Effects of Combined Application of Biocontrol Streptomyces and Cotton Straw Biochar on Soil Microbial Community in Continuously Cropped Cotton Fields: Postprint

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Abstract

Cotton is an important economic crop. Long-term continuous cropping can cause imbalances in cotton soil microecology, exacerbate soil-borne diseases, and consequently lead to declines in yield and quality, thereby affecting the healthy development of the cotton industry. This study investigated soil from continuously cropped cotton fields through laboratory incubation experiments. Different amounts of cotton straw biochar [0 g · kg ^{1} (CK), 25.0 g · kg ^{1}, 50.0 g · kg ^{1}, 100.0 g · kg ^{1}] were added based on the application of the biocontrol actinomycete Streptomyces flavotricini. Using microbial counting and 16S rDNA gene sequence analysis, the effects of combined application on the quantity of biocontrol bacteria, microbial quantity, and species in continuously cropped cotton field soil were examined, providing new insights for the biological control of cotton Verticillium wilt. The results demonstrated that: (1) Combined application of biocontrol actinomycetes and cotton straw biochar significantly affected the microbial flora in continuously cropped cotton field soil. Compared with the treatment of single application of biocontrol actinomycete agent, the combined application significantly increased the quantities of bacteria, actinomycetes, and fungi in soil, wherein the treatment with 25.0 g · kg ^{1} cotton straw biochar increased the bacteria/fungi ratio (B/F) and actinomycetes/fungi ratio (A/F) in soil by 5,271.2% and 30.8%, respectively ($P<0.05$). (2) The quantity of biocontrol actinomycetes in soil increased significantly with increasing cotton straw biochar application rate, with the treatment of 100.0 g · kg ^{1} cotton straw biochar showing a significant increase of 2,672.8% ($P<0.05$). Cotton straw biochar possesses the potential to serve as an excellent carrier for biocontrol actinomycetes. (3) Combined application of biocontrol actinomycetes and cotton straw biochar also altered the quantity and proportion of dominant microorganisms in soil, particularly increasing the quantity and proportion of Bacillus among bacteria;

the combined application of 100.0 g · kg ^{1} cotton straw biochar and the microbial agent resulted in significantly higher quantity and proportion of Streptomyces in soil compared with the control, but decreased the quantity of Micromonospora; it increased the quantities of Aspergillus oryzae, Aspergillus niger, and Trichoderma among fungi, but decreased their proportions. These findings indicate that combined application of biocontrol actinomycetes and cotton straw biochar can increase the quantity of biocontrol actinomycetes in continuously cropped cotton field soil, enhance the disease suppression and growth promotion effects of biocontrol agent preparations, improve the soil microbial community structure of continuously cropped cotton fields, and holds substantial application potential in preventing and controlling cotton continuous cropping obstacles.

Full Text

Effect of Cotton Stalk Biochar Application on Soil Microflora of Continuous Cotton Cropping Under Use of Antagonistic Actinomycetes*

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Abstract: Cotton is an important cash crop, and long-term continuous cropping can cause imbalances in soil microecology, increase soil-borne diseases, and consequently reduce yield and quality, thereby affecting the healthy development of the cotton industry. This study investigated continuously cropped cotton field soil through laboratory incubation experiments. Based on the application of the biocontrol actinomycete *Streptomyces flavotricini*, different amounts of cotton stalk biochar were added [0 g · kg ^{1} (CK), 25.0 g · kg ^{1}, 50.0 g · kg ^{1}, 100.0 g · kg ^{1}]. Using microbial counting and 16S rDNA gene sequence analysis, we examined the effects of combined application on the quantity and diversity of biocontrol agents and microorganisms in continuously cropped cotton soil, providing new insights for the biological control of cotton Verticillium wilt. The results showed that: (1) The combined application of biocontrol actinomycetes and cotton stalk biochar significantly affected the soil microflora of continuously cropped cotton fields. Compared with the treatment applying biocontrol actinomycetes alone, the combined application significantly increased the numbers of bacteria, actinomycetes, and fungi in soil. Specifically, the treatment with 25.0 g · kg ^{1} biochar increased the bacteria/fungi ratio (B/F) and actinomycetes/fungi ratio (A/F) by 5,271.2% and 30.8%, respectively ($P<0.05$). (2)

