

Integrated Biological Control of Tobacco Bacterial Wilt (*Ralstonia solanacearum*) and Its Effect on Rhizosphere Microbial Community

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Abstract

Biological control agents and soil amendments have been applied to control tobacco bacterial wilt, but the mechanism is not well-known. In the present study, a field experiment was performed to investigate the soil physicochemical properties, disease index (DI) and disease incidence of tobacco bacterial wilt, and rhizosphere microbial community. The results show that the control efficacy of single application of antagonistic bacteria and calcium cyanamide was 46.43% and 51.92%, respectively. While the combined control efficacy of antagonistic bacteria and calcium cyanamide was 65.79%. Besides, the combined application of antagonistic bacteria and calcium cyanamide could increase soil pH, total N alkaline N, and exchangeable Ca, which were negatively associated with the microbial diversity, soil-borne pathogenic microorganisms, and incidence of tobacco bacterial wilt. Additionally, the combination of antagonistic bacteria and calcium cyanamide can improve the proportion of some antagonistic microbial species, and these antagonistic microbial species were inversely associated with the DI of tobacco bacterial wilt. In conclusion: The integrated measure may influence soil microbial communities through enhancing soil physicochemical properties and rebuild healthy soil microbial community structure to mitigate tobacco bacterial wilt. The current study presented valuable insights into the mechanisms enhancing soil health in the integrated measure.

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Keywords

Biological Control, Tobacco Bacterial Wilt, Alkaline Fertilizer, Physicochemical Property, Rhizospheric Microorganism

1. Introduction

Tobacco bacterial wilt is a soil-borne bacterial infection triggered by *Ralstonia solanacearum*, which seriously damages the growth of tobacco [1] [2]. Nowadays, tobacco bacterial wilt is distributed in almost all the flue-cured tobacco growing areas in the world, especially in tropical and subtropical tobacco areas. It has been a major devastating disease threatening world tobacco production, which in turn causes great economic loss [3].

Tobacco bacterial wilt has received extensive attention, and many research studies have been reported about how to restrain the tobacco bacterial wilt in breeding resistant varieties, agricultural control, and chemical control. However, these conventional approaches have a partial effect and are associated with many problems, such as lack of resistant varieties, easy loss of resistance, and the poor control effect of chemical agents, environmental pollution, and so on [4].

The occurrence of tobacco bacterial wilt is not only related to high temperature and high humidity climate but also related to soil types, soil nutrients and continuous cropping [5] [6]. It was reported that continuous harvesting obstacles lead to the imbalance of the number and proportion of microbial populations in the soil and the relative increase of *R. solanacearum* [7]. Besides, the incidence of tobacco bacterial wilt shows a significant positive correlation with pH values of the soil [8] [9]. Therefore, tobacco bacterial wilt is difficult to be effectively controlled, thus, new methods should be developed for effective control. In recent years, biological control technology has become a research hotspot because of its environmental and ecological safety and its ability to achieve sustainable development of tobacco [10]. Studies show that culturing of antagonistic into bioorganic fertilizers has helped in control of tobacco bacterial wilt. It can not only increase the population of beneficial microorganisms in soil and enhance the disease resistance of tobacco plants but also improve the rhizosphere micro-ecological environment, thus promote root growth and nutrient absorption [11] [12]. Besides, soil amendments can change the physicochemical properties of soil, improve and repair soil ecological environment, affect soil microbes, and reduce the number of pathogens [13] [14]. Due to the complexity of the environment and the limitation of single control methods, the prevention, and control effect of the disease-affected field is not an ideal strategy. Therefore, an integrated approach based on the combination of biological control and soil amendments was proposed as a new method to control tobacco bacterial wilt.

In this study, the antagonistic bacteria and calcium cyanamide were used to repair the tobacco field from bacterial wilt. An effective control mechanism of tobacco bacterial wilt was explored by studying the physicochemical properties

of root-soil and microbial community structure in rhizosphere soil. To provide further theoretical basis for integrated biological control of the tobacco bacterial wilt.

2. Results

2.1. Incidence and Disease Index of Tobacco Bacterial Wilt

The disease incidence and DI of bacterial wilt in the BA, CC, and BC groups were found remarkably lower than those in the CK group. Moreover, the incidence and DI in the BC group were also found remarkably lower than those in the BA and CC groups (Figure 1). The control efficacy of the BC group (65.79%) was observed higher than the BA group (46.43%) and CC group (51.92%). These outcomes showed that merging the antagonistic bacteria and calcium cyanamide could control tobacco bacterial wilt more effectively than a single application of antagonistic bacteria and calcium cyanamide.

2.2. Rhizospheric Soil Physicochemical Properties in Different Treatments

The physicochemical properties of rhizospheric soil were analyzed (Table S1). The pH, alkaline N, available K, exchangeable Ca, and exchangeable Mg of rhizospheric soil in the BA, CC, and BC groups were found remarkably higher ($p < 0.05$) than that in the control group. The total N of rhizospheric soil in the CC and BC groups was also observed higher ($p < 0.05$) than that in the control group. However, the organic matter, available P of rhizospheric soil in the CC, and BC groups were observed lower ($p < 0.05$) than those in the control group. These results show that the soil physicochemical properties were significantly affected by a single application of antagonistic bacteria, calcium cyanamide, and a combination of the application of antagonistic bacteria and calcium cyanamide. Moreover, applying calcium cyanamide could effectively increase the pH, total N, alkaline N, available K, exchangeable Ca, and exchangeable Mg of rhizospheric soil.

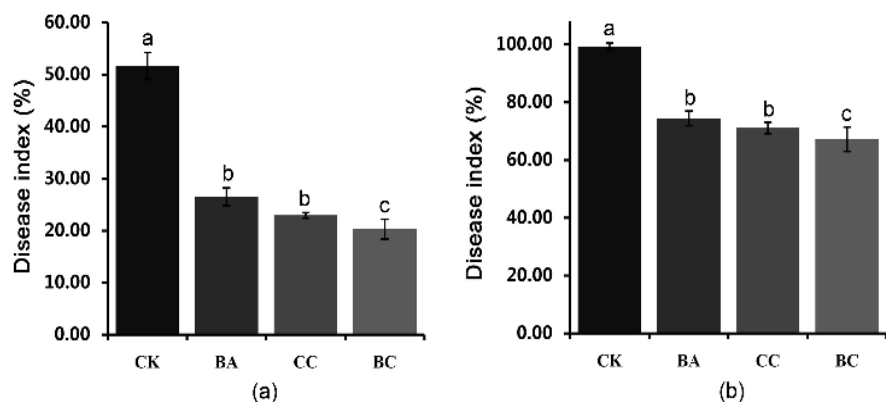


Figure 1. Disease index (a) and incidence (b) of tobacco bacterial wilt. Data are presented as the means and standard errors amid the samples. Values denoted by the bars with different letters are significantly different at $p < 0.05$.

