Continuous-cropping tobacco caused variance of chemical properties and structure of bacterial network in soils

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Abstract
Continuous cropping of the same crop leads to land degradation. This is also called the continuous-cropping obstacle. Here, we investigated how long-term continuous cropping of tobacco influences soil biochemical properties and bacterial networks in the mountain lands of China. Two different fields were sampled: one with 25 years of continuous cropping tobacco and one with noncontinuous cropping tobacco. Soil chemical and biological properties were measured including available phosphorus and potassium, soil organic matter, pH, alkali-hydrolysable nitrogen, micronutrients contents, and activity of urease, catalase, invertase, and phosphatase as well as tobacco agronomic characteristics. Bacterial communities of the two different soils were sequenced by metabarcoding of the 16S ribosomal RNA, and, with these data, network analysis was done. Soil chemical properties and tobacco agronomical properties were negatively affected by the continuous-cropping obstacle, and this treatment has a less complex network (less modules, nodes, and connectivity) than the soil with noncontinuous cropping treatment. For continuous cropping, there were less generalists, which were key species that connect network, than noncontinuous cropping. Moreover, the taxonomic composition of bacterial network was different in the two different treatments. In the continuous-cropping network, 40% nodes had negative interactions, suggesting that more competition or antagonism existed among bacterial species. It concluded that continuous cropping has a detrimental effect on soil chemical and that the bacterial network properties under continuous cropping are more sensitive to soil variables (so more unstable and inefficient) because there are less bacterial species that interact each other and this is due to limited nutrients or excessive toxic nutrient.

KEYWORDS
continuous-cropping obstacle, land degradation, microbial interaction, microbial network, soil chemical properties, tobacco cultivation
1 | INTRODUCTION

Land degradation refers to a significant reduction of quality and productive capacity of the land. There are various forms of land degradation such as soil erosion, excess salinity, desertification, compaction, acidification, decline in land fertility, deterioration of soil structure, nutrient loss, and chemical contamination (Chen et al., 2012). About 40% of agricultural land in the world is moderately degraded, and 9% land is strongly degraded that reduces crop yield by about 13% (Helen & Chandra, 2016). Land degradation is usually caused by unsustainable agricultural land management practices (e.g., long-term monoculture, abuse of fertilizers and pesticides, accelerated deforestation, and overgrazing), which negatively affects soil physical and chemical properties and disrupts the balance of soil microorganisms. In China, for obtaining a high yield, the same crop is grown in the same land year after year without interruption, and this usually leads to severe continuous-cropping obstacle. Generally, continuous-cropping obstacle caused by over cultivation is considered as one of the main types of land degradation (Qin et al., 2017), accompanying with a decline in soil quality such as loss of soil carbon, nutrient depletion, reduction in land fertility, decreased biodiversity, soil acidification, salinity, compaction, and changes of soil microbe communities (Aparicio & Costa, 2007; Chen et al., 2012; Guo et al., 2010; Nayyar et al., 2009; Tian, Liu, Zhang, & Gao, 2010). So far, large areas of croplands are threatened by continuous-cropping obstacle with stunted plant growth, declined plant vigour, and low productivity of crops (Aparicio & Costa, 2007; Chen et al., 2012). For instance, long-term monoculture leads to the decrease of nitrogen, phosphorus, and potassium contents in soil and the unbalanced availability of plant nutrients (Aparicio & Costa, 2007; Tian et al., 2010) and accelerates the accumulation of plant autotoxins (e.g., phenolic acids) and promotes the epidemic of plant diseases (Aparicio & Costa, 2007; Kitazawa, Asao, Ban, Pramanik, & Hosoki, 2005).

Tobacco has been used as a model organism in many studies, laying the groundwork for agricultural biotechnology (Sierro et al., 2014). Tobacco is also a major crop for millions of Chinese farmers and widely planted in Hubei Province, China. Due to limited cultivated lands and lacking of reasonable planting concept, the majority of tobacco is usually planted by a continuous cropping (CC) pattern that potentially enriches the crop-specific microorganisms in soil. Therefore, CC can lead to the reduction in abundances of beneficial bacteria (e.g. Arthrobacter and Lysobacter) and the multiplication of detrimental bacteria (e.g., soil-borne pathogens) in soil (She et al., 2017; Wu et al., 2017). In our previous studies, we found that the abundances of beneficial bacteria and fungi were significantly decreased in the soils with monoculture of tobacco (Wang et al., 2017). Similarly, monoculture of potato also causes the shift of overall bacterial communities and the decrease of abundances of Acidobacteria and Nitrospirae in soils (Liu et al., 2014). However, until now, the focus of many studies has been put on the diversity measures and community composition through analysis of metabarcoding data without taking into account the interrelation between bacterial taxa (network analysis), and few studies have investigated the changes of microbial network in the CC land. The complex interactions among different microbial species and the key microbes changed by continuous-cropping obstacle are still unknown.

In soils, various microorganisms form a complex network of interactions. Different soil microbial species interact with each other through various types of interactions including predation, parasitism, competition, amensalism, commensalism, or mutualism (Olesen, Bascompte, Dupont, & Jordano, 2007). All of these interactions among different microbial species together shape the overall structure of a microbial community and determine the ecosystem stability (Hibbing, Fuqua, Parsek, & Peterson, 2010; Zhou, Deng, Luo, He, & Yang, 2011). In the complex ecosystems such as soil, the microbial interactions are considered more important to ecosystem function than the species diversity and abundance (Bascompte, 2007). Microbe–microbe interactions are a vital part of the soil microbiome (Zhang et al., 2014). By interacting with each other, soil microbes significantly drive global biogeochemical circulation (Gans, Wolinsky, & Dunbar, 2005; Montoya, Pimm, & Solé, 2006). Knowledge of the microbial interactions can help us to identify microbial environmental niches, reveal microbial ecological roles, and expand our understandings for millions of uncultured microbes in soil. Co-occurrence networks of microbial species can ascertain which microbial species share the same niche and play the key roles in the complex networks. The key microorganisms that maintain the stability and operation of microbial community can be discerned by analysing their imbalance, and allelopathy (Yang et al., 2015). For this reason, tobacco was used as a model to study land degradation caused by continuous-cropping obstacle in this study.