The number of biocontrol actinomycetes in soil increased significantly with increasing biochar application rate, with the 100.0 g · kg ^{1} biochar treatment increasing it by 2,672.8% (P<0.05). Cotton stalk biochar demonstrates potential as an excellent carrier for biocontrol actinomycetes. (3) The combined application also altered the numbers and proportions of dominant microorganisms, particularly increasing the quantity and proportion of *Bacillus* among bacteria. The 100.0 g · kg ^{1} biochar treatment significantly increased the number and proportion of *Streptomyces* compared to the control but reduced *Micromonospora* numbers. It increased the numbers of *Aspergillus oryzae*, *Aspergillus niger*, and *Trichoderma* while decreasing their relative proportions. These results indicate that combining biocontrol actinomycetes with cotton stalk biochar can increase biocontrol actinomycete populations, enhance disease prevention and growth-promoting effects, improve soil microbial community structure in continuously cropped cotton fields, and shows great potential for alleviating continuous cropping obstacles in cotton production.

Keywords: Cotton stalk biochar; Antagonistic actinomycete; Continuous cropping cotton; Soil microflora

Introduction

Continuous cropping is widespread in China' s commercial cotton production regions. Under intensive cultivation conditions, long-term continuous cropping of cotton (*Gossypium* spp.) has led to increasing trends in continuous cropping obstacles and soil-borne diseases such as Fusarium and Verticillium wilts, becoming a major issue urgently needing resolution in cotton cultivation. The essence of continuous cropping obstacles lies in the disruption or deterioration of the soil rhizosphere micro-ecosystem. Currently, the extensive use of chemical pesticides has caused problems including drug residues, environmental pollution, and pathogen resistance accumulation, which no longer align with requirements for sustainable agricultural development. Biological control and ecological regulation are environmentally friendly approaches without drug residues that may fundamentally prevent and control soil-borne diseases, though their efficacy depends on the population size and colonization ability of biocontrol agents in soil.

Numerous studies have reported on the effects of biocontrol actinomycetes on crop rhizosphere soil microecology and their disease-preventing and growth-promoting functions. However, biocontrol agents that demonstrate clear antimicrobial effects in laboratory settings often face challenges in pot and field applications due to influences from the biocontrol preparation itself, pathogens, host plants, and environmental factors (such as nutrient sources, temperature, salinity, permeability, and pH in soil), resulting in unstable colonization ability and disease control efficacy. Adding inorganic or organic materials as nutrients and immobilization carriers for biocontrol agents can help them effectively col-

onize soil, establish dominant populations, and achieve better control effects. Commonly used microbial carriers include inorganic materials such as peat, vermiculite, diatomite, and sodium alginate, as well as various organic wastes like mushroom substrate and manure, though these also have negative impacts.

Biochar is a stable carbon-rich solid produced through low-temperature pyrolysis of biomass under oxygen-limited conditions, which can be manufactured from agricultural and forestry wastes such as crop straw and wood chips. With well-developed porosity, huge specific surface area, and strong nutrient adsorption capacity, biochar provides habitats for soil microorganisms, improves soil microbial community structure, promotes microbial diversity, and enhances microbial activity. Biochar has attracted attention from scholars worldwide as an excellent carrier for functional microorganisms. Adding biochar can increase populations of beneficial microorganisms such as mycorrhizal fungi, nitrogen-fixing bacteria, *Enterobacter cloacae*, and *Bacillus mucilaginosus*. However, Aeron et al. compared soil, wood charcoal, wood chips, and wood chip soil, finding that wood chip soil was the most effective carrier for *Pseudomonas fluorescens* PS1. Therefore, in-depth research on biochar' s function as a microbial carrier is needed.

