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The Changes in Rhizosphere Metabolome and Microbiota Are the Main Direct Obstacles to Continuous Cropping in Tobacco (*Nicotiana tabacum* L.)

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Abstract: Continuous cropping obstacles (CC), typical of negative plant–soil feedback, have become a non-negligible constraint to the development of agriculture. In order to investigate the main direct drivers influencing the formation of CC soils from the rhizosphere of CC, tobacco fields were collected and their physicochemical properties, microbial community, and metabolomics were measured and analyzed. We also built a mixed linear model to evaluate the impact of these factors on CC. The results suggested that the pH, available potassium (AK), and zinc (Zn) were significantly higher in CC than in normal tobacco (NOR). However, the content of available nitrogen (AN) decreased significantly. Alpha diversity of the bacterial community was significantly reduced. Bacterial community structure also varied significantly in CC. The study identified an ecological cluster with a significant negative correlation with the above-ground biomass of plants. In this cluster, the pathogenic microbiome increased and the beneficial microbiome decreased. The orthogonal partial least squares discriminant analysis (OPLS-DA) indicated clear variations in the metabolomic profiles of the rhizosphere soil between the CC and the NOR. There was an accumulation of toxic compounds and a decrease of beneficial compounds in rhizosphere soils with CC. The mixed linear model showed that only microbiome and metabolites, rather than the soil's physicochemical properties, significantly affected plant above-ground biomass. According to the model's standardized coefficients, metabolites contributed more to the continuous crop obstacles than the microbial community. The soil's physicochemical properties do not directly cause the emergence of CC. The allelochemicals and microbial community are the main direct obstacles to continuous cropping in tobacco, and allelochemicals contribute more than the microbial community.

Keywords: continuous cropping obstacles; ecological cluster; metabolome; microbiota

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1. Introduction

Plants modify the environment of the soil to overcome ecological challenges and optimize their fitness [1–4]. However, this process can affect the growth of the plants themselves [5]. This plant–soil feedback is divided into positive and negative. In the primary succession stage of the community, positive feedback promotes the development of the ecosystem and plays an important role in the formation of the community [6]; the negative feedback promotes the coexistence of species and avoids the emergence of dominant species in the community, which is of great significance to the stability and species diversity of the community [7,8]. However, negative feedback brings adverse factors to agriculture. For example, continuous cropping obstacles, as a classic plant–soil negative feedback, considerably restrict the potential for sustainable agriculture [9].

Many agricultural plants exhibit strong continuous cropping obstacles, such as crop, medicinal, and Solanaceae plants. Continuous cropping obstacles have become a non-negligible constraint to the development of Chinese agriculture. Much research has been conducted to determine the causes and mechanisms of continuous cropping obstacles. In

general, soil fertility unbalance, rhizosphere autotoxin enrichments, and microbial community variation were considered to be the three main factors of continuous cropping obstacles [10,11]. For example, continuous strawberries were accompanied by variations in soil's physicochemical properties [12]; *Saussurea lappa* rhizosphere soil extracts inhibited self and lettuce growth due to ROS enrichment and damage to membranes [13]; continuous cropping modified the rhizosphere microbial community of the sweet potato, decreasing the microbes that were beneficial and increasing those that were harmful [14]. However, the direct contribution of these three factors to continuous cropping obstacles remains understudied.

Tobacco is a model organism and an important crop for millions of farmers in China. However, tobacco depletes some nutrients (for instance: phosphorus, nitrogen, and potassium) selectively from the soil, causing decreased soil fertility and imbalanced soil nutrients. More attention has been paid to the secondary metabolites' aroma components in tobacco than in other crops. Most allelochemicals are secondary metabolites. Generally, tobacco is also a plant prone to continuous cropping obstacles. Meanwhile, because of limited farmland and the absence of appropriate cultivation methods, tobacco in southwestern China is generally grown in a continuous cropping pattern, which often leads to continuous cropping obstacles with quality deterioration and yield decline [15]. In this study, we used tobacco to explore the direct contribution of three factors to continuous cropping obstacles. The physicochemical properties of continuous cropping obstacles and normal tobacco rhizosphere soil were detected. The microbial community was analyzed using Illumina MiSeq sequencing technology, and the metabolomics of the rhizosphere soil were analyzed using GC-MS technology. Our study provides useful information for the research of continuous cropping obstacles and has some guidance for agricultural production.

2. Materials and Methods

2.1. Sample Collection

The sampling site was located in Gucheng, Lijiang, Yunnan Province, China (26°43′13″ N, 100°15′35″ E). The mean annual temperature in the region is between 13 and 20 °C, and the mean annual rainfall is 1000 mm. We sampled Yunyan301 as a material. Yunyan301 is a variety that tobacco companies like to use for its high quality, and also the main variety in Lijiang. At the time of our sampling, the sample field had been growing tobacco for six years.

When removing flower buds from tobacco, we selected six typical continuous cropping obstacle tobacco (CC) fields and their adjacent normal tobacco (NOR) fields. We took five tobacco rhizosphere soil samples from each field and mixed them into one sample, for a total of twelve samples. The above-ground biomass (OB) of these tobacco plants was also measured. The five rhizospheres in each field were sampled in the "S" shape. The collection of rhizosphere soil was carried out according to Zheng, et al. [16]. We passed the samples through a 2-mm sieve and divided them into three parts. They were dried for physical and chemical characterization and stored at −80 °C for microbiological and metabolomic analysis.