Soil microorganisms are involved in many key processes of soil ecosystem including nutrition cycling, organic matter turnover, soil structure maintenance, and toxin degradation (Brussaard, Ruiter, & Brown, 2007). Due to their quick response to environmental changes and agricultural practices, soil microorganisms are considered as efficient biological indicators for soil fertility and land management (Avidano, Gamalero, Cossa, & Carraro, 2005; Wu et al., 2017). Many studies found that long-term monocropping can result in a decrease of microbial activity and diversity and change the composition and structure of soil microbial community (Nayyar et al., 2009; van Elsas, Garbeva, & Salles, 2002). In the CC soil, microbial community is continuously exposed to root exudates of the same crop year after year that potentially enriches the crop-specific microorganisms in soil.
roles in the networks (Barberán, Bates, Casamayor, & Fierer, 2012). Recently, phylogenetic molecular ecological networks (pMENs) have been used to investigate the ecosystem, which can provide us with new understanding of microbial ecology (Barberán et al., 2012; Ding et al., 2015; Lu et al., 2013; Shi et al., 2016; Zhou et al., 2011). By pMENs, Zhou et al. (2011) found that the network compositions and structures under ambient CO₂ are substantially different from the network under elevated CO₂ level. The network structure of microbial communities subjected to long-term warming is affected by environmental traits including temperature and soil pH (Deng et al., 2012). Lu et al. (2013) found that the healthy soils, in which the crops grew well with a high yield, had more interrelated operational taxonomic units (OTUs) than the microbial network of diseased soils, in which the crops grew poorly with a low yield. Despite the importance of microbial interactions for soil ecosystem, the relationship between land degradation and ecological clusters of soil microbial taxa (a group of microbial species that strongly interact each other) has not been previously investigated at a level of network analysis.

As we suspected, land degradation such as continuous-cropping obstacle may result in significant changes in the correlation network of soil microorganisms; however, empirical evidence for such assumptions is currently lacking. By analysis of soil microbial network, we can forecast the variation tendency of soil ecosystem and their responses to land degradation. To investigate the bacterial networks in response to land degradation caused by long-term CC, we analysed the bacterial 16S ribosomal RNA gene sequences by high-throughput sequencing technologies from the soil samples of CC fields and noncontinuous cropping (NCC) fields, and then the ecological networks of soil microbial communities under NCC and CC were constructed by the random matrix theory-based approach, respectively (Luo et al., 2007). These networks could provide us more information about the potential effects of land degradation on microbial interactions and microbial communities than the previous studies that mainly focused on the diversity and composition of microbial community.

2 | MATERIALS AND METHODS

2.1 | Study sites and soil sampling

Experimental sites are yellow-brown soil (classified as Alfisols) with subtropical humid climate located in Enshi State (29°93′–29°98′N, 109°34′–109°39′E), Hubei Province, China. The mean annual temperature is 16°C, and the mean annual precipitation is 1,400–1,500 mm. General topography is mostly high mountains, with elevations ranging from 959 to 1,140 m in this area.

The soil samples included two groups: soils with CC tobacco and soils with NCC tobacco. For CC soils, farmers’ fields were annually mono-cultivated with tobacco for more than 25 years without rotation with other crops. The intensive agricultural management practices accompanying with increased chemical fertilizer applications (97.5 kg/ha chemical fertilizers including N:P₂O₅:K₂O = 1:1.5:2.5 was applied annually) and non-application of organic amendments had caused severe land degradation in these fields. For NCC soils, farmers’ fields were cultivated with tobacco–corn rotation for more than 25 years (1 year for tobacco and another year for corn). In the NCC fields, 1,950 kg/ha organic fertilizer (35% organic matter, 2% N, 0.4% P₂O₅, 1.5% K₂O) and 58.5 kg/ha chemical fertilizers (N:P₂O₅:K₂O = 1:1.5:2.5) were applied annually. Other soil management measures (such as irrigation, planting, and pesticide application) were same for the NCC and CC fields. Tobacco grew dwarf and stunted in the CC soils but grew vigorous and robust in the NCC soils (Table 1).

Soil samples were collected from 18 plots before transplanting tobacco. One composite soil sample was collected for each plot. In each plot (667m²), soils were randomly sampled at five different sites from tillage layer soils (0– to 15-cm depth and 10-cm diameter) and then well mixed together to form a composite soil sample. Totally, 10 CC soil samples and eight NCC soil samples were obtained. Samples were homogenized though a 2-mm sieve following divided into two subsamples: one for DNA extraction and another for soil chemical properties and enzymatic analysis.

2.2 | Analysis of soil chemical properties

Soil chemical properties were measured as described previously (Bao, 2013). Briefly, contents of available P and available K (AK) were determined photometrically by a flame spectrophotometer. Soil organic matter (SOM) content was assayed with acidified potassium dichromate (K₂Cr₂O₇–H₂SO₄). Soil pH was determined using a pH metre. Alkali-hydrolysable nitrogen content was determined by alkaline hydrolysis diffusion method. Contents of micronutrients (Ca, Mg, Na, Fe, Mn, Zn, and B) were measured as previously described in the protocol of Zheng (1996).