Studies have shown that biochar can alleviate continuous cropping obstacles and control certain plant and soil-borne diseases by regulating the soil microecological environment, though some controversies remain. Consequently, combining biocontrol agents with biochar application has become a new research direction for alleviating continuous cropping obstacles and soil-borne diseases. However, few studies have reported on how combining biocontrol agents with cotton stalk biochar affects the soil microecology of continuously cropped cotton fields. Therefore, investigating the effects of combined application on biocontrol actinomycete populations and soil microflora in continuously cropped cotton fields holds significant importance. Given the complexity and variability of field conditions that are difficult to control precisely, this study employed a simulation method to investigate changes in biocontrol actinomycetes and microbial flora in continuously cropped cotton soil following the application of different amounts of cotton stalk biochar on top of a fixed application rate of biocontrol actinomycetes, exploring the feasibility and technical measures for increasing biocontrol actinomycete populations and enhancing their disease-preventing and growth-promoting effects.

Materials and Methods

Biochar and Soil Materials

Cotton stalk biochar was provided by the Institute of Soil, Fertilizer & Agricultural Water Saving, Xinjiang Academy of Agricultural Sciences. Cotton straw was processed under anaerobic conditions at 500 °C for 3 hours, then crushed and passed through a 0.5 mm sieve. The particle size distribution was: 52% between 0.25–0.5 mm and 48% <0.25 mm. Its basic physicochemical properties

were: organic carbon 771.80 g · kg ¹, total nitrogen 15.72 g · kg ¹, total phosphorus 15.31 g · kg ¹, total potassium 25.04 g · kg ¹, pH 10.27, and electrical conductivity 4.38 mS · cm ¹.

The test soil was collected from a cotton field in Jing County, Hebei Province, that had been under continuous cropping for six years. Soil was sampled from six points in the cotton root zone at 0–20 cm depth, air-dried, mixed thoroughly, and ground to pass through a 0.5 cm sieve. The biocontrol actinomycete used was strain 25# of *Streptomyces flavotricini*, isolated and screened by the Microbial Resources Laboratory of the College of Natural Resources and Environment, Northwest A&F University, which showed good antagonism against the cotton Verticillium wilt pathogen. The powdered live bacterial preparation used in the experiment was produced through solid-state fermentation and low-temperature drying, with a viable count of 5.3×10 cfu · g ¹.

Soil Inoculation Simulation Experiment

The experiment consisted of four biochar application rates: 0 g · kg ¹ (CK), 25.0 g · kg ¹, 50.0 g · kg ¹, and 100.0 g · kg ¹. All treatments were inoculated with strain 25# biocontrol agent at a rate of 5.3×10 cfu · g ¹ (dry soil). A 100 g soil sample was weighed for each treatment, mixed with the appropriate amount of biochar in a 300 mL wide-mouth bottle, and then inoculated with the biocontrol agent. Each treatment had three replicates. Each bottle received 26 mL of sterile water, was sealed, and incubated at 28 °C. During incubation, an equal-weight water addition method was used to maintain consistent weight across all bottles. After 15 days of incubation, the soil in each bottle was thoroughly mixed, and 10.0 g was sampled for soil microbial quantity determination and species analysis.

Soil Microbial Isolation and Counting

All soil samples from different treatments were processed using the dilution plate method for isolation and counting. The media used were beef extract peptone agar (BPA) for bacteria, Gause's No. 1 medium (GA) for actinomycetes, and potato dextrose agar (PDA) for fungi. To prevent bacterial growth, 1 g · L ¹ potassium dichromate was added to GA medium before sterilization to a final concentration of 80 g · mL ¹, and sterile lactic acid was added to PDA medium to a final concentration of 3 mL · L ¹. Bacteria, actinomycetes, and fungi were counted after 2–3 days, 5–7 days, and 3–5 days of incubation, respectively. Colony morphological characteristics were observed and analyzed; colonies with identical morphological features within a plate were designated as the same taxonomic unit. Microorganisms with high abundance (approximately 5% of the total) were identified as dominant species. After counting each dominant species, single colonies were selected for purification, preservation, and identification.

