16%) was annually applied at 280 kg ha<sup>-1</sup> in each experimental plot in late May. The alfalfa fields were managed with conventional cultivation techniques without grazing. The alfalfa was mowed to the soil surface twice and removed from the field in June and August every year except in the first year when the alfalfa was seeded. At other times of year, the alfalfa grew freely.

## 2.2 Soil sampling and soil property determination

The soil samples were collected at a soil depth of 0 - 15 cm on 25 June 2015 when the alfalfa was blooming. Each soil sample was a mixture of more than 5 individual soil cores collected from an area of 300 m<sup>2</sup> (one-third of the total area) of each treatment. A total of 21 soil samples were obtained from seven continuous cropping alfalfa fields. The soil samples were sieved through a 2 mm mesh to thoroughly homogenize them, and the visible plant roots, residues and stones were manually removed. All samples were transferred to the laboratory in an ice-cooled box and divided into two groups: one was placed into a 50 mL centrifuge tube and kept at -80 °C for soil DNA analysis, and the other was dried in the room for determination of soil properties, except for ammonium nitrogen and nitrate nitrogen, which were tested with fresh soil.

For the measurement of soil basic properties, we adopted the methods described in our previous paper (Yao et al., 2017). Briefly, the soil pH was determined using a pH meter in a soil water suspension (1:2.5 w/v). The soil moisture content was measured gravimetrically by drying 15 g of fresh soil to a constant weight in a drying oven at 105 °C for 12 h. The soil total carbon (TC) and total nitrogen (TN) contents were measured using an elemental analyzer (VarioEL III, Germany) (Jones & Willett, 2006). Soil total phosphorus (TP) digested with H<sub>2</sub>SO<sub>4</sub>-HClO<sub>4</sub>, available phosphorus (AP) extracted with 0.5 M NaHCO<sub>3</sub>, and ammonium nitrogen (NH<sub>4</sub><sup>+</sup>-N) and nitrate nitrogen (NO<sub>3</sub><sup>-</sup>-N) extracted with 2.0 M KCl were assayed using a continuous flow analytical system (SKALAR SAN<sup>++</sup>, The Netherlands) (Miranda, Espey & Wink, 2001). Soil total potassium (TK) digested with HNO<sub>3</sub>-HClO<sub>4</sub>-HF and available potassium (AK) extracted with 1.0 M CH<sub>3</sub>COONH<sub>4</sub> were quantified using inductively coupled plasma-atomic emission spectrometry (ICPS-7500, Shimadzu, Japan) (Lu, 1999).

### 2.3 Soil DNA extraction, PCR amplification and Illumina MiSeq sequencing

Soil DNA was extracted from the frozen soil samples (0.5 g wet weight) using a Fast DNA<sup>®</sup> Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions and diluted in DES buffer (DNA Elution Solution-Ultra Pure Water). After DNA extraction, the fungal ITS rRNA was amplified using the primers ITS1F/ITS2R (White et al., 1990), with the forward primer modified with a unique 6 nt barcode at the 5' end. PCRs were performed using 25 µL PCR mixture containing 0.5 µL of each primer at 10 µM, 1.0 µL of template DNA (10 ng), and 23 µL of Platinum PCR SuperMix (TransGen Biotech Co. Ltd., Beijing, China). The amplification was performed at 94 °C for 3 min, followed by 35 cycles at



94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by an extension at 72 °C for 10 min (Liu et al., 2015). Each sample was amplified for three technical replicates. The PCR products were checked in a 1.5% agarose gel with the Gold View™ nucleic acid stain (Beijing Solarbio Science & Technology Co. Ltd., Beijing, China) and were then purified using the agarose gel DNA purification kit (Takara, Dalian, China). The amplicons from all samples were normalized to equimolar amounts and were sequenced using the Illumina MiSeq platform at the Shanghai Majorbio Biotechnology Company, Shanghai, China.

## 2.4 Processing of fungal ITS sequencing data

The raw sequence data obtained from Illumina MiSeq sequencing were processed and analyzed using QIIME Pipeline Version 1.8.0 (<http://qiime.org/tutorials/tutorial.html>) (Caporaso et al., 2010). Briefly, low-quality sequences with a quality score < 20 and shorter than 200 bp in length were removed. Chimeric sequences were detected and eliminated using the Uchime algorithm (Edgar et al., 2011). The remaining high-quality sequences were clustered into Operational Taxonomic Units (OTUs) at 97% similarity using USEARCH (Edgar, 2010). The representative sequence of OTUs was aligned using the Python Nearest Alignment Space Termination (PyNAST) (DeSantis et al., 2006; Caporaso et al., 2010) with a phylogenetic tree built using Fast Tree (Price, Dehal & Arkin, 2009). The taxonomic classification of each representative OTU was assigned using a BLAST comparison against sequences within the GenBank database. In order to analyze the fungal communities at the same sequencing depth, the lowest sequencing number of 29000 sequences was randomly selected per sample. All sequences have been deposited in GenBank with accession number PRJNA509700.

## 2.5 Statistical analysis

The Chao1 richness, Shannon index, Simpson index and Phylogenetic diversity were calculated in QIIME and used to compare the fungal alpha diversity among treatments. Significant differences in soil parameters, fungal alpha diversity and the fungal relative abundances of different taxonomic levels among treatments were determined by one-way analysis of variance (ANOVA), and the correlations between fungal relative abundances and soil parameters and continuous cropping years were tested by Pearson's correlation analysis using SPSS software (Version 22.0). Nonmetric multidimensional scaling (NMDS) analysis was performed to compare the fungal beta diversity between treatments, and canonical correspondence analysis (CCA) was conducted to determine which soil parameters were most frequently related to fungal communities. The NMDS and CCA analyses were all conducted using the "vegan" package in the R environment (R v.2.8.1) (R Development Core Team, 2006). The fungal OTU taxonomic information was uploaded to FUNGuild (<http://www.stbates.org/guilds/app.php>) for functional prediction (Nguyen et al., 2016).

## 3. Results

### 3.1 Soil physicochemical properties

The variations in soil physicochemical properties are displayed in Table 1. Overall, soil moisture, TC, TN, NO<sub>3</sub>-N and AK contents decreased with the extending time from 1 year to 9 years and then increased from 9 years to 35 years in continuous cropping alfalfa soils (Table 1). In contrast, the contents of TP and AP increased and decreased with cropping years in the

treatments of less and more than 10 years, respectively. The soil pH value and TK content fluctuated with the cropping year. The ammonium nitrogen content ( $\text{NH}_4^+\text{-N}$ ) did not significantly change under alfalfa continuous cropping fields.

### 3.2 Fungal community composition

In total, 792,738 high-quality sequences were obtained from all soil samples, ranging from 29,798 to 44,636 per soil sample (mean = 37749) (Table 2). When grouped at the 97% similarity level, 1911 different phylotypes (OTUs) were obtained across all soil samples, with a mean of 653 phylotypes per soil sample.

The phyla Ascomycota, Zygomycota and Basidiomycota were dominant fungi with relative abundances ranging from 64.37% to 76.15%, from 8.61% to 17.98% and from 6.47% to 15.45% across all samples, respectively (Fig. 1; Table S1). The relative abundance of Ascomycota was significantly and negatively correlated with TC, whereas it was positively correlated with TK. Basidiomycota was significantly and negatively correlated with pH, whereas it was positively correlated with TP and AP. However, Zygomycota was significantly and positively correlated with TC, whereas it was negatively correlated with TP and AP (Table S2).

At the class level, Sordariomycetes was dominant, with a relative abundance of more than 30% across all samples. In addition, two classes, Tremellomycetes and Dothideomycetes, were less abundant (relative abundance >10% in at least one sample) (Table S3), and they had positive or negative correlations with soil pH, TC, TP and continuous cropping year (Table S2).

More than 60 fungal orders were detected across all samples. Among them, Hypocreales, Mortierellales, Sordariales and Pleosporales were abundant orders, with a relative abundance of

more than 5% (Table S4). The relative abundance of the most abundant order, Hypocreales, which belongs to the Sordariomycetes class of Ascomycota, was positively and negatively correlated with cropping year and TP, respectively, while the order Sordariales had the opposite correlations (Table S2 and S4).