2.2. Soil Physicochemical Indicators

The rhizosphere soil pH and available nitrogen were measured according to Roberts, et al. [17]. The electric conductivity was measured according to Anderson and Ingram [18]. In addition, we measured the available potassium, available phosphorus, and organic matter according to Liu, et al. [19]. The soil's available trace elements Ca, Mg, K, Fe, Cu, Mn, and Zn were analyzed by atomic absorption spectrophotometry [18]. Principal component analysis (PCA) based on significantly different ($p < 0.05$) soil physicochemical properties was done. We used the PC1 to represent the entire differential physicochemical properties for multiple linear regression analysis (CPC1).

2.3. Microbial Community by Illumina MiSeq Sequencing

Soil samples were transported on dry ice to the Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). Sequencing of the microbial communities was performed using Illumina MiSeq sequencing (Illumina Inc., San Diego, CA, USA) according to the standard protocols. Briefly, microbial community genomic DNA was extracted using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). Primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the V3-V4 hypervariable region of the bacterial 16S rRNA gene. Primer pairs ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) were used to amplify the fungal internal transcribed spacer 1 (ITS1)-ITS2 region. The PCR mixture of genes was configured according to manufacturer's instructions in TransGen AP221-02. Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

The sequencing data quality was controlled by fastp (v0.19.6) and spliced using FLASH (v1.2.7). Amplicon Sequence Variant (ASV) was obtained by noise reduction using the DADA2 method in the qime2 (2022.2) software [20]. The richness, diversity indexes, and the principal coordinate analysis (PCoA) were generated based on qime2 (2022.2). Naive Bayes classifier, which was trained on weighted Silva (v138, 99% full-length sequences, 16S) or UNITE (ver8 dynamic 10.05.2021, ITS) were used to explore the taxonomic composition of the rhizosphere soil [21].

We constructed the co-occurrence network based on ASVs. Briefly, we removed some low-abundance ASVs (occurs greater than five times in at least two samples) first of all. Then we normalized the data and calculated the pairwise spearman correlation matrix. In addition, we converted the correlation matrix into a similarity matrix. The threshold value for the correlation was set at 0.8, and the threshold value for the significance was set at 0.05. We used multi-level pattern analysis and likelihood ratio tests to verify the differential species implementations in indicspecies and edgeR. The ASVs that were detected by both methods were identified as continuous cropping obstacle susceptible ASVs (csASVs). We used the fast greedy method to cluster the nodes (ASVs) in the network into modules.

2.4. Chemical Composition of Rhizosphere Soil by GC-MS

The metabolites of rhizosphere soil were determined by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). An Agilent 8890B gas chromatography system connected to an Agilent 5977B mass selective detector (single quadrupole) was used to make the determination (Agilent Technologies Inc., Santa Clara, CA, USA). The instrument is equipped with an inert electron impact (EI) ionisation source with an ionisation voltage of 70 eV. Samples were separated on a DB-5MS capillary column (40 m × 0.25 mm × 0.25 μm) using 99.999% helium as carrier gas at a constant flow rate (1 mL/min). The temperature of the GC column was programmed to hold at 60 °C for 30 s and rise to 310 °C at a rate of 8 °C per minute for 6 min. The sample injection volume was 1 μL and injected in split mode (15:1) with an inlet temperature of 260 °C. The mass spectrometry conditions were as follows: the ion source temperature was 230 °C and the quadrupole temperature was 150 °C. The scan mode is full scan mode, the quality scan range is m/z 50–500, and the scan frequency is 3.2 scans/s.

After mass spectrometry detection, the raw GC/MS data were pre-processed using the standard procedure of Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Raw data obtained from mass spectrometry detection of GC/MS were pre-processed using MassHunter Workstation Quantitative Analysis software (version v10.0.707.0). False positive peaks (including noise, column bleed, and derivatized reagent peaks) were removed as deredundant and peak pooled. At the same time, metabolites were identified by searching public databases: NIST (version 2017), Fiehn (version 2013), and MS-DIAL (version 2021). A Log10 logarithmization was performed on the raw data matrix to obtain the transformed data matrix for further analysis.

The OPLS-DA was performed on MetaboAnalyst 5.0 (www.metaboanalyst.ca, accessed on 11 November 2022). We also used the variable influence on projection (VIP) to filter for important variables (VIP > 1). We used PCoA based on the differential metabolites. We also used PC1 to represent the entire differential metabolite for multiple linear regression analysis (MPC1).

2.5. Statistics and Analysis

The GraphPad Prism 8.0.2 was used for graphing. The R (http://www.r-project.org, v4.2.0) packages involved in data analysis are edgeR, indicspecies, igraph, Hmisc, sciplot, and reshape2. Multiple linear regression (the independent variable enters the analysis via stepwise) was done using SPSS® 23. The PC1 of PCA was based on significantly different soil physicochemical properties (CPC1), the abundance of module #1 of the network (M1), MPC1, and OB. The OB was considered a dependent variable. All data for which modeling was performed were min-max normalization transformed.

3. Results

3.1. The Biomass of Continuous Cropping Obstacle and Normal Tobacco

The mean above-ground biomass of tobacco was 2.02 kg in NOR and 0.53 kg in CC. This difference was statistically significant (Figure 1, *t*-test, $p < 0.05$).