Bacterial Strain Identification

Selected bacteria were identified based on morphological characteristics combined with 16S rDNA sequence analysis. Total bacterial DNA was extracted using the enzymatic lysis method. The primers used were forward primer PA: 5 -AGAGTTTGATCCTGGCTCAG-3 and reverse primer PB: 5 -AAGGAGGTGATCCAGCCGCA-3 . Amplification conditions were: pre-denaturation at 94 °C for 4 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 2 min; final extension at 72 °C for 10 min; and storage at 4 °C. Amplified products were gel-purified and sent to Nanjing GenScript Biotechnology Co., Ltd. for sequencing. Obtained sequences were compared with those in the GenBank database.

Data Calculation and Statistical Analysis

In the experimental data, the biochar effect (ΔCK%) represented the increase percentage of different microbial groups in biochar-amended soil relative to CK. PB(%) represented the proportion of dominant bacteria in soil relative to the total isolated bacteria, PA(%) represented the proportion of *Micromonospora*, *Streptomyces*, and biocontrol agents relative to the total isolated actinomycetes, PF(%) represented the proportion of dominant fungi relative to the total isolated fungi, and CB(%) represented the increase multiple of dominant bacteria in biochar-amended soil. All experimental data were analyzed using SAS statistical software, and Duncan's new multiple range test was used for significance testing.

Results

Effects of Biocontrol Agent Combined with Biochar on Microbial Quantity and Ratios in Continuously Cropped Cotton Soil

As shown in Table 1 , the combined application of biocontrol agent and cotton stalk biochar significantly increased the numbers of bacteria, fungi, and actinomycetes in continuously cropped cotton soil. Compared with the biocontrol agent alone (CK), the 25.0 $g \cdot kg^{1}$ and 100.0 $g \cdot kg^{1}$ biochar treatments significantly increased bacterial numbers by 7,103.8% and 8,640.7%, respectively ($P<0.05$). The numbers of fungi and actinomycetes increased gradually with increasing biochar application rate. The 25.0 $g \cdot kg^{1}$, 50.0 $g \cdot kg^{1}$, and 100.0 $g \cdot kg^{1}$ treatments significantly increased fungal and actinomycete numbers by 34.2% and 75.5%, 469.6% and 165.1%, and 2,289.4% and 366.3%, respectively ($P<0.05$).

Table 2 shows that the combined application altered the microbial composition in continuously cropped cotton soil. Compared with the biocontrol agent alone, the 25.0 $g \cdot kg^{1}$ biochar treatment increased the bacteria/fungi (B/F), actinomycetes/fungi (A/F), and bacteria/actinomycetes (B/A) ratios by 5,271.2%, 30.8%, and 3,996.0%, respectively. The 50.0 $g \cdot kg^{1}$ treatment decreased B/F

and A/F ratios by 32.0% and 53.4%, respectively, while increasing B/A by 45.7%. The 100.0 g · kg ^{1} treatment increased B/F and B/A ratios by 266.0% and 1,768.8%, respectively, while decreasing A/F by 80.5%.

Effects of Biocontrol Agent Combined with Biochar on Bacterial Community Distribution

Based on the species and quantities of bacteria isolated from soil, *Bacillus* was the dominant genus. Five abundant *Bacillus* strains were selected for molecular identification (Table 3). Table 4 shows that applying different biochar rates with the biocontrol agent altered the species, quantities, and proportions of dominant bacteria in continuously cropped cotton soil. In the biocontrol agent alone treatment, strains YB1 and YB3 each accounted for 3.8% of total isolated bacteria. Biochar addition increased the numbers of these two dominant bacteria, with the 25.0 g · kg ^{1} and 100.0 g · kg ^{1} treatments increasing YB1 and YB3 numbers by 170.6-fold and 150.9-fold, and 18.6-fold and 3.9-fold, respectively ($P<0.05$). However, the 50.0 g · kg ^{1} treatment showed minimal increase in YB1 and even decreased YB3 numbers. Strains YB5, YB6, and YB7 were not detected in the control soil but were present in biochar treatments, with YB5 accounting for 5.1%, 5.7%, and 7.4%; YB6 for 6.7%, 11.0%, and 3.2%; and YB7 for 4.1%, 7.2%, and 2.8% of total bacteria in the 25.0 g · kg ^{1}, 50.0 g · kg ^{1}, and 100.0 g · kg ^{1} treatments, respectively.