2.3. Tobacco Rhizosphere Microbial Community Richness and Diversity

In total, 6,168,190 fine raw sequences with an average length of 390 bps for bacteria and 350 bps for fungi were obtained after quality filtering (Table S2). The difference between OTUs, Sobs, Chao1, and Shannon index of microbial richness and diversity were analyzed (Table 1). As for the bacteria, the Chao1 and Sobs index from the rhizosphere soil of the BC group was observed higher ($p < 0.05$) than those from other groups (BA, CC, and CK). OUT numbers from the rhizosphere soil of the BC and CC group showed no significant difference, but all significantly higher ($p < 0.05$) than CK and BA groups. The same trend was observed for the Shannon index. OUT numbers and Sobs, chao1, Shannon index from the bacterial rhizosphere soil of the BC group was higher than other groups. As for the fungal community, the four indexes from the rhizosphere soil of the BC group were the highest. OUT numbers and Sobs, chao1, Shannon index from the fungal rhizosphere soil of the BC group was higher than other groups. These results suggested that the effects of antagonistic bacteria combined with calcium cyanamide were higher than the single application of antagonistic bacteria or calcium cyanamide.

2.4. The Composition and Structure of Tobacco Rhizosphere Microbial Community

The leading bacterial phyla found in every treatment were *Proteobacteria* (37.80% - 72.28%), *Actinobacteria* (6.15% - 16.18%), *firmicutes* (2.07% - 11.714%), *Acidobacteria* (7.75% - 15.70%), *Gemmatimonadetes* (2.90% - 7.91%), *Chloroflexi* (3.23% - 5.19%), *Bacteroidetes* (1.01% - 3.96%), and *Nitrospirae* (0.71% - 2.50%) (Figure 2(a)). The relative population of *Gemmatimonadetes*, *Acidobacteria*, *firmicutes*, *Actinobacteria*, and *Proteobacteria* made up almost 81.51% - 91.16% amid the 10 predominant bacterial phyla. The relative population of *Proteobacteria* in the BA, CC, and BC groups was higher than that in the CK group, while the relative population of *Actinobacteria*, *firmicutes*, and *Gemmatimonadetes* in the BA, CC, and BC groups were lower than that in the CK group, demonstrating that single application of antagonistic bacteria, calcium cyanamide and the combination of antagonistic bacteria and calcium

Table 1. Alpha diversity index of bacteria and fungal in different treatments.

Treatment	Bacterial				Fungal			
	OTUs	observed_species (Sobs)	chao1	shannon	OTUs	observed_species (Sobs)	chao1	shannon
CK	2396.0 ± 84.7c	2208.3 ± 90.4d	2184.6 ± 51.1d	7.6 ± 0.2c	659.0 ± 11.3d	334.7 ± 13.8d	343.2 ± 11.7d	3.5 ± 0.4b
BA	4122.3 ± 480.9b	2988.0 ± 86.7c	3056.5 ± 78.2c	9.0 ± 0.2b	1214.3 ± 54.2c	432.0 ± 6.0c	449.1 ± 4.7c	2.9 ± 0.2c
CC	4719.3 ± 97.6a	3796.7 ± 52.4b	3859.0 ± 51.6b	9.7 ± 0.1a	1341.3 ± 29.6b	926.0 ± 34.0b	956.5 ± 36.8b	6.1 ± 0.5a
BC	4944.3 ± 88.4a	4188.7 ± 195.6a	4273.2 ± 236.5a	9.7 ± 0.2a	1465.3 ± 27.6a	1091.3 ± 16.2a	1125.3 ± 11.5a	6.3 ± 0.3a

Values are means of SD (n = 3). The different letters in the same column indicate significant differences as determined by LSD test ($p < 0.05$).

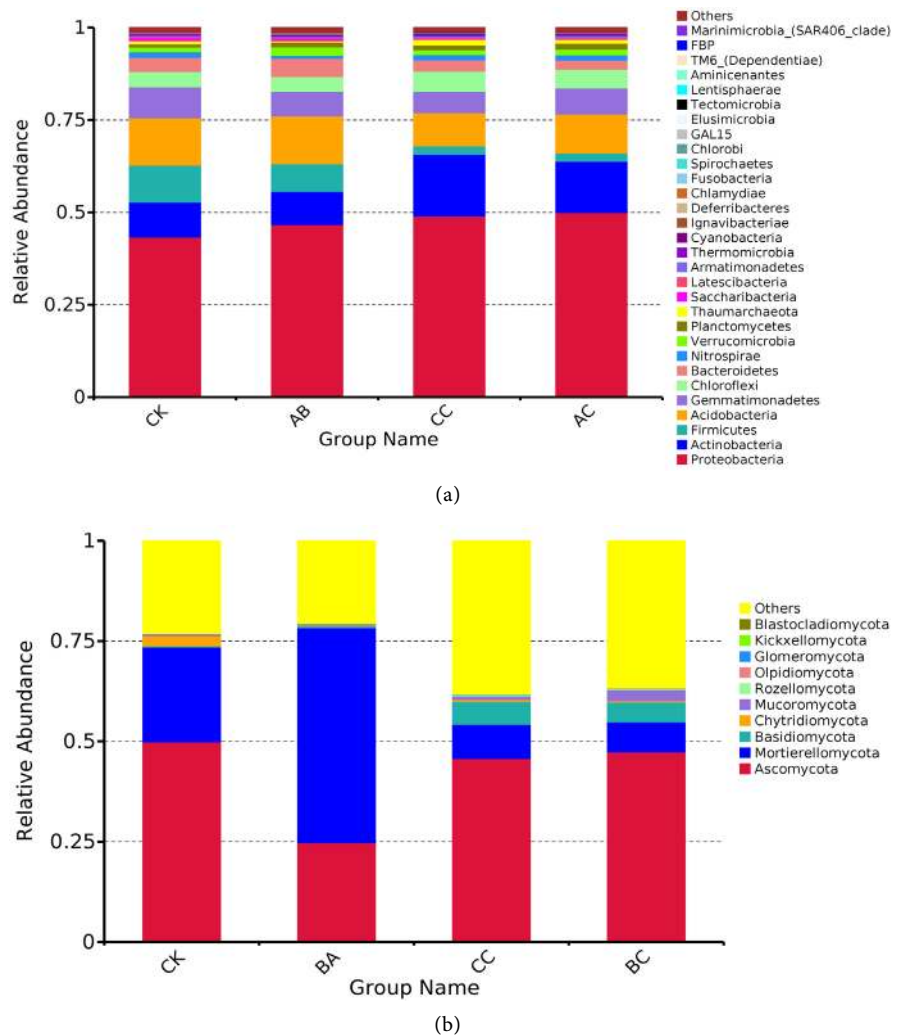


Figure 2. The relative abundance of bacterial phyla (a) and fungal phyla (b) in different treatments.

cyanamide affected the bacterial community structure.