More than 300 fungal genera were detected across all samples. Among them, 38 abundant fungal genera (relative abundance >0.3%) accounted for more than 80% of the fungal sequences (Table S5). *Guehomyces* and *Mortierella* were dominant genera, and their relative abundances varied from 0.42% to 17.19% and 8.42% to 16.74%, respectively (Table S5). The relative abundance of *Guehomyces* was positively correlated with soil TP and AP but negatively correlated with soil pH and alfalfa continuous cropping year ( $r = -0.595$ ,  $P = 0.004$ ) (Table S2, Fig. 2A). The relative abundance of *Mortierella* was negatively correlated with soil TP and AP but positively correlated with soil TC (Table S2). In addition, the relative abundances of three less abundant genera, *Chaetomium* ( $r = -0.645$ ,  $P = 0.002$ ), *Phaeomycoentrospora* ( $r = 0.864$ ,  $P < 0.0001$ ) and *Paecilomyces* ( $r = 0.839$ ,  $P < 0.0001$ ), were negatively and positively correlated with alfalfa continuous cropping year, respectively (Table S2, Fig. 2B-D), and they also had significant correlations with some soil properties, such as soil pH, TN, TP, AP and AK (Table S2).

### 3.3 Fungal functional groups

Among the 1911 OTUs detected in this study, 866 OTUs (45.32% of the total OTUs) were annotated to 14 functional groups based on the FUNGuild database (Fig. 3, Table S6). The relative abundances of plant pathogens and plant saprotrophs were significantly different

between the alfalfa continuous cropping treatments of less than and more than 10 years, except for some treatments (Table S6). In addition, the highest abundance of fungal parasites appeared in ACC9y (1.12%) (Table S6).

Eighty-four OTUs were assigned as the functional group of plant pathogen (Table S6). Among them, the top 6 OTUs belonged to the phylum Ascomycota, with relative abundances ranging from 0.01% to 2.62% (Fig. 4, Table S7). Of these, the relative abundances of OTU211 (*Haematonectria haematococca*) and OTU1311 (*Cyphellophora* sp.) under continuous cropping alfalfa for more than 10 years were significantly higher than those under continuous cropping for less than 10 years (Fig. 4C, F). Their abundances were positively correlated with soil TN and continuous cropping year and negatively correlated with soil TP and AP (Table S7). The relative abundances of OTU1176 (*Fusarium incarnatum*) in ACC13y and ACC35y were significantly higher than that in other treatments (Fig. 4B). OTU1176 abundance had a negative correlation with soil TP and a positive correlation with soil  $\text{NO}_3^-$ -N, AK and alfalfa continuous cropping year (Table S7). However, the relative abundances of the other three dominant OTUs (OTU1786, OTU1880 and OTU1028) did not change regularly with continuous cropping year (Fig. 4A, D, E), but they had significant correlations with some soil properties, such as soil pH, TN and AK (Table S7).

### 3.4 $\alpha$ -diversity pattern of soil fungal communities

To compare the  $\alpha$ -diversity of soil fungal communities, the same survey effort level of 29,000 sequences was randomly selected from each sample. The coverage values of all the samples were more than 99% (Table 2), indicating that the current sequencing depth was sufficient to capture

the fungal diversity. The number of phylotypes ranged from 555 to 746, and phylogenetic diversity ranged from 216 to 300 across all soil samples (Table 2). Pairwise analysis showed that both phylotype richness ( $r = 0.780$ ,  $P < 0.0001$ ) and phylogenetic diversity ( $r = 0.731$ ,  $P < 0.0001$ ) of the soil fungal community increased with the cropping years (Fig. 5A, B). Among soil properties, the soil TP and AP contents were significantly and negatively correlated with both the phylotype richness and phylogenetic diversity (Fig. 5C-F). In addition, other  $\alpha$ -diversity indices, Chao1 richness, Shannon index and Simpson index, also differed among different treatments (Table 2).

### 3.5 $\beta$ -diversity pattern of soil fungal communities

Based on Bray-Curtis distance dissimilarity, the  $\beta$ -diversity of the fungal communities was evaluated with NMDS analysis (Fig. 6A). The NMDS plot exhibited the best separation of fungal communities of alfalfa continuous cropping for less than 10 years from those for more than 10 years, and thus all samples were separated into two major groups (Fig. 6A). A similar result was also found by the clustering analysis (Fig. 1). Within each group, the fungal communities under alfalfa continuous cropping for less than or more than 10 years were not well separated.

A CCA plot was employed to identify the major soil variables that affected the fungal community structure. Based on the results of the Mantel test (Table S8), soil parameters significantly correlated with the fungal community structure were selected for the CCA analysis (Fig. 6B). Of all the tested soil variables, soil TP ( $r = 0.463$ ,  $P = 0.001$ ) had the longest arrow along the CCA1 axis, which indicated that soil TP was the most important soil parameter in shifting the soil fungal communities. The second most important soil factor was soil AP ( $r =$

0.399,  $P = 0.001$ ) (Fig. 6B, Table S8). In addition, soil pH ( $r = 0.175$ ,  $P = 0.020$ ) also made an important contribution to the change in soil fungal community structure (Fig. 6B, Table S8).

## 4. Discussion

### 4.1 Variation in soil properties under alfalfa continuous cropping

Soil properties are influenced by long-term planting or continuous cropping (Fu et al., 2017; Chen et al., 2018). In this study, alfalfa continuous cropping resulted in a change in soil properties in the arid Songnen Plain of Northeast China (Table 1). The soil moisture, TC, TN and  $\text{NO}_3^-$ -N contents first decreased and then increased with the extension of cultivation years. This result is consistent with the report of Jiang et al. (2007), who found that these soil parameters under alfalfa continuous cropping fields, which were influenced by nutrient uptake and litter input to soil, decreased with the greater amount of dry alfalfa grass removed from the field from 3 years to 9 years, while the soil parameters increased from 15 years to 25 years in the semiarid Loess Plateau of Northwest China. In addition, the contents of soil TP and AP in our study increased with planting years under short-term alfalfa continuous cropping conditions (<10 years) (Table 1). This result was basically consistent with the finding of Dong et al. (2016), who stated that the soil TP and AP contents in alfalfa soils increased significantly after applying fertilizer in a short-term period in the Heihe River Basin of an arid region of Northwest China. In addition, the soil AP content in the treatments of alfalfa continuous cropping for less than 10 years was significantly higher than that in the treatments of continuous cropping for more than 10 years. This result was also found in a long-term investigation by Fan et al. (2011), who reported that

after alfalfa grew for more than 10 years, harvesting of the alfalfa with higher density removed more phosphorus and returned less plant residue to soil, resulting in a decrease in soil AP content in the Loess Plateau of northern China. In short, alfalfa continuous cropping led to changes in soil properties, and 10 years may be the optimal length for the alfalfa continuous cropping system in view of soil quality.

#### 4.2 Variation in soil fungal community composition under alfalfa continuous cropping

In our study, we found that Ascomycota, Zygomycota and Basidiomycota were the dominant phyla, while Chytridiomycota was a minor phylum across all soil samples (Fig. 1, Table S1). A similar result was also reported in a *Panax notoginseng* continuous cropping field (Tan et al., 2017), suggesting that Ascomycota, Zygomycota and Basidiomycota are abundant fungal phyla in continuous cropping systems. In addition, continuous cropping significantly elevated and reduced the relative abundances of Ascomycota and Basidiomycota, respectively, especially compared to their abundances in ACC1y (Table S1). This result is basically consistent with the report of Luo et al. (2018), who found that planting alfalfa significantly changed the soil properties (e.g., pH, TC, TP and AP) for microbial growth and subsequently increased the relative abundance of Ascomycota and reduced that of Basidiomycota in alfalfa fields in eastern China.