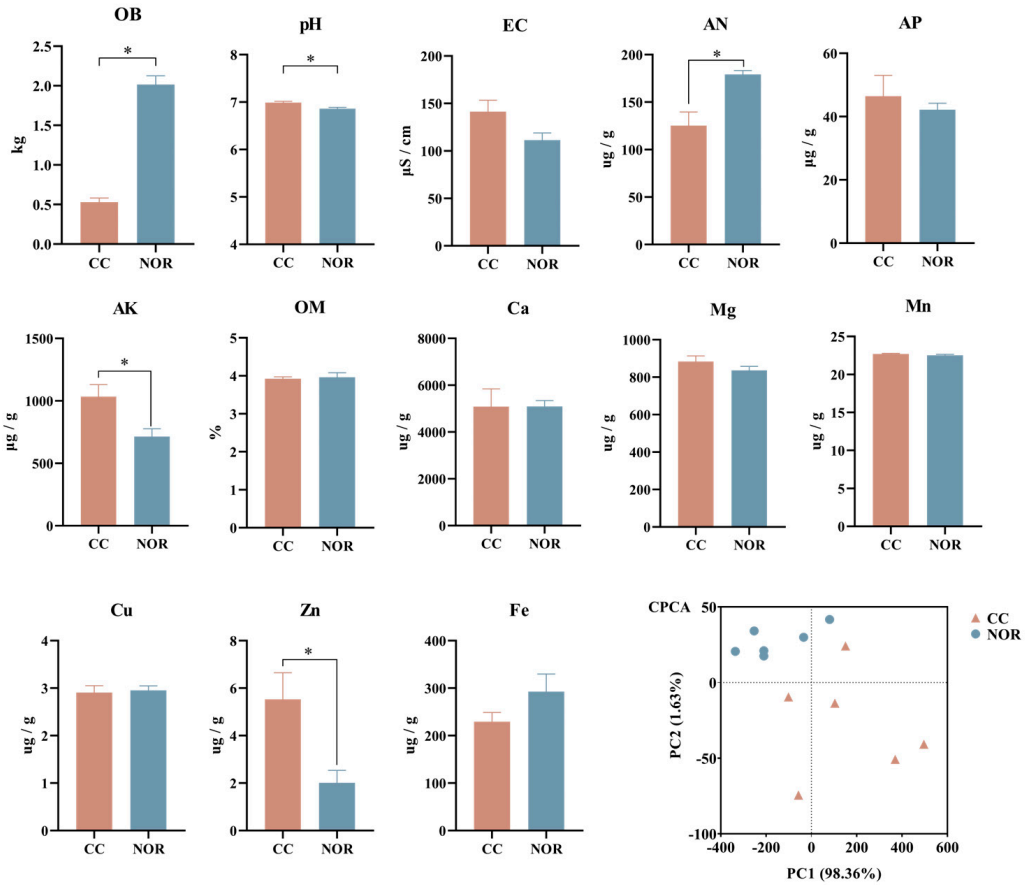

Figure 1. Above–ground biomass of plants and physicochemical properties of rhizosphere soil. OB: above–ground biomass of tobacco; pH: hydrogen ion concentration; EC: electric conductivity; AN: available nitrogen; AP: available phosphorus; AK: available potassium; OM: organic matter; Ca: calcium; Mg: magnesium; Mn: manganese; Cu: copper; Zn: zinc; Fe: iron; CPCA: principal component analysis (PCA) based on pH, AN, AK, and Zn was based on the variance–covariance matrix; CC represents continuous cropping obstacle field tobacco; NOR represents normal field tobacco; Asterisk (*) indicates significant differences (*t*-test, $p < 0.05$). Error bars are standard errors.

3.2. Soil Physicochemical Properties of Continuous Cropping Obstacle and Normal Tobacco

Physicochemical properties were measured in two rhizosphere soils. The CC had a significantly higher pH, available potassium (AK), and zinc than NOR (Figure 1, $p < 0.05$). However, the contents of available nitrogen (AN) were significantly lower in CC than NOR (Figure 1, $p < 0.05$). Principal component analysis (PCA) based on significant difference indicators profiles showed separation between CC and NOR. The first component (PC1) of PCA explained 98.36%.

3.3. Rhizosphere Soil Microbial Communities

To assess the continuous cropping obstacle rhizosphere soil's microbial community, 16S and ITS rRNA pyrosequencing were applied. Across all samples, a total of 652,827 (16S) and 815,338 (ITS) features were obtained, with at least 46,919 (16S) and 59,126 (ITS) features per sample. Compared to NOR, bacteria that belong to Planctomycetota and Acidobacteriota increased significantly in CC, while those belonging to Gemmatimonadota, Methylomirabilota, and Firmicutes decreased significantly ($p < 0.05$, *t*-test, Figure 2).