The top 6 fungal phylum accounted for 68.79% - 87.06% of the fungal community, and *Ascomycota* (36.40% - 60.98%), *Mortierellomycota* (6.21% - 48.60%), *Basidiomycota* (0.25% - 9.07%), *Mucoromycota* (4.23% - 3.62%), *Chytridiomycota* (0.25% - 0.63%) and *Rozellomycota* (0.04% - 0.35%) were the 6 dominant fungal population (**Figure 2(b)**). The relative population of *Basidiomycota* in the CC and BC groups was found higher than those in the CK and BA groups. The relative population of *Ascomycota* in the CC and BC groups was observed higher than those in the BA group but lower than those in the CK group. Moreover, the relative population of *Mortierellomycota* in the CK group was significantly higher than that in CC and BC groups, but lower than those in the BA group. The findings demonstrate that the application of antagonistic bacteria combined with the calcium cyanamide influenced the composition of the fungal community.

The different distributions of the microbial community at the genus level be-

tween the other treatment groups and CK group are illustrated in **Figure S1**. *Terracidiphilus* was one genus that found a significant difference between the BA and the CK groups (**Figure S1(a)**). There were 44 significantly different genera between the CC and the CK groups, and 42 significantly different genera between the BC and the CK group (**Figure S1(b)** and **Figure S1(c)**). The abundance of antagonistic bacteria *Bacillus*, *Paenibacillus*, *Lysobacter*, and *Streptomyces* in the BC and CC groups were higher than that in the CK group, while the abundance of nitrogen cycling bacterial *Gaiella*, *Microvirga*, and *Nitrosospora* in the BC and CC groups were higher than that in the CK group. Moreover, the population of increasing plant health and growth bacteria *Novosphingobium* in BC and CC groups was higher than that in the CK group. The population of carbon cycling bacteria *Steroidobacter* and *Solirubrobacter* in BC and CC groups were both higher than that in the CK group, but the population of *Chthonomonas* in CC and BC groups was significantly lower than that in the CK group (**Figure S1(b)** and **Figure S1(c)**). At the genus level of fungal community, there was no significant difference of fungal proportions between the BA and CK groups, while *Aspergillus*, and *Trichoderma* as antagonistic fungal, were more abundant in the BC and CC groups than in the CK group (**Figure S1(d)** and **Figure S1(e)**).

For further exploring the distribution of the pathogenic and antagonistic microorganisms in different treatments, the abundances of these three types of microorganisms were calculated based on the absolute taxa abundance (**Figure 3**). Four pathogenic microorganisms were found in all the treatments, three of them were fungal pathogens, including *Alternaria*, *Fusarium solani*, and *Fusarium oxysporum*, and one was bacterial pathogens (*R. solanacearum*). The population of *F. oxysporum*, *F. solani*, and *R. solanacearum* were higher ($p < 0.05$) in the CK group than that in other treatments. Moreover, the population of pathogenic microorganisms in the BC group was the lowest of all treatments (**Figure 3(a)**). Twelve genera of antagonistic bacteria, including *Streptomyces*, *Paenibacillus*, *Sphingomonas*, *Stenotrophomonas*, *Flavobacterium*, *Bacillus*, *Actinospica*, *Catenulispora*, *Haliangium* and *Lysobacter*, and two genera of antagonistic fungal, including *Aspergillus* and *Trichoderma* were found in all treatments (**Figure 3(b)**). The population of most antagonistic microorganisms in the CK group was significantly lower ($p < 0.05$) than other treatments, excepting *Flavobacterium*, and *Catenulispora*. Therefore, the population of most antagonistic microorganisms was improved by loading antagonistic bacteria, calcium cyanamide, and combination of antagonistic bacteria and calcium cyanamide. Especially, *Sphingomonas* was significantly improved by loading antagonistic bacteria, and *Streptomyces*, *Flavobacterium*, *Aspergillus*, and *Trichoderma* were significantly improved by loading calcium cyanamide.

