



Full Length Article

Evaluation of Antagonistic *Bacillus* and Biochar against Soybean Root Rot Caused by *Fusarium oxysporum*

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Abstract

Soybean root rot is a worldwide soil-borne fungal disease threatening soybean production, causing huge losses in yield and quality of soybean. *Fusarium* species are well recognized as the important causal agent of *Fusarium* root rot. To screen the beneficial *Bacillus* strains with capability of suppressing soybean root rot and evaluate the impact of *Bacillus* combined with biochar against soybean root rot, a pot experiment was conducted with different treatments. In this study, as potential biological control measures, antagonistic *Bacillus* isolates and different types of biochar were added to soil separately and excellent antagonistic strains mixed with bamboo biochar were applied to the soil. The results showed that seven *Bacillus* strains promoted the growth of soybean seedlings and reduced root rot severity by 33 to 61%. *Bacillus amyloliquefaciens* NH2 was associated with the lowest incidence of soybean root rot, indicating its bio-control potential. The value of plant height, root length and plant dry weight of soybean in the sterilized soil mixed with biochar separately treatment were superior to those of soybean in the inoculated with pathogen treatment, especially the bamboo biochar treatment reduced soybean root rot caused by *Fusarium* significantly and which bio-control efficacy was 77.41%. The soybean plants shoot and root dry weights in the biochar mixed *B. amyloliquefaciens* NH2 or *B. subtilis* DBK treatments were increased by 17.1, 10.7% and 19.51, 19.64%, respectively, which were significantly higher than those of the inoculated pathogen treatment. Compared to antagonistic strain or biochar individual treatments, the disease control efficiency on soybean root rot was up to 64.86% in NH2 strain mixed with bamboo biochar treatment, which reduced root rot severity significantly and showed a synergistic effect. These results suggest that antagonistic *Bacillus* strains mixed with biochar can be used as an effective alternative in managing soybean root rot. © 2021 Friends Science Publishers

Keywords: Antagonistic *Bacillus*; Biochar; Control efficacy; Soybean *Fusarium* root rot

Introduction

Soybean root rot is a soil-borne fungal disease with worldwide distribution, causing a serious damage to soybean production (Chang *et al.* 2015). There are many species of pathogens causing soybean root rot, among which *Fusarium* species are well recognized as the main pathogens reported in different soybean-producing regions around the world (Arias *et al.* 2013a, b). In China, soybean field of the Heilongjiang province accounted for approximate 41% of total cultivated area and 37% of gross output (<http://www.zzys.moa.gov.cn>). As *Fusarium* root rot complex, it can infect soybean, causing large losses in yield

and quality of soybean (Broders *et al.* 2007; Chang *et al.* 2018; Wang *et al.* 2020).

At present, the conventional controlling method, seed coating and field application with chemical fungicide have been widely utilized to reduce and suppress some *Fusarium* diseases (Wang *et al.* 2020). Extensive and unjudicial use of fungicides has certain deleterious effects such as evolved resistance in pathogen, environmental pollution and hazard to human health (Wang *et al.* 2020). Therefore, it is urgent to find a harmless and feasible strategy to suppress the development of *Fusarium* root rot (Anillo *et al.* 2019). With the sustainable development of agricultural production, the application of microbial pesticides in agricultural production

is expanding. Remarkable achievements have been made in the research on rhizospheric growth-promoting bacteria controlling soil-borne diseases of crops and promoting plant growth, among which antagonistic *Bacillus* strains showed a good potential (Mates *et al.* 2019; Javed *et al.* 2021; Sharf *et al.* 2021).

Bacillus strains are kinds of biological bacteria of plant disease with the widest application and broad application prospect. They not only widely exist in external environments including soil and plant root surface, but also serve as common endophytic bacteria in plant (Xiong *et al.* 2018). They have both biofertilizer and biocontrol functions, and are the most commonly used in agriculture production (Wu *et al.* 2019). It is reported that *Bacillus* with other biocontrol agents brings benefits to the plant, both for growth and nutrition, minimizing the effects of adverse conditions. Therefore, the use of beneficial agents that play on multiple fronts can be considered a useful technological resource for farmers, either by promoting plant growth or by its disease-preventing action (Kalantari *et al.* 2018; Moreira *et al.* 2021).