At the genus level, the relative abundance of *Chaetomium* decreased with the extension of continuous cropping time (Fig. 2B). *Chaetomium* belongs to Ascomycota and has the ability to degrade cellulose with efficient cellobiose dehydrogenase activity (Harreither et al., 2011; Abdelkader & Hamed, 2013). Moreover, *Chaetomium* was reported as a potential biocontrol



agent that can resist some soil-borne pathogens by producing antifungal compounds (Huang et al., 2016). Meng et al. (2018) found that the abundance of the pathogen *Fusarium oxysporum* was negatively correlated with the relative abundance of the antagonist *Chaetomium*. The decrease in the relative abundance of *Chaetomium* in our study indicated that alfalfa continuous cropping may suppress the growth of antagonistic fungi. In addition, we observed that the relative abundance of *Paecilomyces* increased with prolonged alfalfa continuous cropping time (Fig. 2D) and had a positive correlation with soil moisture (Table S2). This result was consistent with that in a previous study by Mackie et al. (1999), who stated that dry conditions could restrict the expression of fungal disease, in view of the many species in the genus *Paecilomyces* that are pathogens (Israel et al., 2018; Piekarska, Trusz & Szczesniak, 2018). Meanwhile, some species of this genus were demonstrated as biocontrol agents, such as *Paecilomyces lilacinus* (Wei et al., 2015; Abd-Elgawad & Askary, 2018) and *Paecilomyces fumosoroseus* (Rui, 2018). Furthermore, some species of *Paecilomyces* could cause food contamination and spoilage (Ismail, 2001) as well as play an important role in soil carbon turnover (Kluczek-Turpeinen et al., 2007). We also found that the relative abundance of *Paecilomyces* in alfalfa soils was positively correlated with soil TN and AK content and negatively correlated with soil TP and AP content (Table S2). These soil properties were significantly influenced by alfalfa continuous cropping (Table 1). In this case, the genus *Paecilomyces* of Ascomycetes has a complex role in the environment, and our future study should be deeply focused on its role in the alfalfa continuous cropping system.

At the final phylogenetic resolution level, 84 OTUs were assigned as plant pathogens by FUNGuild analysis, and the relative abundance of plant pathogens increased with the extension of the alfalfa continuous cropping time (Fig. 3, Table S6). This result was consistent with the findings of Li et al. (2014), who stated that continuous cropping increased the abundance of pathogenic fungi in peanut soil. Similarly, the abundances of soil-borne pathogens were increased in continuous cropping fields of cucumber (Feng et al., 2016), tomato (Fu et al., 2017) and potato (Liu et al., 2014). These findings suggested that the environmental conditions under long-term continuous cropping were likely to be prone to pathogen proliferation. In particular, we found that the relative abundance of OTU211, which was assigned to *Haematonectria haematococca* of Ascomycota phylum, was significantly increased when the alfalfa continuous cropping time was longer than 10 years (Fig. 4C). *Haematonectria haematococca* is a plant pathogen, and its teleomorph is *Fusarium solani*, which is virulent and causes alfalfa root rot (Li, Li & Meng, 2005; Cao et al., 2008; Kong et al., 2018). Moreover, the relative abundance of OTU211 (*Haematonectria haematococca*) had significant correlations with soil moisture, TN, TP, AP and AK (Table S7), indicating that alfalfa continuous cropping may cause the occurrence of root rot that influenced the alfalfa growth indirectly through the changes in soil edaphic properties. In addition, the relative abundance of OTU1311, which was assigned to *Cyphellophora* sp., was also significantly higher in the treatments with continuous cropping for more than 10 years than that in the treatments of less than 10 years (Fig. 4F). Some species of the fungal genus *Cyphellophora* are potential pathogens (Decock et al., 2003; Ma et al., 2018), and the relative abundance of OTU1311 was correlated significantly with some soil properties, such

as soil TC, TN, TP and AP (Table S7), suggesting that continuous cropping may promote the proliferation of pathogens (Guo et al., 2011; Ranzi et al., 2017).

### 4.3 Variation in soil fungal diversity under alfalfa continuous cropping

Previous studies showed that the taxonomic richness and diversity of soil microorganisms were strongly influenced by continuous cropping (Jie, Liu & Cai, 2013; Li et al., 2014; Bainard et al., 2017; Tan et al., 2017). In our study, we observed that the number of phylotypes and the phylogenetic diversity were significantly increased with continuous cropping time (Table 2; Fig. 5A, B), which was consistent with a previous report in cropping wheat field soils (Bainard et al., 2017). Similarly, a study in soybean fields of Northeast China indicated that continuous cropping increased the fungal community diversity (Bai et al., 2015). However, soil fungal community diversity in a long-term experiment of monoculture soybean detected by DGGE pattern was not significantly influenced by continuous cropping (Li et al., 2010). The discrepancy may be mainly due to the sensitivity and limitations of the molecular methods (Bai et al., 2015). In addition, the soil fungal community diversity in our study was negatively correlated with soil TP and AP contents (Fig. 5C-F), which were significantly influenced by continuous cropping (Table 2), indicating that continuous cropping could indirectly affect the soil fungal community diversity by changing the soil properties.

### 4.4 Variation in soil fungal community structure under alfalfa continuous cropping

In this study, alfalfa continuous cropping significantly changed the soil fungal community structure (Fig. 6A). This result agreed with the findings of Bai et al. (2015), who stated that the fungal community structure in soybean soils was influenced by continuous cropping using

high-throughput sequencing analysis. Similarly, Song et al. (2018) found that changes in soil nutrients and pH caused by the continuous cropping of *Coptis chinensis* affected fungal survival and growth, thereby significantly altered fungal community composition. In addition, the communities under continuous cropping for more than 10 years were obviously different from those of less than 10 years (Fig. 6A), indicating that 10 years may be a cut-off point in the variation of soil fungal communities under alfalfa continuous cropping. Moreover, the structure of the microbial community could also be altered by soil properties (Yao, Jiao & Wu, 2006; Pulleman et al., 2012; Tan et al., 2017; Chen et al., 2018). In this study, the CCA plot showed that soil pH, TP and AP were the dominant factors in shifting the soil fungal community structure in continuous cropping alfalfa fields (Fig. 6B). Similar results were also reported by Song et al. (2018), who stated that some soil properties, such as soil pH and AP, displayed significant effects on the fungal community composition. These soil properties in our study were markedly influenced by continuous cropping (Table 1), indicating that continuous cropping altered soil characteristics and then changed soil fungal community structure (Tan et al., 2017).

## 5. Conclusions

In summary, long-term continuous cropping of alfalfa altered the soil properties and soil fungal community structure and increased the soil fungal alpha diversity. In particular, alfalfa continuous cropping influenced the relative abundances of some plant pathogens, such as *Haematonectria haematococca* and *Cyphellophora* sp. The soil TP and AP contents, which were significantly affected by alfalfa continuous cropping, were not only negatively correlated with

soil fungal community diversity but also significantly correlated with soil fungal community structure and the relative abundance of specific fungi at the different classification levels. In the end, we suspected that the optimal length of alfalfa continuous cropping may be approximately 10 years according to the variation in soil basic properties and soil fungal community composition, and the further isolation and identification of plant pathogens detected in this study will be required in future research.

## References

- Abd-Elgawad M, Askary TH. 2018. Fungal and bacterial nematicides in integrated nematode management strategies. *Egyptian Journal of Biological Pest Control* 28:74 DOI: 10.1186/s41938-018-0080-x.
- Abdelkader S, Hamed M. 2013. In vitro studies on wood degradation in soil by soft-rot fungi: *Aspergillus niger* and *Penicillium chrysogenum*. *International Biodeterioration & Biodegradation* 78:98–102 DOI: 10.1016/j.ibiod.2012.12.013.
- Bagavathiannan MV, Gulden RH, Van Acker RC. 2011. Occurrence of alfalfa (*Medicago sativa* L.) populations along roadsides in southern Manitoba, Canada and their potential role in intraspecific gene flow. *Transgenic Research* 20:397–407 DOI: 10.1007/s11248-010-9425-2.
- Bai L, Cui JQ, Jie WG, Cai BY. 2015. Analysis of the community compositions of rhizosphere fungi in soybeans continuous cropping fields. *Microbiological Research* 180:49–56 DOI: 10.1016/j.micres.2015.07.007.