2.5. The Correlation among the Microbial Community, Disease Index, and Soil Physicochemical Properties

The Spearman correlation among the soil physicochemical, DI, and microbial

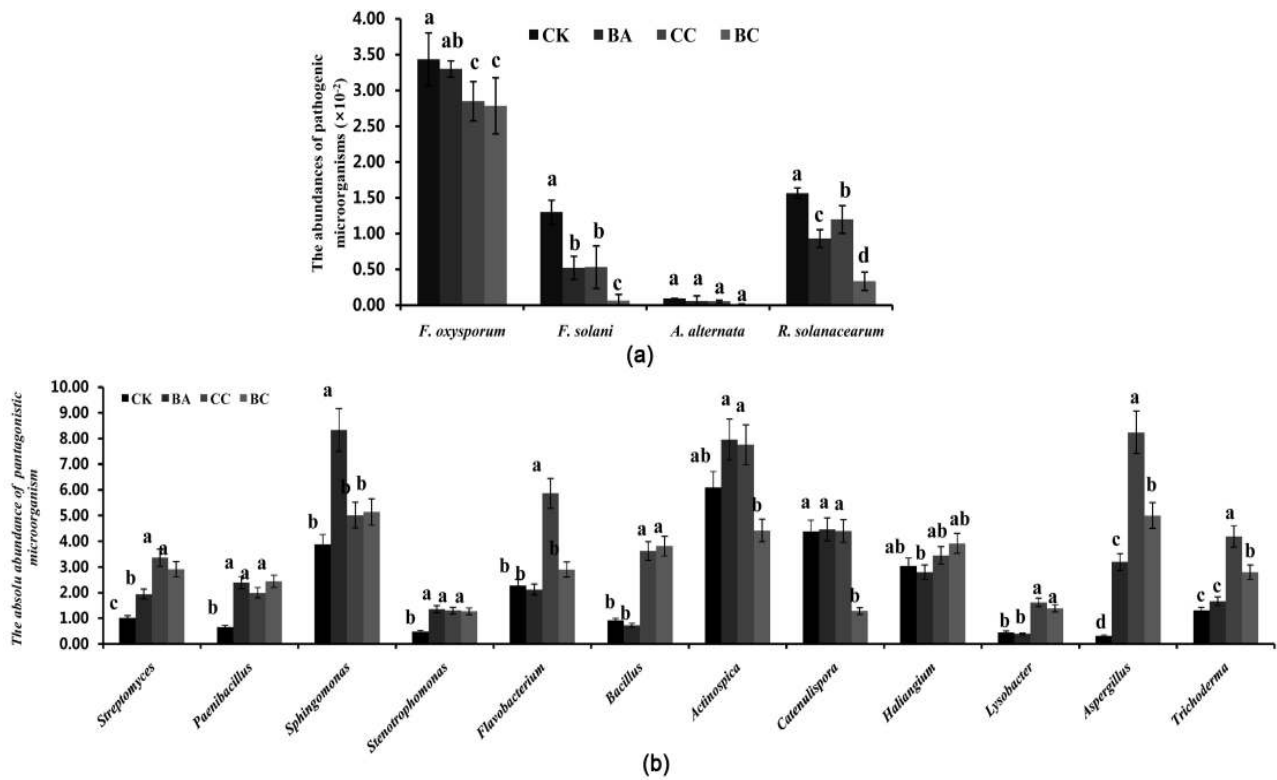


Figure 3. The absolute abundances of pathogenic microorganisms (a) and antagonistic microorganism (b) in different treatments. Values are means of SD (n = 3). Bars with different letters are significantly different at $p < 0.05$ by LSD test.

community were measured to evaluate association amid them (Table S3). The microbial richness and diversity were found in positive correlation with pH, total N, alkaline N, available K, exchangeable Ca, and exchangeable Mg. Additionally, the abundances of antagonistic microorganisms *Streptomyces*, *Paenibacillus*, *Bacillus*, *Haliangium*, *Lysobacter*, and *Aspergillus* were significantly correlated with soil physicochemical properties. Whereas pathogenic microorganisms *F. solani*, *A. alternata*, and *R. solanacearum* showed negatively correlated with soil physicochemical properties. According to the Spearman correlation, the microbial diversity, and the abundances of antagonistic microorganisms *Streptomyces*, *Paenibacillus*, *Flavobacterium*, *Bacillus*, *Haliangium*, *Lysobacter*, and *Aspergillus* showed a significantly negative relationship with the DI of tobacco bacterial wilt. However, pathogenic microorganisms *F. solani*, *A. alternata*, and *R. solanacearum* had a significantly positive relationship with the DI of tobacco bacterial wilt (Table S4). Besides, pH, total N, alkaline N, available K, exchangeable Ca, and exchangeable Mg showed a significantly negative relationship with the DI of tobacco bacterial wilt, while organic matter and available P showed a significantly positive relationship with the DI of tobacco bacterial wilt (Table S5). These results indicated soil physicochemical properties had stronger effects on the microbial community. Furthermore, the microbial community structure affects the DI of bacterial wilt, especially the antagonistic microorganisms and pathogenic microorganisms.

3. Discussion

The knowledge about the mechanism and action of integrated biological control of bacterial wilt will help out the restraining of soil-borne disease in agriculture. Current study aimed to investigate the microbial community, the occurrence and DI of tobacco bacterial wilt, and physicochemical properties of rhizosphere soil. The single application of antagonistic bacteria, calcium cyanamide, and the combination of antagonistic bacteria and calcium cyanamide were confirmed to have an effect on the microbial community and successfully control the soil-borne disease. Additionally, the combination of antagonistic bacteria and calcium cyanamide showed to be more effective than the single application of antagonistic bacteria or calcium cyanamide, suggesting the integrated biological control method may be an applied approach for restraining tobacco bacterial wilt.

Biocontrol agents can increase the number of beneficial microorganisms and enhance the disease resistance of tobacco plants. They also work as bioorganic fertilizers because they help in the root growth and nutrient absorption. Control of bacterial wilt by integrated biological agents including antagonistic bacteria has been found effective in several reports [11] [15] [16]. Besides, soil amendment calcium cyanamide, a nitrogen fertilizer, can increase soil pH, total N, alkaline N, and exchangeable Ca. Furthermore, high soil pH is imperative for restraining bacterial wilt, higher N could enhance the competence of useful microorganisms against pathogens, and exchangeable Ca as fertilizer can increase Ca concentrations to lessen the severity of bacterial wilt [17] [18] [19]. Although antagonistic bacteria and calcium cyanamide were extensively used in the restraining of bacterial wilt, the mechanism of combined application of antagonistic bacteria and calcium cyanamide to control tobacco bacterial wilt is not known. In this study, the control efficacy of single application of antagonistic bacteria and calcium cyanamide was 46.43% and 51.92%, respectively. However, the control efficacy of the combined application of antagonistic bacteria and calcium cyanamide reached 65.79%. Besides, the pH, total N alkaline N, and exchangeable Ca were significantly higher for the combined application of antagonistic bacteria and calcium cyanamide than the single application of antagonistic bacteria. Moreover, Spearman correlation analysis showed pH, total N alkaline N, and exchangeable Ca were negatively correlated with microbial diversity, the soil-born pathogenic microorganisms, and DI of bacterial wilt, which were identical with previous investigations [20] [21]. Therefore, we suggest that the integrated measure reduces the occurrence of bacterial wilt by regulating soil physicochemical properties.

As is known, microbial communities are significant biological indicators for healthy soil. Additionally, soil microbial diversity, composition, and function have been considered to be important factors for maintaining long-term ecosystem balance and controlling plant soil-borne disease outbreaks [22] [23] [24]. Our findings of the current study show that Sobs, Chao1, and Shannon index of

fungus and bacterial communities in the combined application of antagonistic bacteria and calcium cyanamide treatment group were remarkably higher than that in the single application of antagonistic bacteria and calcium cyanamide treatment groups. Additionally, the microbial diversity had a negative relationship with DI of bacterial wilt. This is similar to a previous report [24]. The results indicated that the integrated measure has a significantly positive effect on the diversity and richness of the microbial community of rhizospheric soil, thus reducing tobacco bacterial wilt. Moreover, it was also found that the integrated measure influences the structure of the microbial community. In the present investigation, compared with the control group, certain genera of microorganisms *Gaiella*, *Microvirga*, *Nitrosospora*, *Steroidobacter*, *Solirubrobacter*, and *Novosphingobium* were found in a larger population in rhizospheric soil subject to the combined application of antagonistic bacteria and calcium cyanamide. Previously reports showed that these genera have mostly beneficial effects on recycling nutrients in the soil, the genera *Gaiella*, *Microvirga*, and *Nitrosospora* played an important role in nitrogen cycling [9] [25]. Some genera of *Chthonomonas*, *Steroidobacter*, and *Solirubrobacter* were found to participate in organic carbon cycling in the soil [26]. Besides, *Novosphingobium* was reported to assist plant growth by increasing plant health and growth [27]. In the current study, these beneficial genera had a significantly negative relationship with DI of tobacco bacterial wilt. These genera can directly and effectively control soil-borne diseases by competing for nutrients, space, and induced systemic resistance [28]. Therefore, the integrated measure can affect soil microbial community and rebuild healthy soil microbial community structure to mitigate tobacco bacterial wilt.