2.3 | Analysis of soil enzymes activity

Soil urease activity was measured by the colorimetric determination of ammonium (Kandel & Gerber, 1988). Catalase activity was also determined by the colorimetric assay with K₂Cr₂O₇/acetic acid reagent (Sinha, 1972). Soil invertase activity was assayed by 3,5-dinitrosalicylic acid colorimetry (Schinner & Mersi, 1990). Soil phosphatase activity was detected by p-nitrophenol colorimetry (Tabatabai & Bremner, 1969).

2.4 | Determination of agronomic characters of tobacco

Ninety days after transplanting, a total of 200 tobacco seedlings were randomly selected from each field to investigate the height, stem girth, and leaf number of tobacco. The maximum leaf area and top leaf area in each tobacco seedling were also detected (Wang et al., 2017).

2.5 | High-throughput sequencing of soil microbial communities

For each sample, DNA was extracted from 0.4 g of soils using the FastDNA Spin Kit (MP Biomedicals, USA) according to the manufacturer’s protocol, quantified by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and then used as polymerase chain
reaction templates. Primer pair 338F (5′-ACTCCTACGGGAGGCA GCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used for amplifying V3 and V4 regions of bacterial 16S ribosomal RNA genes, which were routinely used to characterize soil bacterial communities and bacterial species (Kim, Morrison, & Yu, 2011), and then the amplified products were purified by AMPure XP beads. Amplicons were sequenced on Illumina-MiSeq platform (Illumina Inc., USA) at SHBIO Technology Co. Ltd (Shanghai, China). Raw sequence data were preliminarily filtered using FASTX Toolkit 0.0.13 software package to remove low mass base at the tail of sequence (Q value <20) and the sequences less than 35BP, and then the valid reads were chosen with an approximate length about 250BP (Wang et al., 2017). OTUs were assigned at 97% similarity level, and then OTU abundances were normalized to the standardized relative abundances (SRA).

2.6 | Construction and analysis of microbial network

We used the online tool named Molecular Ecological Network Analysis Pipeline (http://ieg2.ou.edu/MENA) to construct microbial networks. Briefly, a matrix of OTUs SRA, a matrix of soil variables, and an OTU annotation file were prepared respectively, and then the SRA matrix was submitted to Molecular Ecological Network Analysis Pipeline to construct networks. Thereafter, modules were detected by the greedy modularity optimization, and three files were generated for network graphs visualization by Cytoscape 3.4.0 software (Su, Morris, Demchak, & Bader, 2014). Network graph was represented using different OTUs (nodes) with positive or negative interactions (edges). Positive interactions indicate that the abundance of OTUs changes along the same trend across different soil samples, whereas negative interactions indicate that the abundance of OTUs changes along the opposite trend in different soil samples (Lu et al., 2013).

pMENs were constructed for the CC and NCC soils, respectively. Each network was separated in different modules, which are different interrelated units in the microbial community. A module is a group of microbial species (nodes) that strongly interact each other among themselves but rarely interact with species in other modules (Bascompte & Stouffer, 2009). Modularity (M) measures the degree on how the network is organized into clearly delimited modules. Various network indexes, including average connectivity (avgK), geodesic distance (avgGD) and clustering coefficient (avgCC), and modularity, were used to describe the topology properties and structure of networks. Connectivity is the number of links of a node with other nodes. Geodesic distance is the shortest path between two nodes. Clustering coefficient describes how well a node is connected with the neighbour nodes. By calculating correlations among module eigengenes, eigengene network analysis can reveal the high-order organization of networks structure and identify the key populations of networks. Module membership (MM), the square of Pearson correlation between the abundance profile of a given OTU and a given module eigengene, shows the key OTUs within a module. The groups of eigengenes with considerable correlations are referred as a meta-module (Langfelder & Horvath, 2007).
In network, different OTUs play distinct roles. Topological roles of different OTUs (nodes) are defined by two parameters, within-module connectivity (Zi) and connectivity among modules (Pi; Olesen et al., 2007). Zi describes how well a node connects to other nodes within its own module, and Pi describes how well a node connects to other modules. According to Zi and Pi, the nodes can be divided into four categories: (a) peripheral nodes (specialists, low Zi and Pi) with few links and almost always connected to the nodes within their own modules, (b) connectors (generalists, low Zi but high Pi) with many links to the nodes within other modules, (c) module hubs (generalists, high Zi but low Pi) with great links to many nodes in their own modules, and (d) network hubs (supergeneralsists, high Zi and Pi) with great links to the nodes both in their own modules and other modules. Peripheral nodes represent specialists. Connectors (generalists), module hubs (generalists), and network hubs (supergeneralsists) are key microorganisms in network.

2.7 | Statistical analysis

One-way analysis of variance was performed using SPSS 20.0 software to test all parameters both at 0.05 and 0.01 significance level. Statistics of networks was calculated including network properties, module eigengene, and randomization of network structure. For assessing statistical significance of networks indexes, a total of 100 randomly rewired networks were generated, and then the differences of indexes between pMEN and random networks were detected by Z test for the NCC and CC networks, respectively. Correlation coefficients for networks were calculated on the basis of global network properties, individual nodes’ centrality, module separation, and modularity (Clauset, Newman, & Moore, 2004). Mantel test was conducted to discern the relationships between module eigengenes and soil properties using R vegan package. Singular value decomposition was used for data reduction in eigengene network analysis (Langfelder & Horvath, 2007).

3 | RESULTS

3.1 | Difference of soil and plant properties between CC and NCC

Soil chemical and biological properties and tobacco agronomical properties were all negatively affected by the land degradation caused by continuous-cropping obstacle. Contents of soil available P, alkali-hydrolysable nitrogen, and SOM in the NCC soils were all significantly higher than those in the CC soils. No significant difference was observed for soil pH and AK content between NCC and CC soils (Table 1a). Fe content in the NCC soils was significantly higher than that in the CC soils, whereas B content in the CC soils was significantly higher than that in the NCC soils (Table 1b). No significant difference was observed for other soil micronutrients (Ca, Mg, Na, Mn, and Zn) content between NCC and CC soils. Compared with CC, the activities of soil urease, invertase, phosphatase, and catalase were all higher under NCC. Tobacco height, stem girth, leaf numbers, maximum leaf area, top leaf area, and yield were all significantly greater in the NCC soils than those in the CC soils (Table 1c).