- 417 Bainard LD, Navarro-Borrell A, Hamel C, Braun K, Hanson K, Gan Y. 2017. Increasing the  
418 frequency of pulses in crop rotations reduces soil fungal diversity and increases the  
419 proportion of fungal pathotrophs in a semiarid agroecosystem. *Agriculture Ecosystems &*  
420 *Environment* 240:206–214 DOI: 10.1016/j.agee.2017.02.020.
- 421 Bastida F, Torres IF, Andrésabellán M, Baldrian P, Lópezmondéjar R, Větrovský T, Richnow  
422 HH, Starke R, Ondoño S, García C. 2017. Differential sensitivity of total and active soil  
423 microbial communities to drought and forest management. *Global Change Biology*  
424 23(10):4185–4203 DOI: 10.1111/gcb.13790.
- 425 Beauregard MS, Hamel C, Atul-Nayyar, Starnaud M. 2010. Long-term phosphorus fertilization  
426 impacts soil fungal and bacterial diversity but not am fungal community in alfalfa.  
427 *Microbial Ecology* 59(2):379–389 DOI: 10.1007/s00248-009-9583-z.
- 428 Cao LX, Zhao CH, Bai QJ, Shao ZZ. 2008. Identification of the pathogens causing root rot of  
429 alfalfa in Inner Mongolia. *Acta Agriculturae Boreali-Sinica*, 23(6):105–107 (In Chinese).
- 430 Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena  
431 AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE,  
432 Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Tumbaugh PJ,  
433 Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows  
434 analysis of high-throughput community sequencing data. *Nature Methods* 7:335–336 DOI:  
435 10.1038/nmeth.f.303.

- Chen JS, Gao C, Di GL, Zhu RF, Zhang YX. 2013. Effects of cutting on alfalfa yield and quality in northeast china. *Journal of Animal & Veterinary Advances* 12(2):253–260 DOI: 10.3923/javaa.2013.253.260.
- Chen W, Teng Y, Li Z, Liu W, Ren W, Luo Y, Christie P. 2018. Mechanisms by which organic fertilizer and effective microbes mitigate peanut continuous cropping yield constraints in a red soil of south china. *Applied Soil Ecology* 128:23–34 DOI: 10.1016/j.apsoil.2018.03.018.
- Decock C., Delgado-Rodríguez G, Buchet S, Seng JM. 2003. A new species and three new combinations in *Cyphellophora*, with a note on the taxonomic affinities of the genus, and its relation to *Kumbhamaya* and *Pseudomicrodochium*. *Antonie van Leeuwenhoek* 84(3): 209–216 DOI: 10.1023/A:1026015031851.
- DeSantis Jr TZ, Hugenholtz P, Keller K, Brodie EL, Larsen N, Piceno YM, Phan R, Andersen GL. 2006. NAST, a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Research* 34:394–399 DOI: 10.1093/nar/gkl244.
- Dong SK, Long RJ, Hu ZZ, Kang MY, Pu XP. 2003. Productivity and nutritive value of some cultivated perennial grasses and mixtures in the alpine region of the Tibetan Plateau. *Grass & Forage Science* 58(3):302–308 DOI: 10.1046/j.1365-2494.2003.00382.x.
- Dong WH, Zhang S, Rao X, Liu CA. 2016. Newly-reclaimed alfalfa forage land improved soil properties comparison to farmland in wheat–maize cropping systems at the margins of oases. *Ecological Engineering* 94:57–64 DOI: 10.1016/j.ecoleng.2016.05.056.
- Doran JW, Parkin TB. 1994. Defining and assessing soil quality. *SSSA Special Publications* 35:3–21.

- 457 Edgar RC. 2010. Search and clustering orders of magnitude faster than blast. *Bioinformatics*  
458 26(19):2460–2461. DOI: 10.1093/bioinformatics/btq461
- 459 Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and  
460 speed of chimera detection. *Bioinformatics* 27:2194–2200 DOI:  
461 10.1093/bioinformatics/btr381.
- 462 Fan J, Hao M, Malhi SS, Wang Q, Huang M. 2011. Influence of 24 annual applications of  
463 fertilisers and/or manure to alfalfa on forage yield and some soil properties under dryland  
464 conditions in northern China. *Polish Journal of Ecology* 59(3):437–443 DOI:  
465 10.1071/CP10370.
- 466 Feng T, Wang YY, Zhang YH, Shi XH, Qin CH, Zhang SA, Jin SC, Zhang H, Zhang J. 2016.  
467 Effects of wheat and soybean stubbles on soil sickness in continuous cropping of cucumber.  
468 *Allelopathy Journal* 39(1):43–53.
- 469 Fu H, Zhang G, Zhang F, Sun Z, Geng G, Li T. 2017. Effects of continuous tomato monoculture  
470 on soil microbial properties and enzyme activities in a solar greenhouse. *Sustainability*  
471 9(2):317 DOI: 10.3390/su9020317.
- 472 Guo ZY, Kong CH, Wang JG, Wang YF. 2011. Rhizosphere isoflavones (daidzein and genistein)  
473 levels and their relation to the microbial community structure of mono-cropped soybean soil  
474 in field and controlled conditions. *Soil Biology & Biochemistry* 11:2257–2264 DOI:  
475 10.1016/j.soilbio.2011.07.022.
- 476 Han QF, Jia ZK, Wang JP. 2005. The analysis of current situation and development prospect of  
477 alfalfa industry at home and abroad. *Pratacultural Science*, 22(3): 22–25.



478 Harreither W, Sygmond C, Augustin M, Narciso M, Rabinovich ML, Gorton L, Haltrich D,  
479 Ludwig R. 2011. Catalytic properties and classification of cellobiose dehydrogenases from  
480 ascomycetes. *Applied and Environmental Microbiology* 77:1804–1815 DOI:  
481 10.1128/AEM.02052-10.

482 Huang X, Liu L, Wen T, Zhang J, Wang F, Cai Z. 2016. Changes in the soil microbial  
483 community after reductive soil disinfestation and cucumber seedling cultivation. *Applied*  
484 *Microbiology & Biotechnology* 100(12):1–13 DOI: 10.1007/s00253-016-7362-6.

485 Ismail MA. 2001. Deterioration and spoilage of peanuts and desiccated coconuts from two sub-  
486 Saharan tropical East African countries due to the associated mycobiota and their  
487 degradative enzymes. *Mycopathologia* 150:67–84 DOI: 10.1023/A:1010863507652.

488 Israel NK, Takashi Y, Keisuke S, Dela CTE. 2018. Antibacterial, cytotoxic and trypanocidal  
489 activities of marine-derived fungi isolated from Philippine macroalgae and seagrasses. *Acta*  
490 *Botanica Croatica* 77(2):141–151 DOI: 10.2478/botcro-2018-0016.

491 Jiang JP, Xiong YC, Jia Y, Li FM, Xu JZ, Jiang HM. 2007. Soil quality dynamics under  
492 successional alfalfa field in the semi-arid loess plateau of northwestern China. *Arid Soil*  
493 *Research & Rehabilitation* 21(4):287–303 DOI: 10.1080/15324980701603524.

494 Jie WG, Liu X, Cai BY. 2013. Diversity of rhizosphere soil arbuscular mycorrhizal fungi in  
495 various soybean cultivars under different continuous cropping regimes. *PLoS ONE*  
496 8(8):e72898 DOI: 10.1371/journal.pone.0072898.

- 497 Jones DL, Willett VB. 2006. Experimental evaluation of methods to quantify dissolved organic  
498 nitrogen (DON) and dissolved organic carbon (DOC) in soil. *Soil Biology and Biochemistry*  
499 38:991–999 DOI: 10.1016/j.soilbio.2005.08.012.
- 500 Kluczek-Turpeinen B, Maijala P, Hofrichter M, Hatakka A. 2007. Degradation and enzymatic  
501 activities of three *Paecilomyces inflatus* strains grown on diverse lignocellulosic substrates.  
502 *International Biodeterioration & Biodegradation* 59(4):283–291 DOI:  
503 10.1016/j.ibiod.2006.09.007.
- 504 Kong QQ, Ruan L, Liu DX, Liu ZY, Zhang YZ, Qin F, Yuan JH, Ma ZH, Wang HG. 2018.  
505 Biological characteristics of *Fusarium* causing alfalfa root rot in Hebei Province. *Journal of*  
506 *China Agricultural University*, 23(8): 59–76 (In Chinese).
- 507 Li CG, Li XM, Kong WD, Ying W, Wang JG. 2010. Effect of monoculture soybean on soil  
508 microbial community in the northeast China. *Plant & Soil* 330(1–2):423–433 DOI:  
509 10.1007/s11104-009-0216-6.
- 510 Li WC, Li WM, Meng YR. 2005. Biological characteristics of dry root rot (*Fusarium solani*) of  
511 *Medicago sativa*. *Acta Prataculturae Sinica* 14(4):106–110 (In Chinese).
- 512 Li XG, Ding CF, Zhang TL, Wang XX. 2014. Fungal pathogen accumulation at the expense of  
513 plant-beneficial fungi as a consequence of consecutive peanut monoculturing. *Soil Biology*  
514 *& Biochemistry* 72:11–18 DOI: 10.1016/j.soilbio.2014.01.019.
- 515 Li Y, Huang M. 2008. Pasture yield and soil water depletion of continuous growing alfalfa in the  
516 Loess Plateau of China. *Agriculture Ecosystems & Environment* 124:24–32.