The antagonistic bacterial species such as *Streptomyces*, *Paenibacillus*, *Sphingomonas*, *Stenotrophomonas*, *Bacillus*, *Actinospica*, *Haliangium*, and *Lysobacter* are well recognized as useful rhizosphere microorganisms and can mitigate many soil-borne diseases and promote plant growth and health [29] [30]. These were present abundantly in the combined application of antagonistic bacteria and calcium cyanamide treatment group. The antagonistic fungus *Aspergillus* and *Trichoderma* have been reported to interact directly with roots to produce bioactive substances that help in the growth of plants and fight against abiotic and biotic stress [31] [32]. The abundance of the above antagonistic fungal strains was higher in the single application of calcium cyanamide treatment group and the combination of antagonistic bacteria and calcium cyanamide treatment group. Furthermore, these antagonistic microbial showed a significantly negative relationship with the DI of tobacco bacterial wilt. Therefore, the proportion of antagonistic microbial was improved by the integrated measure treatment group, which is conducive to recruiting antagonistic adherents of the soil microbiome to shield tobacco plants from disease. Studies have reported that diverse microbial communities tend to be less vulnerable to pathogens attack than a simpler microbial community [33]. Our study showed that the abundances of pathogens *R. solanacearum*, *F. oxysporum*, and *F. solani* were signifi-

cantly lower in the combined application of antagonistic bacteria and calcium cyanamide treatment group than the control group. Besides, these pathogens had significantly correlated with soil physicochemical properties and positive correlation with the DI of bacterial wilt. Consistent with the above findings, the combination of antagonistic bacteria and calcium cyanamide treatment group can control tobacco bacterial wilt through increasing the microbial community diversity and resist the invasion of pathogens.

4. Materials and Methods

4.1. Field Experiment

The field trial was performed from March to September 2018 at a tobacco field in Xuanen County (109°26'20"E, 29°59'55"N), Hubei province, China. The experiment field was continuous cropping for 15 years, and the bacterial wilt was serious. Tobacco seedlings (Yunyan87) were grown in the greenhouse for around 60 days earlier being moved to replant in the trial field. The trial field was divided into four blocks according to the experimental design, each 90 m² in size, representing four treatments: 1) the control group (CK); 2) the BA group applied with *Bacillus amyloliquefaciens* ZM9 [34]. A dilute solution of ZM9 cultures was prepared in water-bearing 1.0×10^7 CFU/mL cell concentration and then watered into the tobacco root (100 mL per plant) after tobacco transplanting; 3) the CC group was applied with 22.5 g/m² of calcium cyanamide to the soil at 20 d pre-transplantation (CC); and 4) the BC group was treated with calcium cyanamide and *Bacillus amyloliquefaciens* ZM9 as CC and BA. There were 60 tobacco plants in each section, every plant was apart of 0.55 m in a row, and each row was at a distance of 1.2 m.

4.2. Rhizosphere Soil Samples and Basic Parameter Calculation

Rhizosphere soil samples were collected at 68 d post-transplantation by the five-spot-sampling method and transported to the lab, then stored at -80°C for microbial community analysis, and at 25°C for physicochemical properties analysis. To study the soil characteristics and available nutrients, collected soil specimens were air-dried and mechanically crushed into less than 2 mm mesh sized powder. Exchangeable magnesium (Mg) and calcium (Ca), available potassium (K), available phosphorus (P), alkaline nitrogen (N), total nitrogen (N), organic matter, and pH were determined by atomic absorption spectrophotometry, flame spectrophotometer, sodium bicarbonate method, alkali solution diffusion method, Kjeldahl method, potassium dichromate volumetric method, and potentiometric method, respectively [35].

4.3. Incidence and Disease Index Calculation

The symptoms of tobacco bacterial wilt of all 60 plants in each section were monitored at 15 d post-transplantation. The severity scale from 0 to 9 was used as reported in an earlier study [14]. Disease incidence (I) and disease index (DI)

were measured using the given formula.

$$I = DI = [\sum(\text{the number of bacterial wilt crops in this index} \times \text{severity scale}) / (\text{total number of crops inspected} \times \text{the highest severity scale})] \times 100.$$

Similarly, the control efficacy of BA, CC, and BC was also calculated based on DI.

$$\text{Control efficacy} = [(\text{Control DI} - \text{Treatment DI}) / \text{Control DI}] \times 100\%.$$

4.4. Extraction and Purification of Soil Microbial DNA

For microbial DNA extraction, 0.5 g soil was weighed precisely from each sample stocked for genome analysis. The FastDNA Spin Kit (MP Biomedicals, USA) was used to extract the soil microbial genomic DNA following the prescribed procedures and specifications. The 1% agarose gel electrophoresis was employed to measure the integrity of DNA samples. Subsequently, the Nanodrop was used to evaluate the purity and concentration of DNA.

4.5. PCR Amplification and Sequencing

The microbial DNA isolated from the rhizosphere soil samples was subjected to use as a template for replication of ITS rRNA and 16S rRNA genes. The DNA samples were diluted to 1 ng·μL⁻¹ with millipore water. The primers 806R (5'-GGACTACHVGGGTWCTAAT-3') and 515F (5'-GTGCCAGCMGCCGCGGTAA-3') [16] were used to copy the V4 regions of the 16S rRNA gene, while the primers ITS2-2043R (5'-GCTGCGTTCTTCATCGATGC-3') and ITS5-1737F (5'-GGAAGTAAAAGTCGTAACAAGG-3') [36] were used to copy the ITS1 regions of ITS rRNA gene. The Illumina HiSeq platform was used to conduct all PCR reactions and sequencing libraries were constructed with the use of the TruSeq® DNA (Illumina, USA) following the prescribed procedures and approvals. After that, the quality of the library was assessed using Bioanalyzer 2100 system and the Qubit@2.0 Fluorometer. Finally, the Illumina HiSeq 2500 platform was used to sequence the DNA library preparation and 250 bp paired-end reads were gained.

4.6. Sequence Data Analysis

The FLASH (V1.2.7) software was employed to merge the paired-end reads and assigned to the corresponding samples using the unique barcodes. The QIIME (V1.7.0) software was used at specific filtering parameters to filter the raw tags and gain the fine clean tags. The Uparse (V7.0.1001) software was used for the sequence analysis and sequences with ≥97% similarity were allocated to the same OTUs. Each distinctive sequence was screened for further taxonomic information annotation. Four indices, OTUs number, observed species (Sobs), Chao1, and Shannon index were measured to evaluate diversity and fertility of soil microbial community. The Student's t-test (T-test) was applied to analyze the differences of microbial proportions at the genus level among treatments. The

QIIME software and R software (V2.15.3) were used to measure all indices and displayed, respectively.