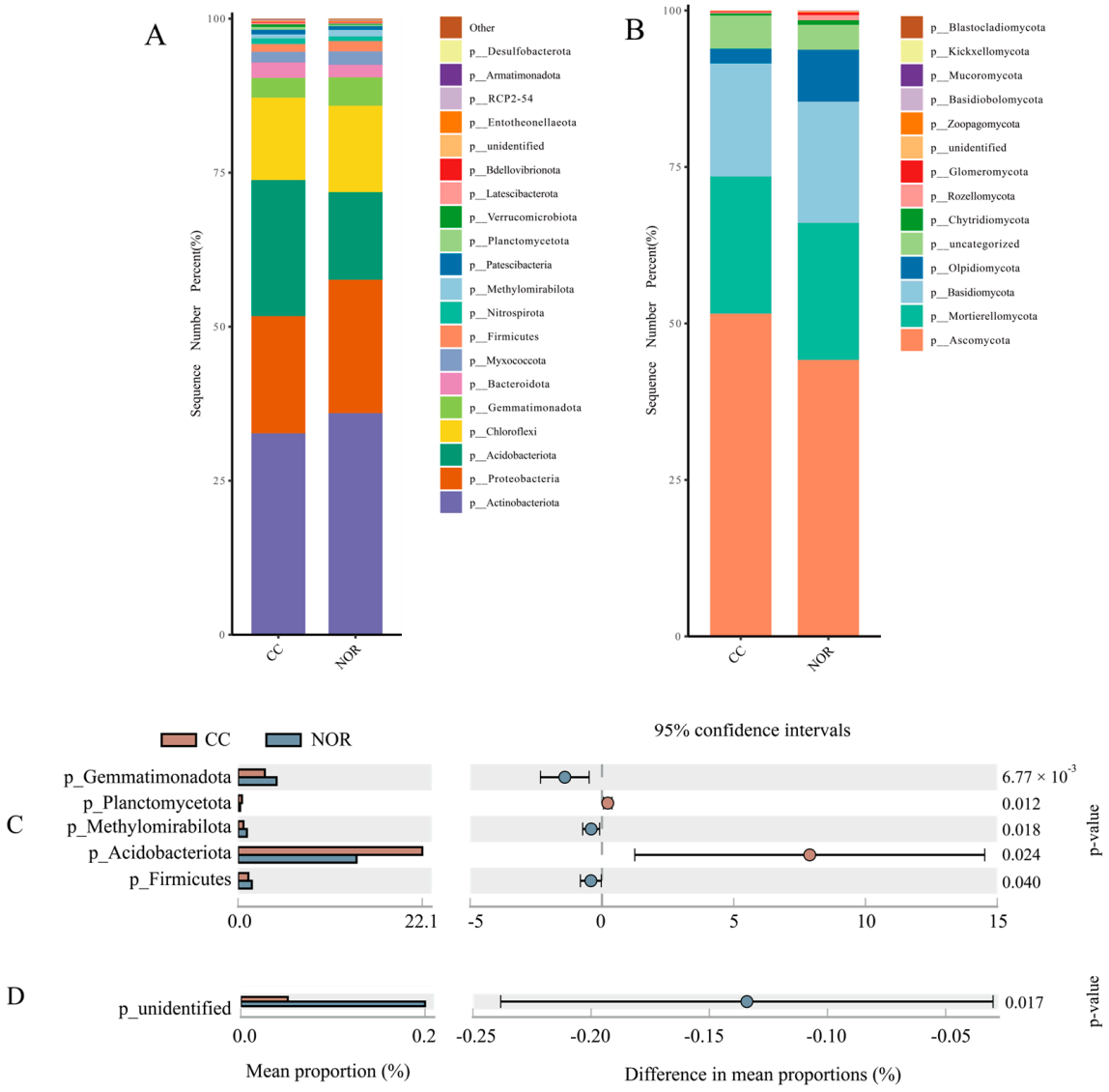

Figure 2. Diversity of rhizosphere microbial community. (**A**): Bacterial phylum level community structure; (**B**): fungal phylum level community structure; (**C**): significantly different bacterial phylum (*t*-test, $p < 0.05$); (**D**): significantly different fungal phylum (*t*-test, $p < 0.05$). Error bars are standard errors.

As a measure of species diversity within a sample, we calculated alpha diversity. The ACE (abundance-based coverage estimator), Shannon, and Simpson indices had been calculated to estimate the richness of each sample. The bacterial community showed that all three indices of diversity were significantly lower in CC than in NOR ($p < 0.05$, Figure 3A). In the fungal community, however, there was no statistically significant difference.