3.2 | Comparing topological properties and structure of networks between CC and NCC

Two networks were constructed for the CC and NCC soils, respectively (Figure 1). The NCC and CC networks consisted of 526 and 332 nodes (OTUs) and 744 and 259 edges (connections), respectively, indicating that the NCC network had a larger size with more nodes and links than the CC network. The NCC network had far more complicated interactions in terms of network size, connectivity, and clustering coefficient compared with the CC network. The networks contained several modules in which different species (nodes) were linked together by edges (i.e., interactions). In the NCC network, more than half of the nodes aggregated into a big module with close interactions among them. Most of links were closer to each other among the nodes of NCC network, suggesting that most of OTUs (nodes) in the NCC network had more frequent interactions when compared with the CC network. In the NCC network, 83.3% (620/744) of the nodes had positive interactions with others, indicating that the microbial community under NCC soils should form a close organization via synergistic interactions among different species. In the CC network, almost 40% (101/259) of the nodes had negative interactions, suggesting that there were more competition or antagonism among different bacterial species in the degraded land caused by continuous-cropping obstacle.

Similar thresholds (Sj) were obtained for the NCC (0.93) and CC (0.92) networks, respectively (Table 2). The connectivity well fitted the power law with R2 values, indicating that these two networks were scale free (Amaral, Scala, Barthélémy, & Stanley, 2000). The avgK of NCC network (2.83) was higher than that of CC network (1.56), suggesting that the NCC network was more complex than the CC network. The average network distance between all pairs of nodes (avgGD) was 7.86 and 5.43 edges for the NCC and CC networks, respectively, suggesting that OTUs in the NCC network were more highly connected with each other when compared with the CC network. The avgCC of NCC (0.24) was higher than the CC (0.08), indicating that nodes in the NCC network tended to better cluster together when compared with those in the CC network. Collectively, the NCC network had higher connectivity, longer geodesic distance, and higher clustering coefficient than the CC network.

The avgCC of empirical networks was higher than the corresponding random networks (0.004-0.011), suggesting that these two networks had both the typical small-world characteristics (Table 2). Modularity (M) values of the NCC network and CC network were both higher than the threshold value for modular structures (0.4), suggesting that these two networks were both modular (Newman, 2006). These results suggested that the topological structures of two networks were quite different. Overall, the soil microbial network of NCC was composed of more highly connected OTUs to form a more clustered topology structure than the CC network.
Comparing composition of networks between CC and NCC

Most of nodes in these two networks belonged to 13 bacterial phyla (Figure 1). Among them, Acidobacteria, Actinobacteria, and Proteobacteria were more dominant than other phyla (see the left panel in Figure 2a). Compared with NCC, the relative proportions of Acidobacteria, Actinobacteria, Firmicutes, and Proteobacteria were all decreased, whereas the relative abundances of Bacteroidetes, Chloroflexi, Gemmatimonadetes, Nitrospirae, and Verrucomicrobia were all increased substantially in the CC network. This result showed that continuous-cropping had changed the relative abundances of bacterial phylogenetic groups that were very different from the NCC soils. Only 14.7% (110/748) of nodes were shared by these two networks, whereas most of nodes (416 for NCC network and 222 for CC network) were unshared and specific in their own networks (see the right panel in Figure 2a). These results suggested that the composition and structure of microbial networks had varied considerably under CC soils when compared with the NCC soils. It seems that the microbial network structure and composition were substantially shaped due to land degradation in this mountain ecosystem.

### TABLE 2  Topological properties of the microbial network under NCC and CC soils and their associated random networks

| Empirical networks | Random networks |
|--------------------|-----------------|
| Network | Size | \(S_t\) | \(R^2\) | \(\text{avgK}\) | \(\text{avgGD}\) | \(\text{avgCC}\) | \(M\) | \(\text{avgGD}\) | \(\text{avgCC}\) | \(\text{avgM}\) |
| CC | 332 | 0.92 | 0.95 | 1.56 | 5.43 | 0.08 | 0.94 (97) | 7.54 ± 1.25 | 0.004 ± 0.002 | 0.93 ± 0.01 |
| NCC | 526 | 0.93 | 0.88 | 2.83 | 7.86 | 0.24 | 0.87 (67) | 4.92 ± 0.09 | 0.011 ± 0.004 | 0.66 ± 0.01 |

Note. The random networks were generated by rewiring all of the links of a phylogenetic molecular ecological network with the identical numbers of nodes and links to the corresponding empirical phylogenetic molecular ecological network. \(S_t\): similar thresholds; \(\text{avgK}\): average connectivity; \(\text{avgGD}\): average geodesic distance; \(\text{avgCC}\): average clustering coefficient; \(M\): modularity; \(\text{avgM}\): average modularity; CC: continuous cropping; NCC: noncontinuous cropping.

3.3 | Comparing composition of networks between CC and NCC

Figure 1: Overview of (a) noncontinuous cropping network and (b) continuous cropping network. Different colours of the nodes belong to different bacterial phyla. Blue lines and red lines indicate negative and positive interactions between different nodes, respectively [Colour figure can be viewed at wileyonlinelibrary.com]
Eigengene network analysis

Eigengene analysis showed that all modules could form MMs in these two networks. A total of 16 modules (>5 nodes per module) including 396 MMs were obtained for the NCC network (Figure 3a, in the left panel), whereas 10 modules (>5 nodes per module) including 116 MMs were obtained for the CC network (Figure 3b, in the left panel). The size of modules varied substantially, ranging from 6 to 59 nodes in the NCC network and from 6 to 26 nodes in the CC network. Also, more modules and MMs were found in the NCC network when compared with the CC network.