- 517 Liu J, Sui Y, Yu Z, Shi Y, Chu H, Jin J, Liu X, Wang G. 2015. Soil carbon content drives the
- 518 biogeographical distribution of fungal communities in the black soil zone of northeast China.
- 519 *Soil Biology & Biochemistry* 83:29–39 DOI: 10.1016/j.soilbio.2015.01.009.
- 520 Liu X, Zhang J, Gu T, Zhang W, Shen Q, Yin S, Qiu H. 2014. Microbial community diversities
- 521 and taxa abundances in soils along a seven-year gradient of potato monoculture using high-
- 522 throughput pyrosequencing approach. *PLoS ONE* 9:e86610 DOI:
- 523 10.1371/journal.pone.0086610.
- 524 Luo CG, Deng YW, Inubushi K, Liang J, Zhu SP, Wei ZY, Guo XB, Luo XP. 2018. Sludge
- 525 biochar amendment and alfalfa revegetation improve soil physicochemical properties and
- 526 increase diversity of soil microbes in soils from a rare earth element mining wasteland.
- 527 *International Journal of Environmental Research & Public Health* 15(5): 965 DOI:
- 528 10.3390/ijerph15050965.
- 529 Lupwayi NZ, Harker KN, Clayton GW, Turkington TK, Rice WA, O'Donovan, J. T. Soil
- 530 microbial biomass and diversity after herbicide application. *Canadian Journal of Plant*
- 531 *Science*, 2004, 84(2): 677–685.
- 532 Lu RK. 1999. *Analytical Methods of Soil Agrochemistry*. Beijing: Chinese Agriculture Science
- 533 and Technology Press.
- 534 Ma M, Jiang X, Wang Q, Ongena M, Wei D, Ding J, Guan D, Cao F, Zhao B, Li J. 2018.
- 535 Responses of fungal community composition to long-term chemical and organic
- 536 fertilization strategies in Chinese Mollisols. *MicrobiologyOpen* 7(2):e00597 DOI:
- 537 10.1002/mbo3.597.

- 538 Mackie JM, Lloyd DL, Ryley MJ, Irwin JAG. 1999. Fungal diseases of temperate annual pasture  
539 legumes in southern Queensland. *Animal Production Science* 39(6):699–707 DOI:  
540 10.1071/EA99012.
- 541 Meng TZ, Yang YJ, Cai ZC, Ma Y. 2018. The control of *Fusarium oxysporum* in soil treated  
542 with organic material under anaerobic condition is affected by liming and sulfate content.  
543 *Biology and Fertility of Soils* 54(2):295–307 DOI: 10.1007/s00374-017-1260-7.
- 544 Miranda KM, Espey MG, Wink DA. 2001. A rapid, simple spectrophotometric method for  
545 simultaneous detection of nitrate and nitrite. *Nitric Oxide* 5:62–71 DOI:  
546 10.1006/niox.2000.0319.
- 547 Nguyen NH, Song Z, Bates ST, Branco S, Tedersoo L, Menke J, Schilling JS, Kennedy PG.  
548 2016. FUNGuild: An open annotation tool for parsing fungal community datasets by  
549 ecological guild. *Fungal Ecology* 20(1):241–248 DOI: 10.1016/j.funeco.2015.06.006.
- 550 Piekarska K, Trusz A, Szczesniak S. 2018. Bacteria and fungi in two air handling units with air  
551 recirculating module. *Energy & Buildings* 178:154–164 DOI:  
552 10.1016/j.enbuild.2018.08.036.
- 553 Price MN, Dehal PS, Arkin AP. 2009. Fast Tree: computing large minimum evolution trees with  
554 profiles instead of a distance matrix. *Molecular Biology and Evolution* 26, 1641–1650 DOI:  
555 10.1093/molbev/msp077.
- 556 Pulleman M, Creamer R, Hamer U, Helder J, Pelosi C, Peres G, Rutgers M. 2012. Soil  
557 biodiversity, biological indicators and soil ecosystem services-an overview of European

- approaches. *Current Opinion in Environmental Sustainability* 4(5):529–538 DOI:  
10.1016/j.cosust.2012.10.009.
- Raiesi F. 2007. The conversion of overgrazed pastures to almond orchards and alfalfa cropping  
systems may favor microbial indicators of soil quality in Central Iran. *Agriculture  
Ecosystems & Environment* 121:309–318 DOI: 10.1016/j.agee.2006.11.002.
- Ranzi C, Camera JN, Deuner CC, Ranzi C, Camera JN, Deuner CC. 2017. Influence of  
continuous cropping on corn and soybean pathogens. *Summa Phytopathol* 43(1):14–19 DOI:  
10.1590/0100-5405/2150.
- Ren XL, Jia ZK, Wan SM, Han QF, Chen XL. 2011. The long-term effects of alfalfa on soil  
water content in the Loess Plateau of northwest China. *African Journal of Biotechnology*  
10(21):4420–4427.
- Ruiu L. 2018. Microbial biopesticides in agroecosystems. *Agronomy* 8(11):235 DOI:  
10.3390/agronomy8110235.
- Song X, Pan Y, Li L, Wu X, Wang Y. 2018. Composition and diversity of rhizosphere fungal  
community in coptis chinensis franch. continuous cropping fields. *PLoS ONE* 13(3),  
e0193811 DOI: 10.1371/journal.pone.0193811.
- Su YZ. 2007. Soil carbon and nitrogen sequestration following the conversion of crop-land to  
alfalfa forage land in northwest China. *Soil & Tillage Research* 92:181–189 DOI:  
10.1016/j.still.2006.03.001.
- Tan Y, Cui YS, Li HY, Kuang AX, Li XR, Wei YL, Ji XL. 2017. Rhizospheric soil and root  
endogenous fungal diversity and composition in response to continuous *Panax notoginseng*

cropping practices. *Microbiological Research* 194:10–19 DOI: 10.1016/j.micres.2016.09.009.

Wei W, Xu YL, Li SX, Zhu L, Song J. 2015. Developing suppressive soil for root diseases of soybean with continuous long-term cropping of soybean in black soil of northeast China. *Acta Agriculturae Scandinavica, Section B-Soil and Plant Science* 65(3):279–285 DOI: 10.1080/09064710.2014.992941.

White T, Bruns T, Lee S, Taylor J. 1990. PCR protocols a guide to methods and applications. In: Innis MA, ed. *PCR Protocols: A Guide to Methods and Applications*. San Diego: Academic Press, 315–322.

Xiao XY, Wang MW, Zhu HW, Guo ZH, Han XQ, Zeng P. 2017. Response of soil microbial activities and microbial community structure to vanadium stress. *Ecotoxicology & Environmental Safety* 142:200–206 DOI: 10.1016/j.ecoenv.2017.03.047.

Yao H, Jiao X, Wu F. 2006. Effects of continuous cucumber cropping and alternative rotations under protected cultivation on soil microbial community diversity. *Plant & Soil* 284:195–203 DOI: 10.1007/s11104-006-0023-2.