4.7. Statistical Analysis

The SPSS version 18.0 (IBM, USA) and Microsoft Excel 2007 were employed to analyze the data. The significant difference amid treatments was measured with a one-way analysis of variance (ANOVA) and the least significant difference (LSD) test ($p < 0.05$). Correlation analysis was conducted by spearman (2-tailed).

5. Conclusion

The findings of the current study suggest that the integrated measure can regulate soil physicochemical properties, and effectively reduce the incidence of tobacco bacterial wilt, thus obviously increase the diversity and richness of soil microorganisms. It can further effectively resist the invasion of pathogens, and promote the transformation of diseased soil ecosystem into healthy and sustainable development through prevention and control of tobacco bacterial wilt.

Author Contributions

Yong Yang and Yanyan Li initiated and designed the research. Xiaoqiong Yang, Chunli Li, Lin Wang and Ji Feng performed the experiments and collected the data. Yun Hu analyzed the data and wrote the manuscript. Yong Yang and Shouwen Chen revised the manuscript. All authors reviewed and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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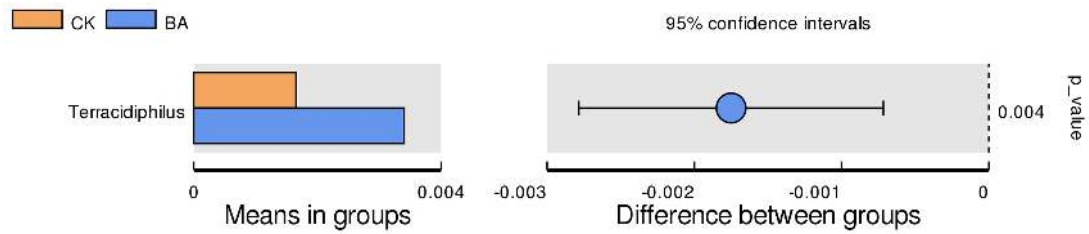
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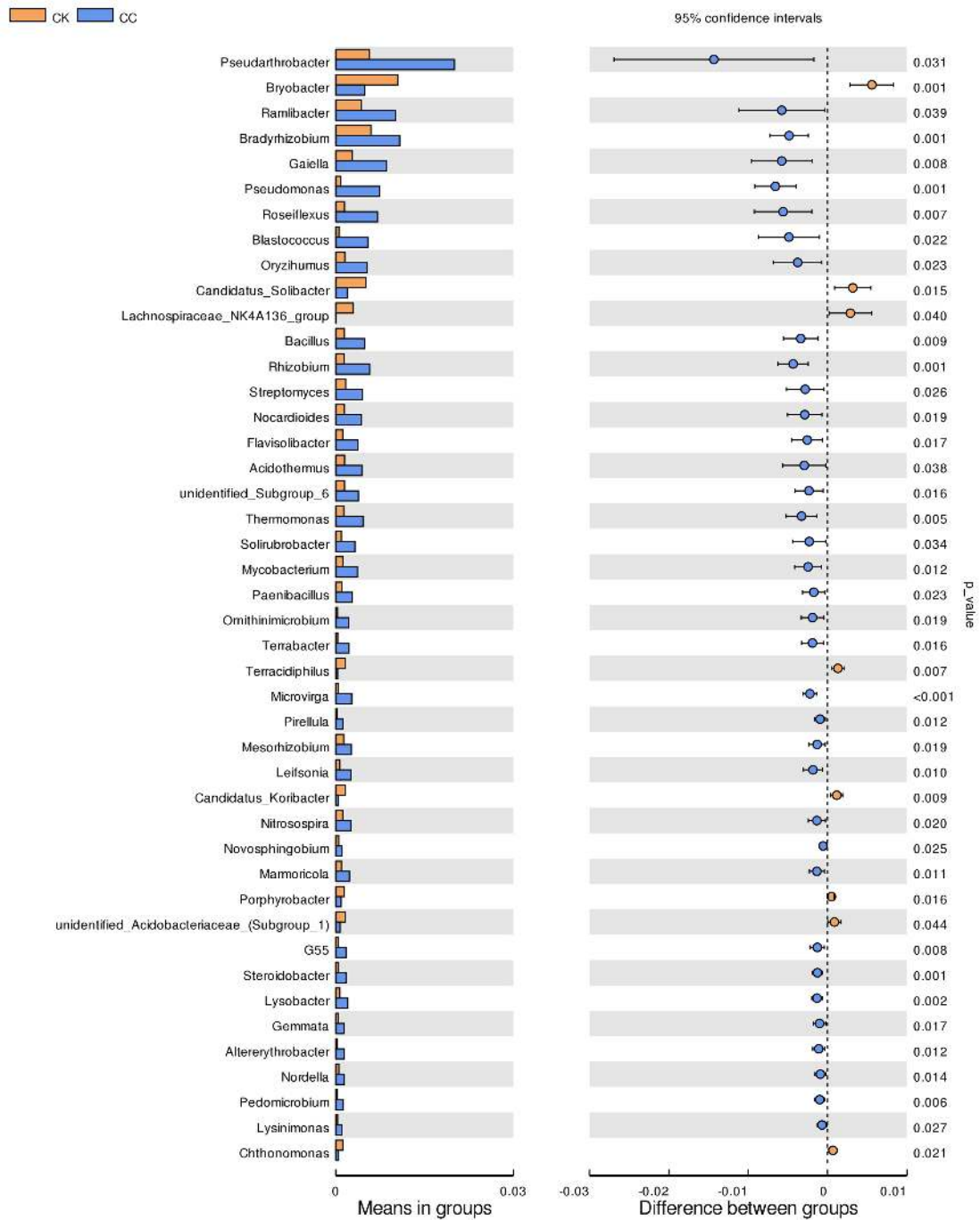
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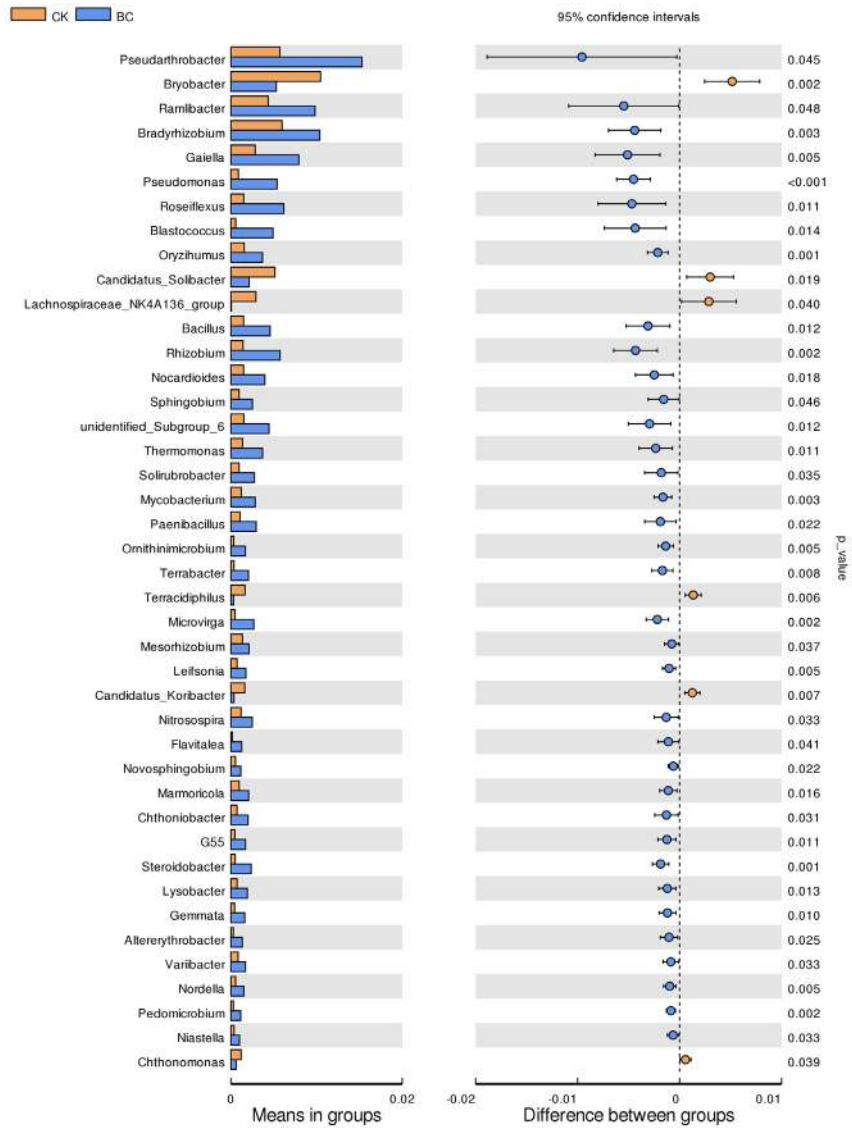
Supplementary Materials