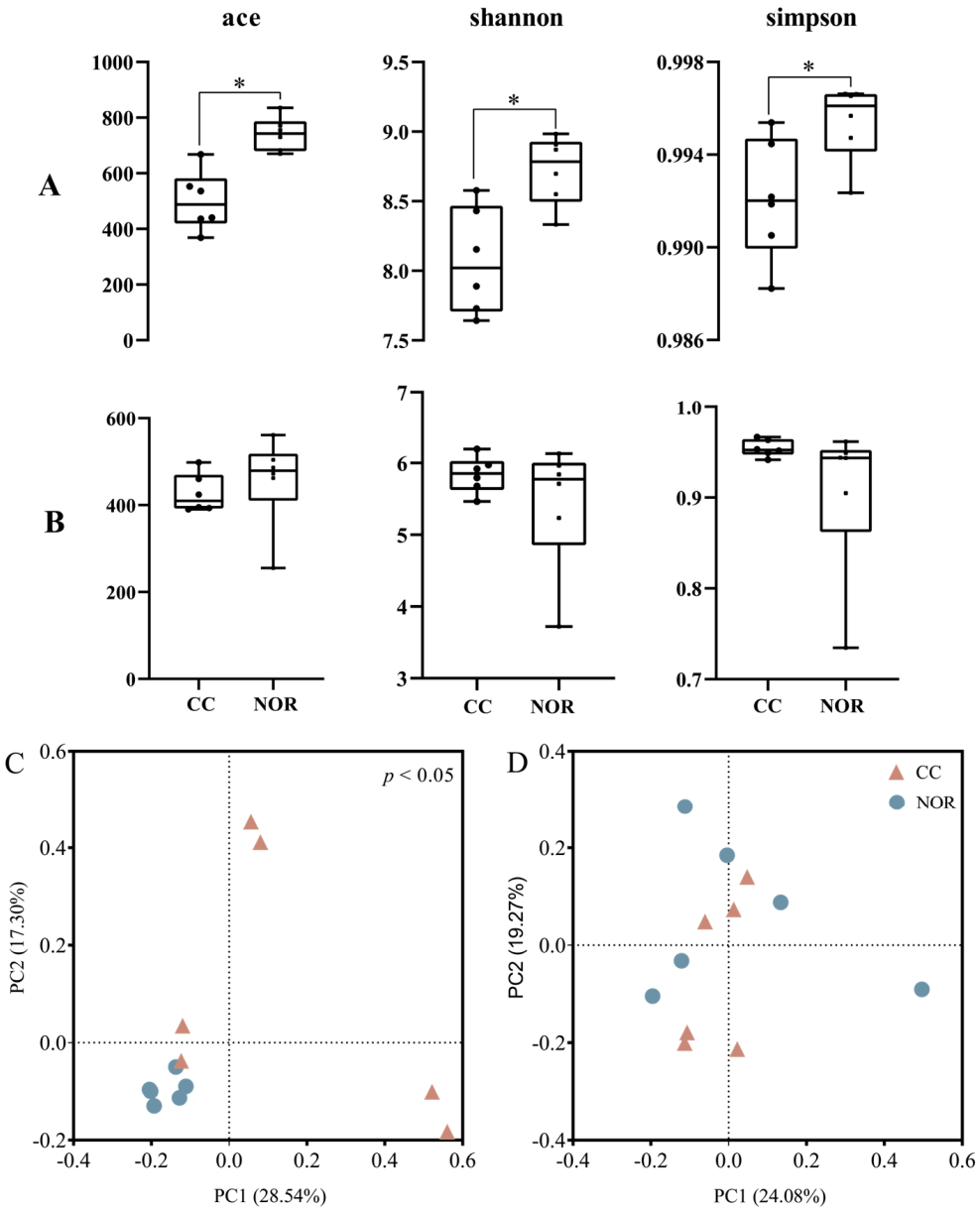

Figure 3. Diversity of rhizosphere microbial community. (**A**): The α diversity of bacterial community; (**B**): the α diversity of fungal community; ACE representative of the abundance-based coverage estimator; Shannon representative of shannon indices; Simpson representative of Simpson indices; CC represents continuous cropping obstacle field tobacco; NOR represents normal field tobacco; asterisk (*) indicates significant differences (*t*-test, $p < 0.05$). Error bars are standard errors; (**C**): the principle coordination analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA, by Adonis) analysis based on the Bray–Curtis distance revealed distinct differences in rhizosphere soil bacterial community structure between CC and NOR ($p < 0.05$); (**D**): the PCoA and PERMANOVA (by Adonis) analysis based on the Bray–Curtis distance revealed distinct differences in rhizosphere soil fungal community structure between CC and NOR.

According to the PCoA and Adonis, there were significant differences in the structure of the soil bacterial community in the rhizosphere between CC and NOR based on the Bray–Curtis distance (Figure 3C, $p < 0.05$). The explanations of PC1 and PC2, the first two components of PCoA, in bacterial community were 28.54% and 17.30%, respectively. The fungal community were 24.08% and 19.27%, respectively.

We investigated the ASV distribution patterns in the rhizosphere's inter-kingdom (bacteria and fungi) co-occurrence network (Figure 4A). We observed that the abundance patterns of the inter-kingdom co-occurrence network were associated with continuous cropping obstacles. The emergence of a continuous cropping obstacle was associated with a variation in the microbial community. Many of the csASVs belonging to CC were mainly located in modules 1 and 2, separated from others in modules containing mainly csASVs belonging to NOR. We also calculated the cumulative relative abundance of the modules in the network. There was a significant difference in the abundance of module #1 (M1) between the CC and NOR (Figure 4B, *t*-test, $p < 0.05$). Furthermore, this module was also significantly negatively correlated with the above-ground biomass of tobacco (Table 1).