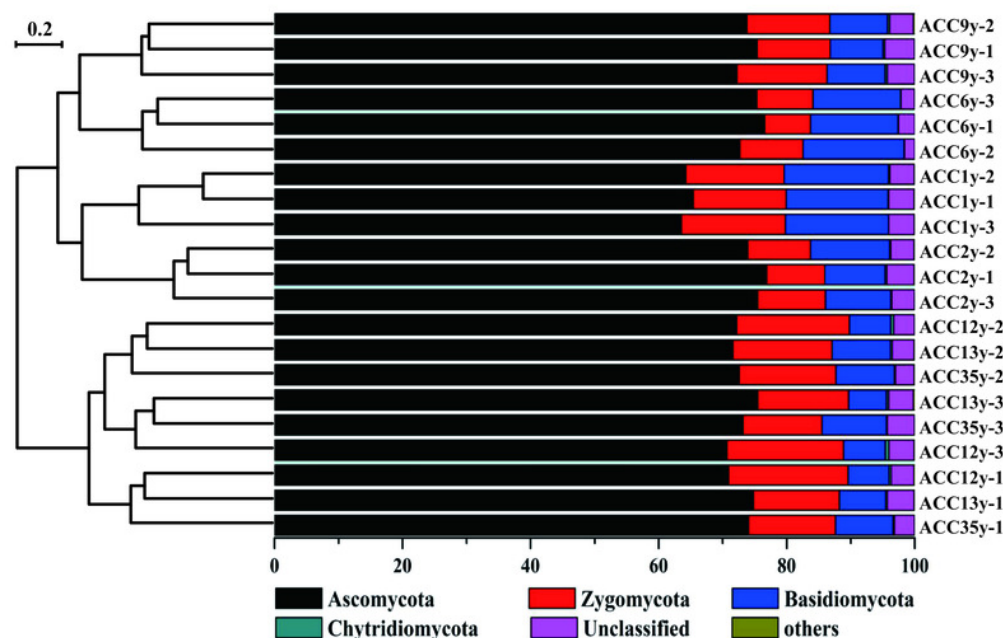
Yao Q, Liu J, Yu Z, Li Y, Jin J, Liu X, Wang G. 2017. Three years of biochar amendment alters soil physiochemical properties and fungal community composition in a black soil of northeast China. *Soil Biology & Biochemistry* 110:56–67 DOI: 10.1016/j.soilbio.2017.03.005.

- 598 Zhang J, Wang Q, Xiao Y, Pang XP, Jia, T. T., Song, R., Liu, H. X. 2016. Effects of alternate  
599 furrow irrigation on the biomass allocation and water use efficiency of alfalfa. *Acta*  
600 *Prataculturae Sinica*, 25:164–171 DOI: 10.11686/cyxb2015232.
- 601 Zhong Y, Wang J, Song Y, Liang Y, Li G. 2012. Microbial community and functional genes in  
602 the rhizosphere of alfalfa in crude oil-contaminated soil. *Frontiers of Environmental*  
603 *Science & Engineering* 6(6):797–805 DOI: 10.1007/s11783-012-0405-z.

# Figure 1

Phylogenetic relationships of fungal communities shown with the relative abundances of different fungal phyla

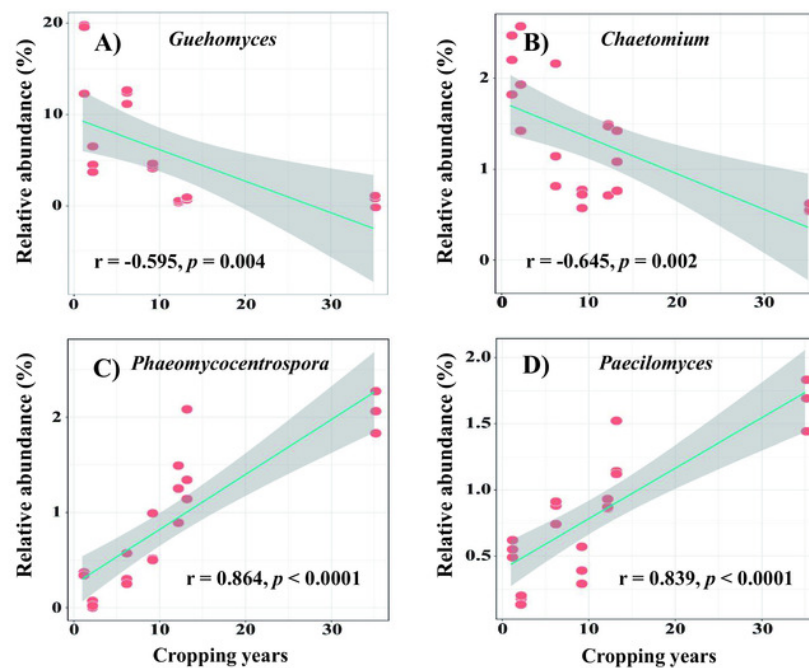
ACC1y, ACC2y, ACC6y, ACC9y, ACC12y, ACC13y and ACC35y represent the treatments of alfalfa continuous cropping for 1, 2, 6, 9, 12, 13 and 35 years, respectively.





# Figure 2

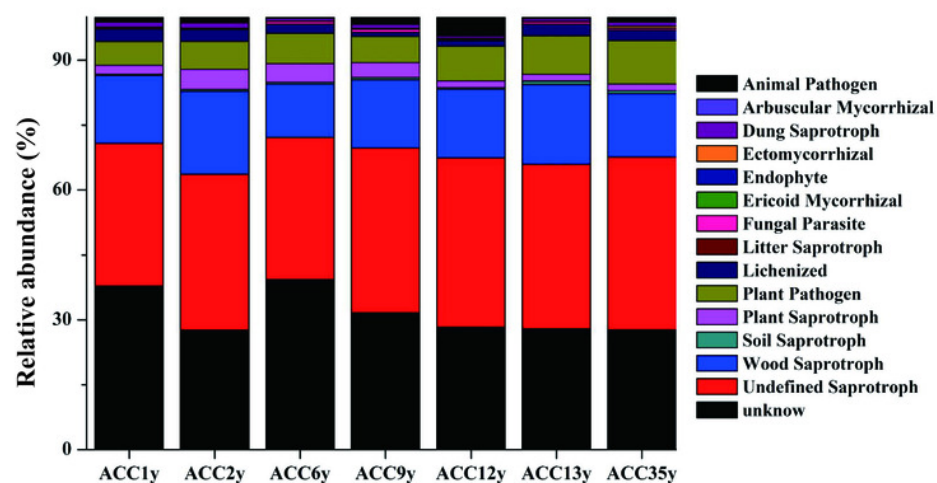
The relationship between relative abundances of dominant fungal genera and alfalfa continuous cropping years