The compositions of MMs were quite different between these two networks. The proportions of MMs belonging to Acidobacteria, Firmicutes, and Proteobacteria were all higher in the NCC network than the CC network, whereas the percentages of MMs belonging to Actinobacteria, Bacteroidetes, Chloroflexi, Gemmatimonadetes, Latiscibacteria, Nitrospirae, Planctomycetes, and Verrucomicrobia were all increased in the CC network when compared with the NCC network (see the left panel in Figure 2b). It suggested that the MMs belonging to Acidobacteria, Firmicutes, and Proteobacteria were enriched in the NCC network and that the MMs belonging to Actinobacteria, Bacteroidetes, Chloroflexi, Gemmatimonadetes, Latiscibacteria, Nitrospirae, Planctomycetes, and Verrucomicrobia were enriched in the CC network. Only 5.1% MMs (25/487) were overlapped between two networks (see the right panel in Figure 2b), and most of MMs (94.9%) were specific in the NCC (371 MMs) or CC (91 MMs) networks.

The high-order structure of two networks was further analysed. Eigengenes from different modules were significantly correlated to form meta-modules. Three meta-modules were clustered in the NCC

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**FIGURE 2** Proportion of (a) phylogenetic lineages of total nodes and (b) significant module memberships in the two networks. Venn diagrams show the nodes shared and unshared by the two networks. CC: continuous cropping; NCC: noncontinuous cropping [Colour figure can be viewed at wileyonlinelibrary.com]
The eigengenes of modules N 2, N 6, N 10, N 11, N 15, and N 16 were found with significant correlations (equivalent to a clustering height at 0.4) to cluster together as one meta-module (Figure 3a, see in the upper right panel). Besides, modules N 1, N 3, and N 13 were clustered together as one meta-module, and modules N 4, N 5, N 7, N 8, N 9, N 12, and N 14 were clustered together as another meta-module. These three meta-modules contained all of MMs (396 MMs) in the NCC network.

Module N 9 in the NCC network was used as an example of eigengene network analysis (Figure 3a). The eigengenes of modules N 2, N 6, N 10, N 11, N 15, and N 16 were found with significant correlations (equivalent to a clustering height at 0.4) to cluster together as one meta-module (Figure 3a, see in the upper right panel). Besides, modules N 1, N 3, and N 13 were clustered together as one meta-module, and modules N 4, N 5, N 7, N 8, N 9, N 12, and N 14 were clustered together as another meta-module. These three meta-modules contained all of MMs (396 MMs) in the NCC network. Two meta-modules were clustered in the CC network (Figure 3b). The eigengenes of modules C 3, C 4, C 5, and C 7 were clustered together as one meta-module and modules C 1 and C 6 as another meta-module (Figure 3b, see in the lower right panel). These two meta-modules composed of 77 MMs, accounting for 66.4% of total MMs in the CC network.

FIGURE 3 Overview of eigengene network for (a) noncontinuous cropping (NCC) network and (b) continuous cropping (CC) network (left panel). Modules (with >5 nodes) are labelled with "N" (NCC network) or "C" (CC network). Different colours of node belong to different bacterial phyla. The correlations and heat map indicate the module eigengenes of (a) NCC network and (b) CC network (right panel). The upper part shows the hierarchical clustering based on Pearson correlations among module eigengenes. The heat map shows the coefficient values (r). Red colour represents high correlation, whereas blue colour means low correlation [Colour figure can be viewed at wileyonlinelibrary.com]

Module N 9 in the NCC network was used as an example of eigengene network analysis (Figure 4). The heat map showed the relative abundances of 14 OTUs in different soil samples, in which OTU 21 and OTU 38 were found with the most dominant relative abundance (Figure 4a). The abundance profile of eigengene was shown in Figure 4b. Among eight soil samples, the sixth soil sample contained the highest abundance of MMs, following by the third one, and the forth soil sample contained the lowest abundance of MMs. The key OTUs with significant MMs in module N 9 were OTU 63 and OTU 338 belonging to Alphaproteobacteria and OTU 21, OTU 239, and OTU 601 belonging to Gemmatimonadetes, Acidobacteria, and Actinobacteria, respectively. Their abundances changed with the same trend in all eight soil samples (Figure 4c).

3.5 Key microorganisms of networks

Topological roles of nodes in two networks were shown in Figure 5. According to the threshold values of \(Zi\) (2.5) and \(Pi\) (0.62; Olesen et al., 2007), all OTUs were divided into peripherals, connectors, and module hubs, but no nodes fell into network hubs. Most of OTUs (97.5% for the NCC network and 98.8% for the CC network) were peripherals. Furthermore, 88.4% of peripherals in the NCC network and 97.6% of peripherals in the CC network had no links to other nodes outside their own modules (\(Pi = 0\)). In the NCC network, 13 generalists (12 module hubs and one connector) belonged to Acidobacteria, Actinobacteria, Firmicutes, \(\alpha\)-Proteobacteria, and \(\gamma\)-Proteobacteria, respectively. OTU 7 (module hub) belonging to Firmicutes was closely related to Bacillus, which is popularly used to control plant diseases and decompose organic matters (Meng, Jiang, & Hao, 2016; Shah et al., 2013). OTU 869 (module hub) was assigned to aerobic denitrifying Hyphomicrobiaceae. Four module hubs (OTU 9, OTU 13, OTU 44, and OTU 164) and one connector (OTU 59) were all belonged to Acidobacteria with a leading position in the network. One connector (OTU 406) and three module hubs (OTU 41, OTU 380, and OTU 1122) were also found in the CC network. Overall, more
generalists were found in the NCC network, indicating that more nodes in the NCC network tended to connect with other nodes both within their own and other modules.