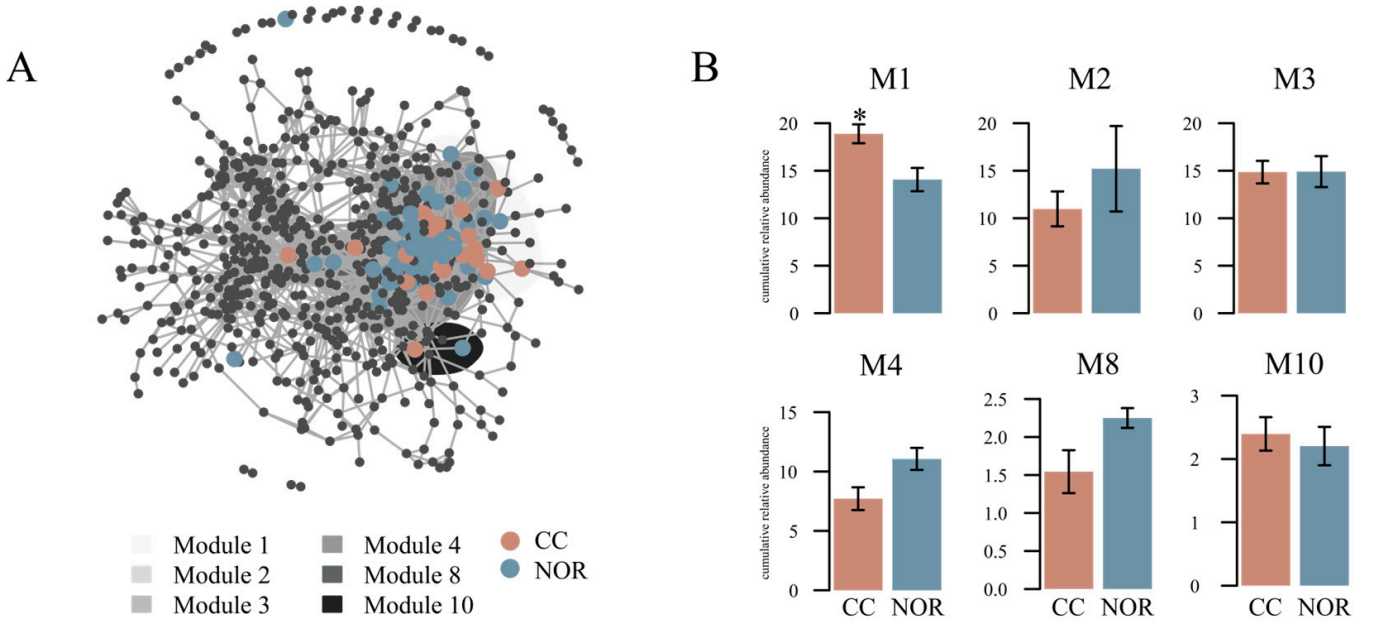

Figure 4. Co–occurrence patterns of continuous cropping obstacle sensitive ASVs and ecological cluster. (**A**): Co–occurrence networks visualizing significant correlations ($r > 0.8$, $p < 0.05$; indicated with gray lines) between bacteria and fungi ASVs in rhizosphere soil communities. (**B**): Cumulative relative abundance (as counts per million, CPM; y-axis in × 1000) of all bacteria and fungi of the continuous cropping obstacle susceptible modules in the networks. Asterisk (*) indicates significant differences (*t*-test method, $p < 0.05$). Error bars are standard errors.

Table 1. Correlation between above-ground biomass of tobacco and co-occurrence networks ecological clusters (module).

		M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
OB	Pearson Correlation	−0.817 *	0.271	−0.350	0.500	0.354	−0.008	0.410	0.465	0.383	−0.324
	p-value	0.001	0.394	0.265	0.098	0.258	0.981	0.185	0.128	0.219	0.304

M1–M10 represents the abundance of the top ten modules in the co-occurrence network. OB represents above-ground biomass of tobacco. Asterisk (*) indicates significant correlation ($p < 0.05$).

3.4. Rhizosphere Soil Metabolomics

A total of 175 metabolites were identified and semi–quantified by GC–MS based rhizosphere soil metabolomics.OPLS–DA of metabolomics data (Figure 5A) showed a clear

separation between CC and NOR. This indicates that continuous cropping obstacles are accompanied by alterations in the soil metabolite profile. Variable influence on projection (VIP) filtered for 59 metabolites with contribution (VIP > 1, Figure 5B). The metabolites are diverse and mainly include organic acids, fatty acids, and phenolic acid compounds such as succinic acid, catechol, 6-hydroxynicotinic acid, thioglycolic acid, 7,4′-Dihydroxyflavone, 2,3-dihydroxybiphenyl, dioctyl phthalate, benzoylformic acid, etc.