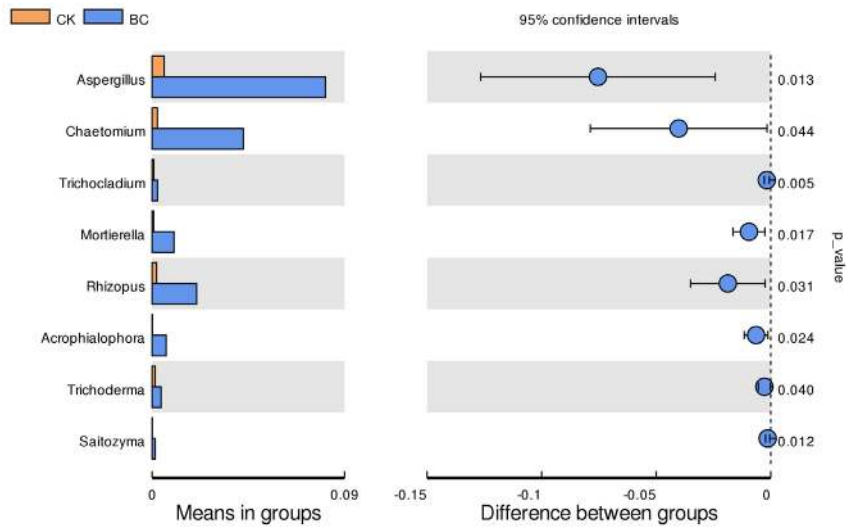
(a)



(b)



(c)



(d)

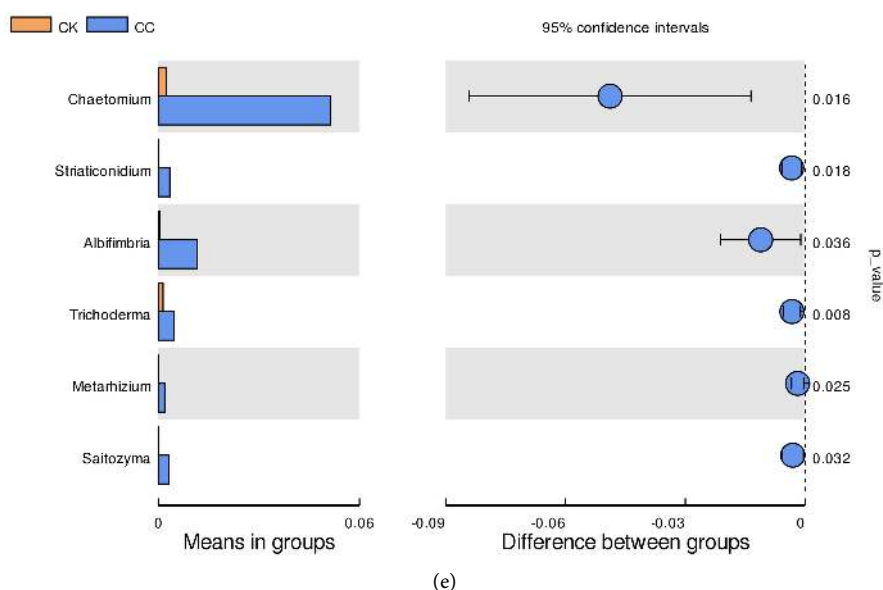


Figure S1. Differential analysis of bacterial proportions at the genus level between CK and BA (a), CK, and CC (b), CK, and BC (c). Differential analysis of fungal proportions at the genus level between CK and BC (d), CK, and CC (e). The abundances of different genera were analyzed by the T-test.

Table S1. Soil physiochemical properties in different treatments.