### 3.6 | OTUs’ response to soil variables in networks

Most of module eigengenes were significantly ($p < 0.05$) correlated with soil variables. In the NCC network, the significantly positive correlations were found for module N 1 versus urease and catalase, N 3 versus urease and Ca, N 5 versus phosphatase, N 7 versus catalase, N 10 versus AK, N 13 versus Ca, N 14 versus invertase and phosphatase, N 15 versus Na, and N 6, N 10, N 11, N 12, and N 16 versus SOM, respectively (Table 3). These modules contained 221 OTUs, accounting for 56% of the total eigengene memberships in the NCC network. Besides, the significantly negative correlations were found for modules N 1, N 3, and N 13 versus SOM; N 6 versus pH; N 11 and N 16 versus urease; N 12 versus Ca; and N 13 versus AK.

In the CC network, the significantly positive correlations were found for module C 1 versus urease, pH, and Ca; C 2 versus Mg; C 4 versus Mg; C 5 versus Mg; C 6 versus pH and urease; C 7 versus Mg; and C 10 versus Mg and Fe, respectively. The significantly negative correlations were found for module C 1 versus Mg; C 3 versus pH and Ca; C 4 versus pH and Ca; C 5 versus urease, pH, SOM, and Ca; C 7 versus pH; and C 10 versus urease and SOM; respectively (Table 3). These modules contained 75 OTUs, accounting for 66% of total eigengene memberships in the CC network. Collectively, the majority of bacteria were positively affected by soil variables in the NCC soils but negatively affected by soil variables in the CC soils. The network interactions among different bacterial populations were changed by continuous-cropping obstacle, and these changes were significantly related to the soil properties such as pH, Ca, Mg, and SOM.

### 4 | DISCUSSION

Soil chemical properties (e.g., SOM, Fe, and B contents) were significantly affected by continuous-cropping obstacle. SOM is important for soil ecology such as water conservation and providing nutrients (N, P, S, and micronutrients) for plants and microbes (Obalum, Chibuike, Peth, & Ouyang, 2017), thus playing a vital role to maintain the soil fertility and crop productivity. Here, SOM content in the CC soils was significantly lower than that in the NCC soils (Table 1a), indicating that the soils fertility of CC was lower than the NCC soils. Thus, continuous-cropping obstacle could lead to the decrease of SOM content and soil fertility. SOM can be degraded by species of Acidobacteria (García-Fraile, Benada, Cajuham, Baldrian, & Lladó, 2015), Firmicutes (e.g., Bacillus; Kumar, Khare, & Dubey, 2012), and Proteobacteria (Zhang et al., 2015). Under NCC, the generalists (OTU 59, OTU 44, and OTU 164) belonging to Acidobacteria might degrade the abundant SOM in soils. In the CC network, they (OTU 59, OTU 44, and OTU 164) were all specialists possibly due to the low SOM content under CC soils. The degraded SOM, especially labile SOM fraction, could serve as an easily available carbon source for bacterial growth that was speculated by the positive correlations between...
SOM and many eigengene memberships (modules N 6, N 10, N 11, N 12, and N 16) in the NCC network (Table 3).

Iron (Fe) is an essential micronutrient both for plants and soil microbes (Raymond, Dertz, & Kim, 2003). Many bacteria belonging to Firmicutes and Proteobacteria (e.g., Bacillus, Burkholderia, Enterobacter, Erwinia, Paenibacillus, Pantoea, Pseudomonas, Serratia, and Stenotrophomonas) can synthesize siderophores to absorb Fe from the surrounding environment (Zhou et al., 2016), hence occupying favourable niches via depriving Fe from the neighbouring competitors (McNally & Brown, 2015). The siderophore producers also play an important role in promoting plant uptake of Fe (Zhou et al., 2016). Here, the MMs belonging to Firmicutes and Proteobacteria were enriched in the NCC network (Figure 2b), possibly promoting the growth of tobacco and soil bacteria by Fe absorption.

Boron (B) is an essential plant micronutrient, but excessive B is toxic to both of plants (Aitken & McCallum, 1988) and of bacteria (Mansour, Bradford, & Venkatesan, 2008; Sayin, Ucan, & Sakmanoglu, 2016). A significant decrease in microbial diversity and richness is observed in the rhizospheric soil with high B content (Nelson & Mele, 2007). Here, B content was significantly higher in the CC soils than the NCC soils (Table 1b). High content of B in the CC soils was possibly detrimental for the bacterial community and tobacco growth and thus might be one of the constraints to limit soil quality that is defined as the capacity of soil to sustain biological productivity, maintain environmental quality, and promote plant and human health (Andrews, Karlen, & Mitchell, 2002). For this reason, reduced application of B fertilizer might be favourable for alleviating continuous-cropping obstacle in this area. These results indicated that long-term CC tobacco had led to land degradation (limited nutrients or excessive toxic nutrient), hence hampering plant growth in the CC fields (Table 1c).

Interactions among different microbial species are very important for ecosystem community's function in the complex soil ecosystem (Barberán et al., 2012; Lupatini et al., 2014). Thus, network analysis has been increasingly used to explore the potential microbial interactions in different ecosystems (de Menezes et al., 2015; Eiler, Heinrich, & Bertilsson, 2012). However, few attempts have been done to study the change of co-occurrence network patterns at degraded lands. In this study, we employed network analysis to visualize and quantify interactions among microbes. Figure 5 shows the distribution of operational taxonomic units (OTUs) based on their topological roles. Each symbol represents an OTU in the continuous cropping (CC) network (blue circle) or noncontinuous cropping (NCC) network (red triangle). The threshold values of Zi and Pi for categorizing OTUs are 2.5 and 0.62, respectively. All of the OTUs identified as generalists are labelled. OTU 59 and OTU 406 are marked as the examples of role shift [Colour figure can be viewed at wileyonlinelibrary.com]
the microbial interactions and co-occurrence patterns between soil bacteria at degraded land caused by continuous-cropping obstacle. Two pMENs were constructed based on random matrix theory approach, which can automatically identify the thresholds for network construction; hence, the constructed networks are considered accurate and reliable (Deng et al., 2012; Luo et al., 2007; Zhou et al., 2010). This study provides a new insight into the crucial roles of microbial interactions that can enhance our understanding of the response of microbial communities to land degradation caused by continuous-cropping obstacle.