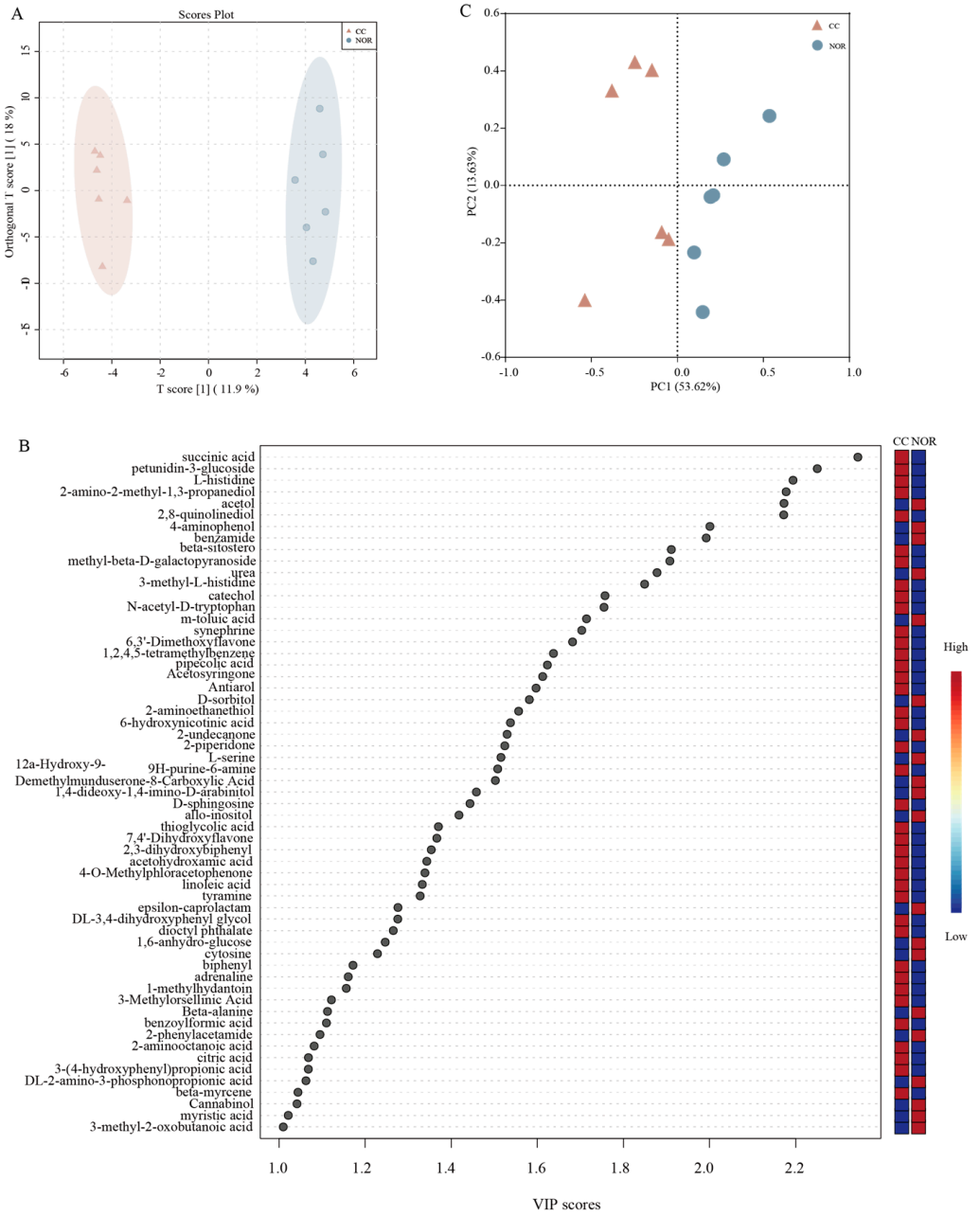

Figure 5. Metabolites in rhizosphere soil. (**A**): OPLS–DA scores plot of metabolites in soil between CC and NOR; (**B**): variable influence on projection (VIP) of compounds; (**C**): dimension reduction of important metabolites. The PCoA based on the Rho distance revealed distinct differences in metabolites when VIP > 1.

The PCoA based on the differential metabolites result revealed distinct differences between CC and NOR (Figure 5C). The first two components (PC1 and PC2) of PCA explained 53.62% and 13.63%. We used PC1 to represent rhizosphere soil metabolomics changes for the multiple linear regression model.

3.5. Multiple Linear Regression Model

We used three indicators (OB, Chemical Properties PC1, M1, metabolomics PC1) to construct the multiple linear regression model (Table S1). The R2 of the final model was 0.82 ($p < 0.05$). Only two variables (metabolomics MPC1 and M1, $p < 0.05$) were ultimately included in the model. The inflation factor (VIF) of each was less than two. The standardized coefficients of the metabolomics PC1 and M1 were 0.53 and −0.46, respectively (Table 2). Changes in metabolomics contribute more to continuous cropping obstacles than microbiota.

Table 2. Multivariate linear regression model variable coefficient.

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Collinearity Statistics	
		B	Std. Error	Beta			Tolerance	VIF
1	(Constant)	−0.127	0.141		−0.900	0.389		
	MPC1	1.219	0.248	0.841	4.909	0.001	1.000	1.000
2	(Constant)	0.378	0.233		1.624	0.139		
	MPC1	0.772	0.270	0.533	2.865	0.019	0.558	1.793
	M1	−0.690	0.277	−0.463	−2.491	0.034	0.558	1.793

MPC1 represents the first components of the PCoA based on the differential metabolites. M1 represents the abundance of module #1 in the co-occurrence network. B represents unstandardized coefficients of an independent variable. Beta represents standardized coefficients of an independent variable. VIF represents variance inflation factor.

4. Discussion

Continuous cropping obstacles are common in agroecosystems, and the mechanisms that cause them are very complex. Therefore, it is essential to investigate the mechanisms that help us to develop agricultural ecosystems sustainably. Continuous cropping led to changes in biological and environmental factors in the soil, and these changes led to an obstacle to continuous cropping [12,22,23].

Soil physicochemical properties changed along with continuous cropping obstacles (Figure 1), which may potentially affect the abundance of microorganisms [24]. The preferential uptake by the plant may have caused a nutrient imbalance, such as a deficiency of AN and an excess of AK and Zn [12]. Thus, continuous cropping could lead to an imbalance of soil's physicochemical properties. However, simply correcting these imbalanced physicochemical factors in soil does not prevent continuous cropping obstacles [25].

Soil microbes are critical in preserving soil health and inhibiting plant disease [26]. Our results showed that CC exhibited a significantly lower $\alpha-$diversity of the bacterial community than NOR (Figure 3A), which is consistent with previous studies [27]. Higher soil bacterial diversity aided in healthy plant growth as well as the creation and maintenance of disease–resistant soil [28,29]. There were also significant differences in the $\beta-$diversity (PCoA analyses and PERMANOVA tests, $p < 0.05$, Figure 3C) of the bacterial communities between the CC and NOR. We found that csASVs clustered in separate modules in the microbial networks, corresponding to the normal or continuous cropping obstacles (Figure 4). In addition, we identified one ecological cluster (module #1) containing high proportions of ASVs that had different abundances in the two types of samples. There was a significant increase in the relative abundance of module #1 in the CC. This may be because the microbes in module #1 were similarly coupled to the continuous cropping obstacles and therefore grouped together in the network. Module #1 was the main ecological cluster to generate the continuous crop obstacles. The csASVs of detoxification and tolerance in module #1 decreased, while the pathogenicity csASVs increased (Table S2). Species such as RB41 sp., Sphingobium sp., unidentified species in the family Gemmatimonadaceae, KD4-96 sp., MND1 sp., Nocardioides sp., Terrabacter sp., and Microvirga sp. decreased in CC. The RB41 belongs to the family Blastocatellaceae, which plays a vital function in sustaining soil metabolism and bio–geochemical functions under prolonged nutrient deficiency stress [30]. Sphingobium sp., Gemmatimonadaceae, KD4-96 sp., MND1 sp.,

Nocardioides sp., Terrabacter sp., and Microvirga sp. are detoxifying bacteria that degrade organic pollutants [31–38]. Unidentified species in the order Chaetothyriales, Didymella sp., Chaetomium sp., Microdochium sp., and unidentified species in family Stachybotryaceae increased in CC. According to many studies, these are pathogenic microorganisms [39–46]. The increased pathogenic microorganisms and decreased beneficial microorganisms promote the formation of continuous crop obstacles [47].