# Figure 3

Variations in composition of fungal functional groups inferred by FUNGuild

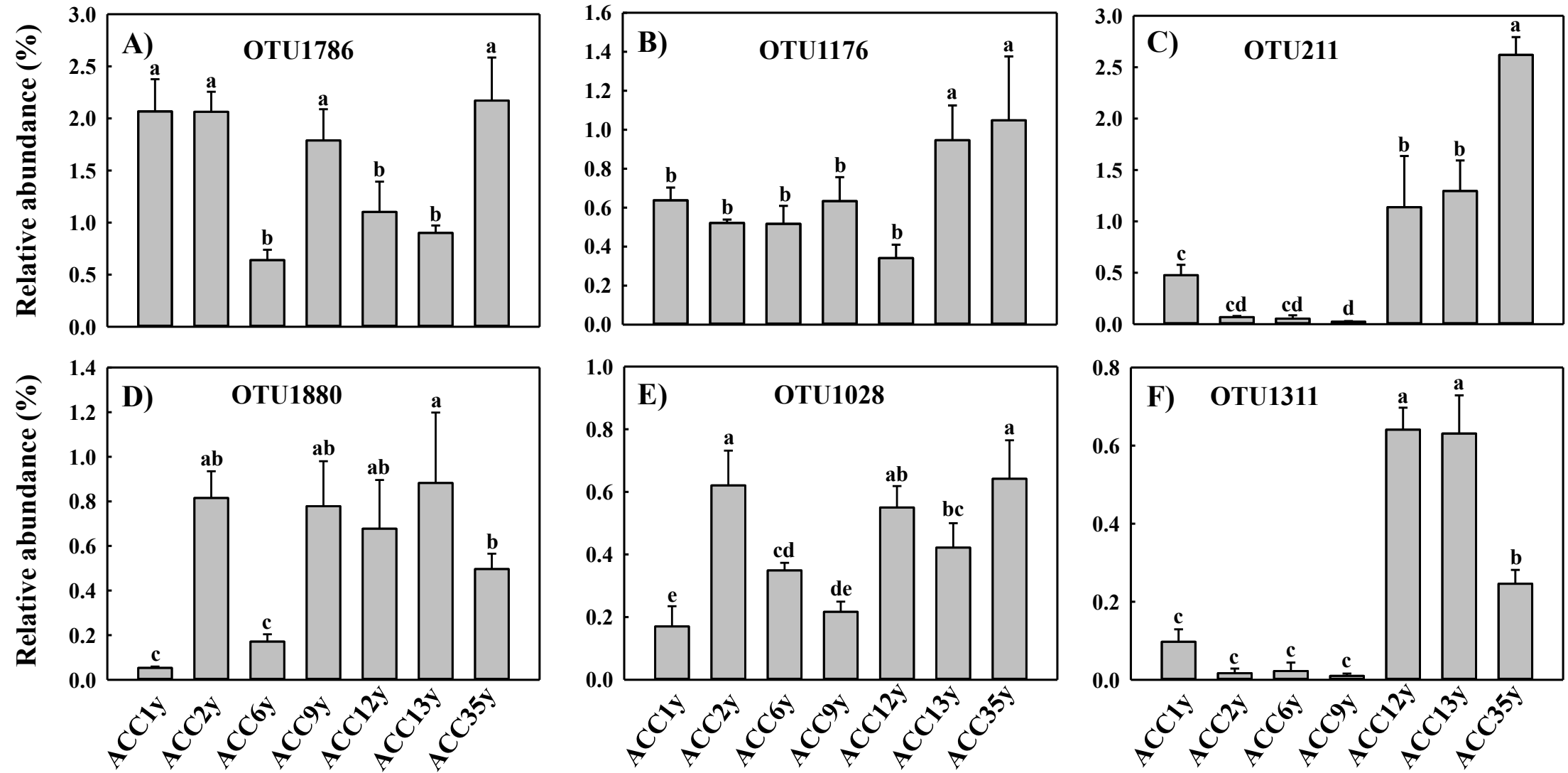
ACC1y, ACC2y, ACC6y, ACC9y, ACC12y, ACC13y and ACC35y represent the treatments of alfalfa continuous cropping for 1, 2, 6, 9, 12, 13 and 35 years, respectively.



# **Figure 4**(on next page)

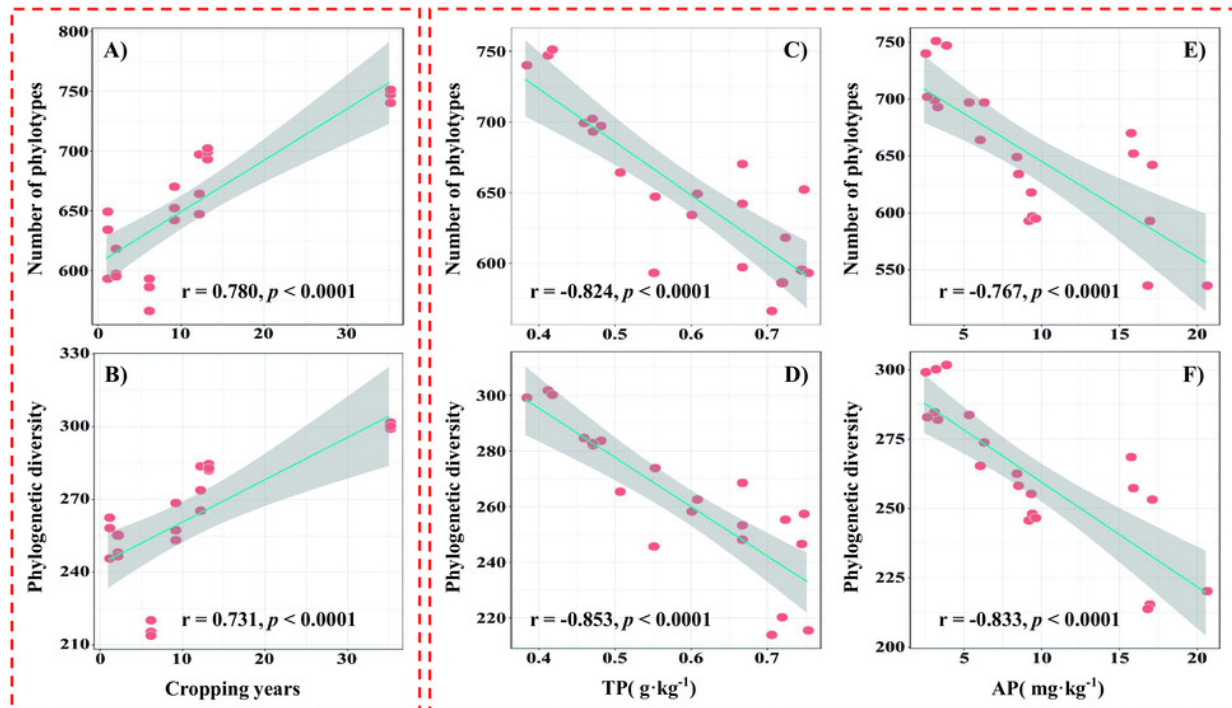
Effect of alfalfa continuous cropping on the relative abundance of dominant plant pathogens at the OTUs level

Different letters above columns indicate significant difference between treatments tested by one-way ANOVA ( $P < 0.05$ ). Error bar show mean  $\pm$  SE (n = 3). ACC1y, ACC2y, ACC6y, ACC9y, ACC12y, ACC13y and ACC35y represent the treatments of alfalfa continuous cropping for 1, 2, 6, 9, 12, 13 and 35 years, respectively.