Tobacco continuous-cropping obstacle had a significantly negative impact on the network interactions of microbial communities and had changed the structures and compositions of microbial network. In the CC soils, the microbial network had fewer interactions among different microbial populations when compared with the NCC soils (Figure 1). Microbe–microbe interactions within communities contribute to cause the complexity in community structure and maintain the stability of ecosystem (e.g., soil microbiome; Barberán et al., 2012; Hibbing et al., 2010; Schmitt et al., 2012; Zhang et al., 2014; Zhou et al., 2011). In the CC soils, the microbe–microbe interactions within communities seemed to be weak for maintaining the complexity of community structure and the stability of ecosystem. The exchanges of message, energy, and nutrient among different microbial species were probably hindered under CC soils, in which the microorganisms had fewer interactions when compared with the NCC soils. On the contrary, the NCC network was more complex with more links among OTUs than the CC network, suggesting that there are more interactions among species in the soil microbial community under NCC. Possibly, in the NCC soils, microorganisms could better cooperate together to deal with environmental impacts when compared with the CC soils.

It was found that 83.3% of nodes in the NCC network had positive interactions with others. Positive interactions reflect cooperative behaviours such as cross feeding, syntrophic interactions, and mutualistic interactions among different microbial species (Raes & Bork, 2008). This indicated that the microbial community under NCC soils formed a close organization via synergistic interactions among different species, whereas in the CC network, almost 40% of nodes had negative interactions, suggesting that many bacterial species in the continuous-cropping soils competed for resources or spaces and repelled each other. These negative interactions might derive from depletion of key nutrients (e.g., phosphorus, nitrogen, iron, and SOM) or accumulation of toxic compounds (e.g., boron) in the soils. It was speculated that different microbes in the CC networks compete the limited nutrients and inhibit the growth of their competitors (Freilich et al., 2010). These results suggested that CC dramatically changed the network interactions of various microbial populations in soils.

Generalists are key organisms that promote exchanges of energy, information, and materials among different species in network, hence playing important roles in maintaining the balance of microbial community. In ecology, generalists can utilize a wide range of food

| Module | Code | No. of node | Urease | Invertase | Phosphatase | Catalase | pH | AK | SOM | Ca | Na |
|--------|------|-------------|--------|-----------|-------------|----------|----|----|-----|----|----|
| N1     | 31   | 0.86 (0.006)| 0.76 (0.03)| -0.81 (0.01) |
| N3     | 25   | 0.85 (0.008)|         | -0.88 (0.004) 0.77 (0.03)|
| N5     | 39   |             | 0.77 (0.03)|          |
| N6     | 6    |             | -0.76 (0.03)| 0.72 (0.04)|
| N7     | 36   |             | 0.78 (0.02)|          |
| N10    | 12   |             | 0.86 (0.006)| 0.74 (0.04)|
| N11    | 12   | -0.89 (0.003)|          | 0.73 (0.04)|
| N12    | 42   |             | 0.74 (0.03) -0.8 (0.02)|
| N13    | 8    |             | -0.76 (0.03) -0.79 (0.02) 0.98 (2e-05)|
| N14    | 6    | 0.84 (0.009) 0.77 (0.02)|          |
| N15    | 6    |             |          |
| N16    | 10   | -0.75 (0.03)|          | 0.84 (0.01)|

| Module | Code | No. of node | Urease | Phosphatase | pH | SOM | Ca | Mg | Fe |
|--------|------|-------------|--------|-------------|----|-----|----|----|----|
| C1     | 26   | 0.81 (0.005)| 0.91 (0.0002)| 0.66 (0.04) | -0.86 (0.001)|
| C2     | 18   |             |          | 0.76 (0.01)|
| C3     | 13   |             | -0.77 (0.009) | -0.8 (0.005)|
| C4     | 11   |             | -0.9 (0.0004) | -0.79 (0.007) | 0.65 (0.04)|
| C5     | 9    | -0.82 (0.004)| -0.8 (0.005) | -0.73 (0.02) | -0.73 (0.02) | 0.87 (0.001)|
| C6     | 8    | 0.65 (0.04) | 0.9 (0.0004)|          |
| C7     | 10   | -0.91 (0.0003)|          | 0.72 (0.02)|
| C10    | 6    | -0.82 (0.004)| -0.65 (0.04) | 0.65 (0.04) | 0.73 (0.02)|