The soil metabolites of the rhizosphere are primarily derived from metabolites derived from roots and rhizosphere microorganisms. We used GC–MS to determine metabolites in rhizosphere soil (Figure 5B). Among the important compounds screened (VIP > 1), the main compounds that increased in CC were organic acids (succinic acid, L-histidine, N-acetyl-D-tryptophan, 6-hydroxynicotinic acid, thioglycolic acid, acetohydroxamic acid, linoleic acid, 3-Methylorsellinic acid, benzoylformic acid, 2-aminooctanoic acid, citric acid, and 3-(4-hydroxyphenyl)propionic acid), phenolics (petunidin-3-glucoside, catechol, Acetosyringone, and DL-3,4-dihydroxyphenyl glycol), flavonoids (6,3′-Dimethoxyflavone, 7,4′-Dihydroxyflavone, and beta-myrcene), alkaloids (2,8-quinolinediol, synephrine, and Antiarol), biogenic amines (tyramine and adrenaline), terpenoids (9H-purine-6-amine), sugars (methyl-beta-D-galactopyranoside) and environmental toxins (1,2,4,5-tetramethylbenzene, 2,3-dihydroxybiphenyl, biphenyl and dioctyl phthalate). Many of them were allelochemicals as well as toxic substances, and some were potentially active substances for plants. For example, succinic acid is a typical allelochemical that inhibits photosynthesis and tobacco seed germination and growth [48,49]. Catechol is also an allelochemical which decreases the germination rate of seeds, the growth of radicles and hypocotyls, and the fresh and dry weight of seedlings [50]. The benzene series 1,2,4,5-tetramethylbenzene, 2,3-dihydroxybiphenyl and biphenyl are environmental toxicants [51,52]. Another toxic substance, dioctyl phthalate, can increase the content of ROS in cells, disrupt the subcellular structure, and inhibit plant growth [53–55]. Some other compounds that are potentially harmful to plants are: thioglycolic acid, which forms thioethers with the benzyl alcohol groups of lignin [56]; 7,4′-Dihydroxyflavone, which inhibits germination [57,58]; and benzoylformic acid, a derivative of benzoic acid, which can inhibit the growth of tobacco [59]. In addition, a synergistic interaction between different harmful components can increase their own toxicity [60,61]. We also observed a decrease in some beneficial compounds in CC. For example, D-sorbitol can help plants resist stress [62]. Compound 2-undecanone can promote seed germination [63]. An increase in harmful compounds and a decrease in beneficial compounds may combine to cause continuous cropping obstacles.

We used a mixed linear model to estimate the relationship between soil physicochemical properties, microorganisms and metabolites, and tobacco above–ground biomass (Table 2 and Table S1). Only microorganisms and metabolites were significantly associated with plant above–ground biomass. Previous studies have found that even the addition of missing nutrients to soils does not improve its resistance to continuous cropping obstacles [25]. Moreover, trends in soil's physicochemical properties were not consistent across different studies related to continuous crop obstacles [12,64]. These show that changes in the soil's physicochemical properties do not directly cause the emergence of continuous crop obstacles. There is an ecological cluster (module #1) in the rhizosphere microorganism community that is significantly associated with the plant's above–ground biomass. This ecological cluster exhibits an increase in pathogenic microorganisms, and a decrease in the detoxification and tolerance microbiome may lead to a decrease in detoxification capacity. These variations promote the occurrence of continuous crop obstacles [47]. Changes in soil metabolites, combined with a loss of detoxifying microorganisms and an increase in harmful microorganisms, may have contributed to the continuous crop obstacles, according to our model. Furthermore, according to the model's standardized coefficients, metabolites contributed more to the continuous crop obstacles than the microorganism community.

5. Conclusions

In conclusion, the soil's physicochemical properties do not directly cause the emergence of continuous crop obstacles. The metabolites and microbiota are the main direct obstacles to continuous cropping in tobacco. We identified an ecological cluster in the rhizosphere that is associated with continuous crop obstacles. Although we assumed that its decrease in detoxification capacity was evident, direct evidence was still lacking. Meanwhile, GC–MS found only a fraction of the substances in the rhizosphere soil. In the future, we will try to explore the mechanism of continuous crop obstacles more deeply through metagenome sequencing technology and high–throughput targeted metabolomics. In tobacco planting, consideration should be given to preserving a healthy tobacco rhizosphere microecology and reducing harmful compounds (for example, through crop rotation or organic fertilizer, while removing plant residues after plant harvest) to alleviate continuous cropping obstacles.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13040964/s1, Table S1: multiple linear regression model summary; Table S2: indicator species information table in module #1.

Author Contributions: Conceptualization, F.Y. and J.S.; methodology, F.Y.; software, Q.D.; validation, Y.Y.; formal analysis, Y.Y.; investigation, C.J.; resources, J.S.; data curation, Q.D.; writing—original draft preparation, F.Y.; writing—review and editing, J.S. and Q.D.; visualization, C.J.; supervision, Y.Y.; project administration, C.Z.; funding acquisition, F.Y. All authors have read and agreed to the published version of the manuscript.

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