Effects of Biocontrol Agent Combined with Biochar on Actinomycete Community Distribution

Table 5 shows that compared with the biocontrol agent alone, all biochar treatments increased *Micromonospora* and *Streptomyces* numbers by 25.3%–85.8% and 44.4%–1,372.9%, respectively. The 100.0 g · kg ^{1} treatment significantly increased *Micromonospora* and *Streptomyces* numbers by 76.0% and 1,372.9%, respectively ($P<0.05$). Biochar application significantly affected the introduced biocontrol actinomycete population, increasing *Streptomyces flavotricini* numbers by 49.8%–2,672.8% compared to the no-biochar control, with its proportion reaching 49.4% of total culturable actinomycetes.

Regarding proportions, biochar treatments showed different effects on the three actinomycete groups relative to total isolated actinomycetes. Compared with the biocontrol agent alone, biochar treatments reduced the *Micromonospora* proportion to 8.6%–16.2%, consistently lower than the control and decreasing with increasing biochar rate. The 25.0 g · kg ^{1} and 50.0 g · kg ^{1} treatments reduced the *Streptomyces* proportion to 16.6% and 17.5% (decreases of 17.4% and 12.9%, respectively), while the 100.0 g · kg ^{1} treatment increased it to 63.5% (a 215.9% increase). The 25.0 g · kg ^{1} treatment reduced the proportion of strain 25# (*S. flavotricini*) to 7.1%, whereas the 100.0 g · kg ^{1} treatment significantly increased it to 49.4%.

Effects of Biocontrol Agent Combined with Biochar on Fungal Community Distribution

Based on the species and quantities of fungi isolated from soil, strains YF1, YF2, and YF3 were identified as dominant fungal groups, each exceeding 5% of total isolates in most cases. Preliminary identification based on colony and spore morphology identified these three strains as *Aspergillus oryzae*, *Aspergillus niger*, and *Trichoderma koningii*. Tables 6 and 7 show that the combined application altered the quantities and proportions of dominant fungi in continuously cropped cotton soil. Compared with the biocontrol agent alone, all biochar treatments except the 50.0 g · kg ^{1} treatment (which decreased YF2 numbers) increased the three dominant fungi, with the 100.0 g · kg ^{1} treatment significantly increasing them by 455.5%, 244.8%, and 8,936.1%, respectively ($P<0.05$). However, the relative proportions of these three dominant fungi generally decreased with increasing biochar application rate, likely due to the overall increase in total fungal numbers.

Discussion

Biocontrol actinomycetes face challenges in developing viable formulations due to poor tolerance and slow growth characteristics, as well as difficulties in reproduction, low sporulation, and reduced control efficacy after multiple generations. Their biocontrol activity is also easily affected by environmental and nutritional factors during application, leading to issues with stability and persistence. Therefore, selecting appropriate nutrients, carriers, or co-application with other fertilizers to maximize biocontrol activity has become a key focus for biocontrol actinomycete applications. Currently, peat, vermiculite, and other inorganic minerals are commonly used as carriers, but these have limited reserves, uneven distribution, and environmental issues from extraction. Organic nutrient carriers also have negative effects including harmful organisms, heavy metal and salt accumulation, and antibiotic residues.

Biochar, produced from organic waste materials such as crop straw, rice husk, wood, and animal manure through pyrolysis at 300–700 °C under oxygen-limited or anaerobic conditions, has attracted attention as a novel biocontrol agent carrier due to its wide range of feedstock sources. Although the benzene rings in biochar structure are highly stable, its aliphatic and oxidized carbon components are easily decomposable, and it contains abundant available nutrients including N, P, K, Ca, and Mg that can be utilized by microorganisms. However, some studies have shown that as a carbon source or substrate, the utilization efficiency of biochar by biocontrol fungi such as *Trichoderma* is not as good as glucose, lactose, or starch, and some biochars may even have potential inhibitory effects on microbial reproduction. Nevertheless, due to its porous nature, biochar can provide attachment sites and ample space for microbial survival, serving as an excellent carrier for plant probiotics and other microorganisms. Its strong

adsorption capacity can also retain soil nutrients, providing a favorable living environment for biocontrol agents. This study demonstrates that combining biocontrol actinomycetes with cotton stalk biochar can improve soil microbial community structure in continuously cropped cotton fields and increase the population of the biocontrol actinomycete *S. flavotricini*, indicating that cotton stalk biochar can serve as a good carrier for biocontrol actinomycetes with significant biocontrol potential. However, biochar type, production conditions, and application rate all affect its performance as a microbial carrier.