Note. Data with $p \geq 0.05$ were not shown. The data in brackets were the $p$ values. AK: available K; SOM: soil organic matter; SOM: soil organic matter.
sources, thus growing well in many habitats and distributing broadly across the soil ecosystem, whereas specialists have special nutritional requirements, thus surviving only in some restricted habitats (Deng et al., 2012; Olesen et al., 2007; Tao et al., 2018). In this study, only four generalists were found in the CC network, whereas 13 generalists were found in the NCC network (Figure 5). More generalists in the NCC network were favourable for maintaining the microbial community more ordered, stable, and efficient by promoting exchange of energy, nutrients, and metabolites among different microbes (Olesen et al., 2007; Székely & Langenheder, 2014). Moreover, roles of some nodes shifted in two networks, suggesting that continuous-cropping obstacle seemed to have changed the ecological roles of key microorganisms in soils. It was noteworthy that some nodes (OTU 44, OTU 59, OTU 102, and OTU 164) were generalists in the NCC network but were specialists in the CC network, possibly due to poor nutrients (lower SOM, Fe, nitrogen, and P contents, etc.) and excessive toxic element (e.g., B) in the degraded land (Table 1). OTU 44, OTU 59, and OTU 164 belonged to Acidobacteria. Acidobacteria, important contributors to ecosystems, are particularly abundant in soils (Foessel et al., 2014; Jones et al., 2009). As generalists in the NCC network, Acidobacteria could effectively connect with other nodes to form a stable ecological system; however, as specialists in the CC network, they rarely connected with other species. Conversely, OTU 406 was identified as a specialist in the NCC network and as a generalist in the CC network. OTU 406 belonged to Phenyllobacterium that can effectively degrade plant autotoxins such as phenolic acids produced by CC plants (Kitazawa et al., 2005). Thereby, OTU 406, as a specialist in the NCC network, suggested that fewer plant autotoxins were possibly accumulated in the soils under NCC whereas, as a generalist in the CC network, suggested that there were more autotoxins secreted by tobacco roots under CC soils.

Eigengene network analysis showed that the high-order organizations of two networks were different. Fourteen MMs were co-occurrence in module N 9 (Figure 4), suggesting that these MMs might interact with each other to occupy the same ecological niches in the soil through synergistic interactions. NCC network (396 nodes) had more eigengenes than the CC network (116 nodes), suggesting that there were more nodes in the co-occurrence pattern under NCC soils. NCC network fell into three meta-modules containing all of the eigengene memberships, whereas CC network fell into two meta-modules containing only partial memberships (66.4%). Meta-module is a group of interrelated modules, suggesting that more bacterial species were interrelated in the NCC network when compared with the CC network. NCC network had a higher high-order organization and formed a more stable community than the CC network (Figure 3 a,b). Complicated interactions among module eigengenes also indicated that the NCC network was more stable and ordered than the CC network (Lu et al., 2013; Tao et al., 2018).

Most of MMs were specific in the NCC or CC network, implying that they likely occupy individual ecological niches just in their own networks. MMs belonging to Actinobacteria, Chloroflexi, Gemmatimonadetes, Nitrospirae, Planctomycetes, and Verrucomicrobia were enriched in the CC network (Figure 2b). Generally, Actinobacteria, Chloroflexi, and Gemmatimonadetes often live in a dry environment (DeBruyn, Nixon, Fawaz, Johnson, & Radoshevich, 2011; Santos-Medellín, Edwards, Liechty, Nguyen, & Sundaresan, 2017). Actinobacteria, Nitrospirae, Planctomycetes, and Verrucomicrobia are oligotrophic microorganisms that usually live in a nutrient-deficient environment (Koeh et al., 2015; Rodriguez, Guerra, Forstner, & Hahn, 2016; Wertz, Kim, Breznak, Schmidt, & Rodrigues, 2012). Deteriorated environment in the CC soils (limited nutrients or excessive toxic nutrient) might specifically select these microorganisms. However, MMs belonging to Acidobacteria, Firmicutes, and Proteobacteria were enriched in the NCC network (Figure 2b). Species of Firmicutes can degrade hemicellulose and cellulose by producing various enzymes (xylanases, β-xylidosidases, α-glucuronidases, and acetyl xylan esterases; Zhu et al., 2016) and inhibit plant pathogens by producing antibiotics (surfactin, iturin, fengycin, etc.; Qi et al., 2010). Proteobacteria can decompose organic matter to oligosaccharides by production of enzymes (cellulases, chitinases, xylanases, and amylases), thus providing carbon sources for other microorganisms (e.g., Acidobacteria; Taha et al., 2015). Overall, Acidobacteria, Firmicutes, and Proteobacteria might play important ecological roles by collaborating with each other in breakdown of plant polysaccharide that could provide carbon sources for other microbial species in the network. This could have been a beneficial factor for maintaining the stability of microbial network under NCC soil.

Microbial community is usually affected by soil chemical properties. We found that the change of network structure and role shift of key microorganisms was significantly correlated with soil chemical variables. Most of module eigengenes (56%) in the NCC network were positively correlated with soil variables, whereas majorities (66%) of module eigengenes in the CC network were negatively correlated with soil variables (Table 3). The results suggested that continuous-cropping obstacle has a detrimental effect on the bacterial network. Possibly, soil variables were more favourable for the exchanges of nutrients, energy, and information among different bacterial populations in the NCC network when compared with the CC network. This study linked land degradation with microbial associations, and the changes of networks can act as indicators or predictors for land degradation in the future study. However, our results only considered the bacterial community, whereas other microorganisms, for example, fungi and archaea, might play important roles in the microbial interactions as well (Kallenbach, Frey, & Grandy, 2016; Pereira e Silva, Dias, van Elsas, & Salles, 2012). Future efforts should consider other microorganisms (fungi, archaea, etc.) to enhance the breadth and depth of inferred interactions.

5 CONCLUSIONS

Continuous-cropping obstacle has strong negative effects on soil chemical properties, tobacco agronomical properties, and microbial networks. This study demonstrated the changes of bacterial network in response to the land degradation caused by continuous-cropping obstacle. Key microorganisms, network organization, and their responses to soil variables were all different between NCC and CC networks. NCC network had longer geodesic distance, higher connectivity and clustering coefficient, and more generalists than the CC
network, whereas CC network with fewer interactions among different bacterial groups seems to be less stable and ordered than the NCC network; 83.3% of nodes in the NCC network had positive interactions, whereas 40% of nodes in the CC network had negative interactions, suggesting that there were more competition or antagonism among bacterial species under CC. Some generalists in the NCC network were shifted to specialists in the CC network.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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