# Figure 5

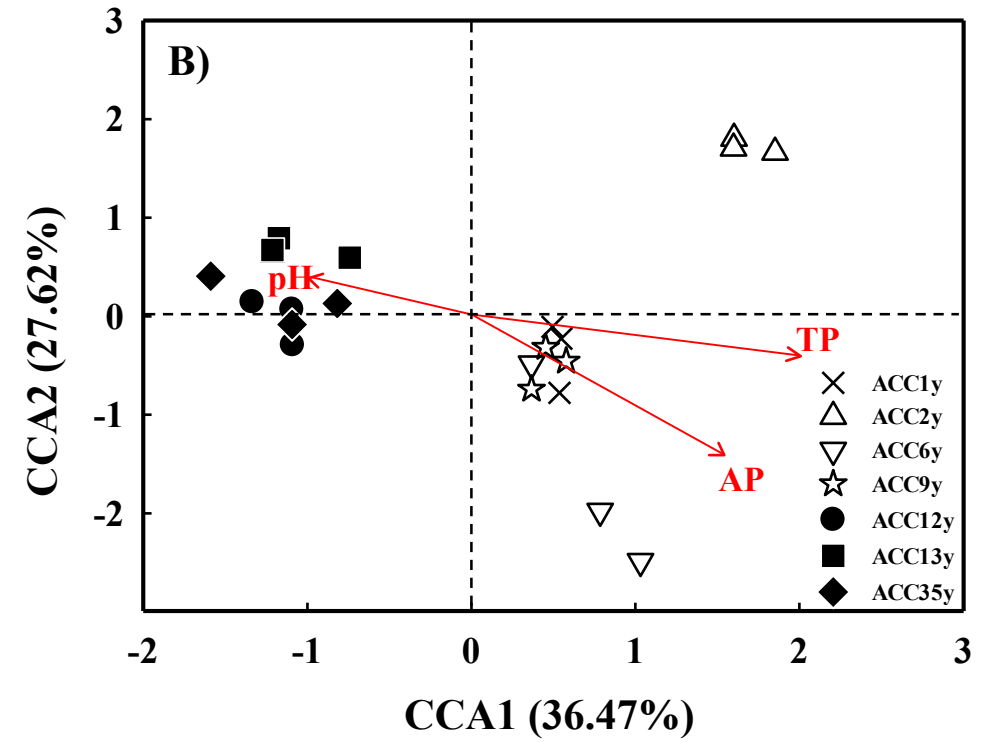
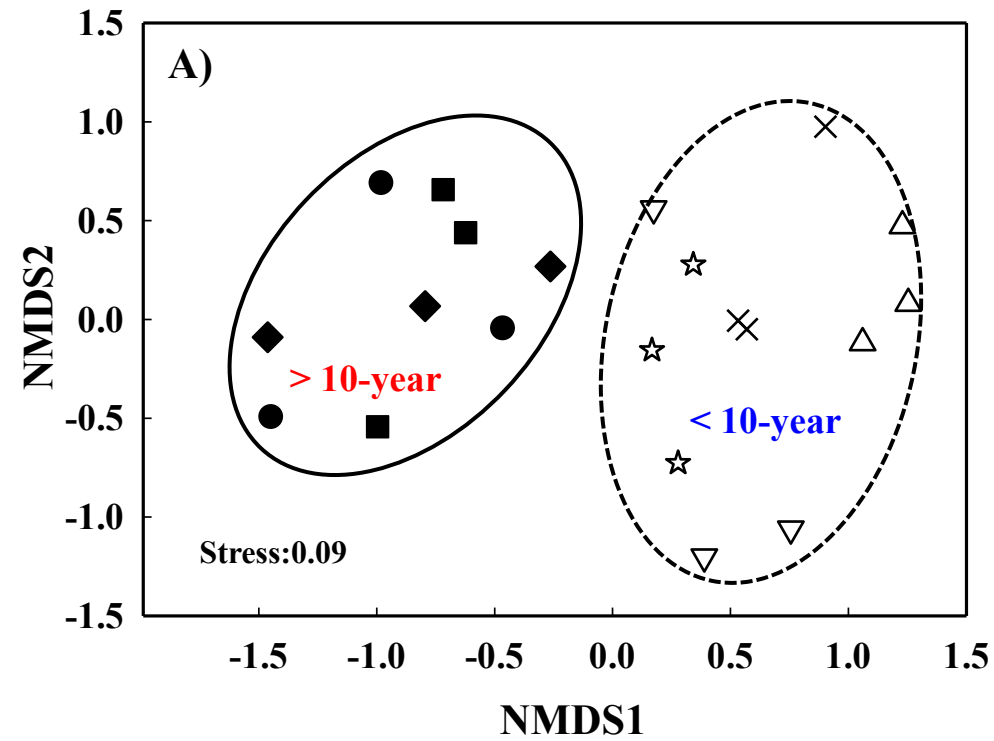
The relationship between soil fungal phylotype richness or phylogenetic diversity and alfalfa continuous cropping year (A, B), soil TP (C, D) or AP (E, F)



# **Figure 6**(on next page)

Nonmetric multidimensional scaling (NMDS) plot of soil fungal communities for different continuous cropping treatments (A) and canonical correspondence analysis (CCA) of fungal community changes with soil variables (B)

ACC1y, ACC2y, ACC6y, ACC9y, ACC12y, ACC13y and ACC35y represent the treatments of alfalfa continuous cropping for 1, 2, 6, 9, 12, 13 and 35 years, respectively.



**Table 1** (on next page)

Soil physicochemical properties under alfalfa continuous cropping



1 **Table 1.** Soil physicochemical properties under alfalfa continuous cropping

Treatment	pH	Moisture (%)	TC <sup>a</sup> (g kg <sup>-1</sup> )	TN <sup>a</sup> (g kg <sup>-1</sup> )	TP <sup>a</sup> (g kg <sup>-1</sup> )	TK <sup>a</sup> (g kg <sup>-1</sup> )	NH <sub>4</sub> <sup>+</sup> -N (mg kg <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> -N (mg kg <sup>-1</sup> )	AP <sup>a</sup> (mg kg <sup>-1</sup> )	AK <sup>a</sup> (mg kg <sup>-1</sup> )
ACC1y <sup>b</sup>	8.34±0.03c <sup>c</sup>	15.41±1.62ab	22.52±0.38a	1.20±0.04b	0.58±0.03b	20.02±0.22bc	57.91±3.21a	5.67±0.46a	8.60±0.42c	128.34±5.25bc
ACC2y	8.43±0.16bc	14.41±0.21b	19.66±0.08bc	1.18±0.02b	0.71±0.04a	20.16±0.46bc	60.17±6.28a	4.17±0.11bc	9.35±0.15c	133.06±5.40b
ACC6y	8.40±0.05c	14.10±0.85bc	18.73±0.62cd	1.19±0.05b	0.72±0.02a	21.19±0.76a	56.47±0.96a	3.92±0.12c	18.05±2.17a	122.36±2.68c
ACC9y	8.60±0.03a	12.73±0.29c	17.39±0.79d	0.83±0.03c	0.69±0.05a	21.46±0.34a	57.51±1.05a	3.93±0.17c	16.16±0.75b	103.93±1.63d
ACC12y	8.54±0.02ab	14.76±0.77ab	24.01±1.49a	1.44±0.14a	0.51±0.04c	19.53±0.59c	56.27±4.16a	4.18±0.14bc	5.79±0.50d	128.58±6.03bc
ACC13y	8.55±0.08ab	14.81±0.48ab	19.08±1.26cd	1.34±0.16ab	0.46±0.01c	20.89±0.02ab	55.91±1.29a	5.45±1.77ab	2.92±0.34e	128.05±0.79bc
ACC35y	8.47±0.01abc	16.26±0.55a	20.88±1.01b	1.54±0.18a	0.40±0.02d	20.78±0.65ab	57.13±2.36a	6.03±0.81a	3.11±0.67e	159.13±8.35a

2 <sup>a</sup> TC, TN, TP, TK, AP and AK indicate soil total carbon, total nitrogen, total phosphorus, total potassium, available phosphorus and available potassium, respectively.

3 <sup>b</sup> ACC1y, ACC2y, ACC6y, ACC9y, ACC12y, ACC13y and ACC35y represent the treatments of alfalfa continuous cropping for 1, 2, 6, 9, 12, 13 and 35 years, respectively.

4 <sup>c</sup> Different letters within the same column indicate significant difference between treatments tested by One-Way ANOVA ( $P < 0.05$ ). Values are the means ± SE (n = 3).

**Table 2**(on next page)

Illumina MiSeq sequenced fungal data and fungal community diversity indices (at 97% sequence similarity) based on the ITS rRNA gene

**Table 2** Illumina MiSeq sequenced fungal data and fungal community diversity indices (at 97% sequence similarity) based on the ITS rRNA gene.

Treatment	Fungal sequences	Number of phylotype <sup>a</sup>	Chao1 Richness <sup>a</sup>	Shannon index <sup>a</sup>	Simpson index <sup>a</sup>	Phylogenetic diversity <sup>a</sup>	Coverage (%) <sup>a</sup>
ACC1y <sup>b</sup>	36757±5250 <sup>c</sup>	625±29de <sup>d</sup>	837±21a	4.40±0.05d	0.030±0.003a	255.41±8.76c	99.42
ACC2y	37751±3436	603±13e	770±28bc	4.56±0.07c	0.020±0.002b	249.91±4.63c	99.47
ACC6y	41112±1377	555±33f	689±34d	4.17±0.06e	0.027±0.005a	216.41±3.31d	99.49
ACC9y	38180±7604	655±14cd	749±42c	4.69±0.14bc	0.018±0.000b	259.63±7.93c	99.51
ACC12y	36652±2427	686±19bc	814±11ab	4.62±0.08bc	0.022±0.000b	274.23±9.17b	99.49
ACC13y	37392±5169	698±5b	860±31a	4.74±0.07ab	0.019±0.000b	283.13±1.32b	99.43
ACC35y	36402±5219	746±6a	845±40a	4.84±0.04a	0.019±0.003b	300.31±1.31a	99.43

<sup>a</sup> Those data were calculated from 29000 fungal sequences per soil sample.

<sup>b</sup> ACC1y, ACC2y, ACC6y, ACC9y, ACC12y, ACC13y and ACC35y represent the treatments of alfalfa continuous cropping for 1, 2, 6, 9, 12, 13 and 35 years, respectively.

<sup>c</sup> Values are the means ± SE (n = 3).

<sup>d</sup> Different letters within the same column indicate significant difference between treatments tested by One-Way ANOVA ( $P < 0.05$ ).