Chemical properties	Treatment			
	CK	BA	CC	BC
pH	5.09 ± 0.09d	6.05 ± 0.22c	7.10 ± 0.12b	7.40 ± 0.23a
organic matter (%)	3.22 ± 0.07a	2.67 ± 0.14b	2.32 ± 0.07c	2.07 ± 0.05d
total N (%)	0.13 ± 0.01b	0.14 ± 0.01b	0.17 ± 0.01a	0.18 ± 0.01a
alkaline N (mg/kg)	107.75 ± 2.45d	126.17 ± 3.37c	140.76 ± 6.07b	163.09 ± 5.37a
available P (mg/kg)	100.72 ± 4.03a	76.20 ± 4.52a	65.27 ± 3.08b	49.381 ± 5.61b
available K (mg/kg)	439.35 ± 22.64b	575.91 ± 16.37a	593.13 ± 37.25a	610.89 ± 35.57a
exchangeable Ca (mg/kg)	1302.75 ± 90.23c	1525.01 ± 50.69b	2325.01 ± 122.06a	2450.71 ± 141.58a
exchangeable Mg (mg/kg)	96.20 ± 1.20d	111.45 ± 5.71c	132.57 ± 6.59b	152.00 ± 10.42a

Values are means of SD (n = 3). The different letters in a row indicate significant differences as determined by the LSD test ($p < 0.05$).

Table S2. Characteristics of effective tags from samples of different treatments.

Sample	Bacterial			Fungal		
	Raw reads	Average length (bp)	Effective (%)	Raw reads	Average length (bp)	Effective (%)
CK1	92,561	389	94.93	98,933	350	97.04
CK2	96,320	390	95.23	93,569	350	96.65
CK3	97,280	388	94.32	98,844	350	96.74
BA1	81,464	390	95.12	98,434	350	94.89
BA2	93,979	390	94.56	81,318	350	95.05
BA3	88,806	389	95.23	89,425	350	95.27
CC1	85,487	390	95.31	99,198	350	91.67
CC2	81,182	390	95.03	99,933	350	91.56
CC3	70,693	390	94.98	97,245	349	93.45
BC1	96,660	390	95.25	86,367	350	94.55
BC2	86,539	389	95.23	82,224	350	94.12
BC3	88,634	390	95.41	83,095	350	95.49

Table S3. Spearman correlation analysis between microbial community and soil physicochemical properties.

		pH	organic matter	total N	alkaline N	available P	available K	exchangeable Ca	exchangeable Mg
Bacteria	Sobs	0.958**	-0.902**	0.787**	0.930**	-0.993**	0.573	0.776**	0.965**
	chao1	0.867**	-0.895**	0.714**	0.895**	-0.874**	0.664*	0.895**	0.874**
	shannon	0.937**	-0.923**	0.808**	0.951**	-0.993**	0.573	0.797**	0.944**
Fungal	Sobs	0.923**	-0.916**	0.879**	0.930**	-0.944**	0.636*	0.783**	0.958**
	chao1	0.671*	-0.699*	0.763**	0.741**	-0.762**	0.476	0.608*	0.727**
	shannon	0.937**	-0.930**	0.872**	0.923**	-0.930**	0.692*	0.818**	0.986**
	<i>Streptomyces</i>	0.832**	-0.797**	0.749**	0.790**	-0.776**	0.469	0.685*	0.769**
	<i>Paenibacillus</i>	0.804**	-0.793**	0.716**	0.867**	-0.800**	0.502	0.772**	0.705*
	<i>Sphingomonas</i>	0.301	-0.287	0.274	0.371	-0.287	0.196	0.224	0.315
	<i>Stenotrophomonas</i>	0.140	-0.224	0.190	0.252	-0.133	0.252	0.210	0.182
	<i>Flavobacterium</i>	0.494	-0.560	0.606*	0.564	-0.483	0.511	0.637*	0.571
	<i>Bacillus</i>	0.727**	-0.776**	0.724**	0.776**	-0.671*	0.490	0.818**	0.650*
	<i>Actinospica</i>	-0.322	0.427	-0.524	-0.434	0.371	0.147	-0.035	-0.350
Antagonistic	<i>Catenulispora</i>	-0.238	0.350	-0.513	-0.364	0.224	0.210	0.105	0.245
	<i>Haliangium</i>	0.762**	-0.818**	0.872**	0.888**	-0.790**	0.580*	0.727**	0.769**
	<i>Lysobacter</i>	0.748**	-0.734**	0.777**	0.741**	-0.720**	0.538	0.734**	0.797**
	<i>Aspergillus</i>	0.706*	-0.643*	0.267	0.545	-0.643*	0.573	0.720**	0.713**
	<i>Trichoderma</i>	0.021	-0.252	0.313	0.252	-0.077	0.455	0.259	0.112
	<i>F. oxysporum</i>	-0.369	0.341	-0.182	-0.411	0.538	-0.264	-0.404	-0.432
	<i>F. solani</i>	-0.735**	0.819**	-0.687*	-0.749**	0.699*	-0.320	-0.573	0.692*
	<i>A. alternata</i>	-0.798**	0.685*	-0.504	-0.770**	0.805**	-0.482	-0.552	-0.721**
	<i>R. solanacearum</i>	-0.847**	0.896**	-0.793**	-0.875**	0.798**	-0.531	-0.685*	-0.819**
	Pathogenic								

* and ** indicate correlation is significant at $p < 0.05$ and $p < 0.01$.

Table S4. Spearman correlation analysis between pathogenic, antagonistic microorganisms, microbial diversity and disease index of tobacco bacterial wilt.

Antagonistic microorganisms	<i>Streptomyces</i>	<i>Paenibacillus</i>	<i>Sphingomonas</i>	<i>Stenotrophomonas</i>	<i>Flavobacterium</i>	<i>Bacillus</i>
Correlation coefficient	-0.804**	-0.846**	-0.350	-0.245	-0.623*	-0.832**
Antagonistic microorganisms	<i>Actinospica</i>	<i>Catenulispora</i>	<i>Haliangium</i>	<i>Lysobacter</i>	<i>Aspergillus</i>	<i>Trichoderma</i>
Correlation coefficient	0.378	0.357	-0.846**	-0.762**	-0.615*	-0.237
Pathogenic microorganisms	<i>F. oxysporum</i>	<i>F. solani</i>	<i>A. alternata</i>	<i>R. solanacearum</i>		
Correlation coefficient	0.334	0.791**	0.678**	0.910**		
microbial diversity	Bacteria Sobs	Bacteria chao1	Bacteria shannon	Fungal Sobs	Fungal chao1	Fungal shannon
Correlation coefficient	-0.895**	-0.902**	-0.916**	-0.923**	-0.671*	-0.930**

* and ** indicate correlation is significant at $p < 0.05$ and $p < 0.01$.

Table S5. Spearman correlation analysis between disease index of tobacco bacterial wilt and soil physicochemical properties.

	pH	organic matter	total N	alkaline N	available P	available K	exchangeable Ca	exchangeable Mg
Correlation coefficient	-0.944**	0.979**	-0.851**	-0.972**	0.902**	-0.629*	-0.874**	-0.909**

* and ** indicate correlation is significant at $p < 0.05$ and $p < 0.01$.