Compared with the biocontrol actinomycete alone, biochar application increased the population of *S. flavotricini* in soil. Since biochar application increased soil pH and biocontrol actinomycete numbers increased with biochar rate, we speculate that biocontrol actinomycetes can grow well in slightly alkaline conditions, thus significantly increasing their populations at high biochar application rates. Biochar input also altered the microbial community structure compared with the biocontrol agent alone, with application rate significantly affecting bacterial, actinomycete, and fungal numbers. While some studies have shown that bacterial numbers increase significantly with biochar application rates of 5–60 $g \cdot kg^{1}$, others have reported that bacterial, actinomycete, and fungal numbers first increase then decrease with increasing biochar rates. This study found that compared with the biocontrol agent alone, combined biochar application significantly increased bacterial, actinomycete, and fungal numbers in continuously cropped cotton soil, showing an increase-decrease-increase trend with rising biochar rates. The significant increase at 100.0 $g \cdot kg^{1}$ may be due to the substantial increase in biocontrol actinomycetes suppressing soil-borne pathogens and improving soil environment, thereby increasing overall microbial numbers. However, whether this high application rate affects cotton growth requires verification through cotton planting experiments.

The combined application increased bacterial and actinomycete numbers relative to fungi, with B/F and A/F ratios decreasing as biochar application rate increased, demonstrating clear regulation of microbial community structure and shifting continuously cropped cotton soil toward a bacterial-dominated state. Whether this change results from increased biocontrol agent populations or direct biochar effects requires further investigation with controls lacking either the biocontrol agent or biochar. As this was a soil incubation experiment without plant roots, field experiments are needed for validation.

The combined application had different effects on different microbial groups. This study isolated five dominant bacterial strains, all belonging to *Bacillus*. *Bacillus oceanisediminis* numbers increased with biochar rate, while *Bacillus bingmayongensis* decreased, and the other three strains showed an initial increase followed by decrease. *Bacillus* species are important components of plant disease biocontrol microorganisms with significant potential for controlling various plant diseases. *Bacillus cereus* was isolated from all three biochar treatments but not from the control, and previous research has demonstrated its disease-preventing, growth-promoting, and antimicrobial effects. With increas-

ing biochar rate, *Micromonospora* numbers and proportions decreased while *Streptomyces* and the introduced biocontrol agent increased. The number of beneficial *Trichoderma* fungi also increased. These findings suggest that combining biocontrol agents with appropriate rates of cotton stalk biochar can promote beneficial bacteria, actinomycetes, and fungi in soil, which may positively impact the alleviation of continuous cropping obstacles, though further analysis of pathogen numbers and dominant microorganisms is needed.

Conclusion

The combined application of biocontrol actinomycetes and cotton stalk biochar significantly affected the soil microflora of continuously cropped cotton fields. Compared with biocontrol actinomycetes alone, the number of biocontrol actinomycetes in soil increased significantly with biochar application rate, demonstrating cotton stalk biochar' s potential as an excellent carrier. The combined application also significantly increased microbial numbers and altered dominant microbial quantities and proportions, particularly increasing *Bacillus* numbers and proportions among bacteria. The 100.0 $g \cdot kg^{1}$ biochar treatment significantly increased *Streptomyces* numbers and proportions compared to the control while reducing *Micromonospora* numbers. These results indicate that combining biocontrol actinomycetes with cotton stalk biochar can increase biocontrol actinomycete populations, enhance disease prevention and growth-promoting effects, improve soil microbial community structure in continuously cropped cotton fields, and shows great potential for controlling continuous cropping obstacles in cotton production.

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