Biochar, a porous form of carbon made from organic waste such as animal dung, animal bones, plant roots, wood chips, crop residues, *etc.* is increasingly being used as a soil amendment (Bonanomi *et al.* 2015; Rogovska *et al.* 2017). The effect of biochar amendments on crop productivity has been still unclear, but has been shown to depend on quality of the biochar (Peterson *et al.* 2013). Recent studies have suggested that biochar applications may alter the severity of disease caused by foliar and soil-borne fungal pathogens (Elmer and Pignatello 2011; Rogovska *et al.* 2017) and activate both induced and systemic resistance mechanisms in plants (Rasool *et al.* 2021). From the first report on the effect of biochar on *F. oxysporum* root rot of lettuce (Matsubara *et al.* 2008), to the impact on corn stalk rot and soybean root rot (Rogovska *et al.* 2017; Liu *et al.* 2019), biochar has shown great potential and strong developmental prospects. This paper aimed to use antagonistic bacteria isolated from the topsoil and rhizosphere soil, and biochar produced from diverse sources, our study is motivated that application of antagonistic *Bacillus* and biochar to pot experiment can improve soybean growth and reduce severity of *F. oxysporum* root rot.

Materials and Methods

Biochar and soil preparation

Four biochars were used in this study. Rice husk biochar A (Pyrolysis temperature, 500°C for 4 h, Shanghai Jiao Tong University). Bamboo biochar B (500°C for 4 h, Thermo Fisher Scientific, Inc., Pittsburgh, USA). Rice husk biochar C (500°C for 20 min, Energy Research Institute of Heilongjiang Academy of Agricultural Sciences (HAAS). Wheat straw biochar D (200°C for 10 min, Energy Research Institute of HAAS). Soil was collected from

Heilongjiang Modern Agricultural Demonstration Area (126.86 N, 45.85 E) in Harbin, Heilongjiang, China. After air-drying the soil and passing it through a 2 mm sieve, the soil was autoclaved for 2 h.

Pathogenic fungi, antagonistic *Bacteria*, inoculums and chemical fungicide preparation

F. oxysporum M3-3xu (isolated from soybean root rot sample in Northeast China in 2018) was incubated on PDA medium (25°C, 5 days). Three mycelia plugs were cut out along the colony edges using a 7 mm hole punch and were inoculated into PDB medium (25°C, 180 rpm for 5 days) to prepare 10^6 spores mL⁻¹ suspension. Similar five plugs were inoculated in a 250 mL sterilized (121°C for 40 min) bottle containing 150 g sorghum grain. The sorghum culture was incubated for 10 days at 25°C and shaken daily to ensure uniform fungal growth.

B. subtilis JHK, *B. subtilis* DBK and *B. subtilis* N5B8, *B. amyloliquefaciens* NH2, *B. amyloliquefaciens* LFY5 and *B. amyloliquefaciens* BQD1 and *B. aryabhattai* FY were identified as antagonistic bacteria and were stored in our biological control laboratory. The antagonistic bacteria were inoculated into LB medium (25°C, 180 rpm for 3 days) to prepare 10^8 cfu mL⁻¹ bacterial suspension. At the end of fermentation, the suspension of bio-control bacteria was adsorbed onto sterile peat (1:1 v/w) and then air-dried to prepare solid bio-control inoculum containing 10^7 cfu g⁻¹ dry soil. As chemical fungicide, Carbendazim 80% WP was considered as a control for chemical treatment.

Evaluation of biocontrol *Bacillus* against soybean root rot

The experiment was performed to study the effect of 7 species *Bacillus* strains on the severity of soybean root rot and promoting the growth of soybean. The soil and vermiculite were thoroughly mixed (1:2 v/v) and poured into plastic pots with diameter of 11 cm and height of 11 cm. Soybean cultivar Sui Nong 53 (Heilongjiang, China) seeds were surface-sterilized with 3% (v/v) sodium hypochlorite for 3 min, rinsed with sterile water for 5 min and then five seeds were sown in each pot. All pots were placed in greenhouse. After 10 days, the seedlings were thinned down to three per pot.

The experiment comprised 16 treatments, with three replicates each. #1 Inoculated pathogen treatment (each pot was irrigated with 30 mL pathogen spore suspension), #2 Pathogen with 7 antagonistic strains respectively (after inoculating with 30 mL pathogen spore suspension 1 day, 40 mL antagonistic strain suspension was used for irrigation and on the seventh day, the same amount of antagonistic strain suspension was irrigated), #3 Antagonistic strain (same as in #2) without pathogen spore suspension, #4 Non-inoculated control (each pot was irrigated with 40 mL sterile LB medium). Plants were watered as needed to maintain moisture at 60% of field capacity. Thirty days after seeding,

the shoots and roots were oven-dried at 80°C for 48 h and dry weight was determined. Disease severity was rated using the 0–5 scale. The disease severity index was calculated according to the formula (1), and the disease control efficiency was calculated by formula (2).

$$\text{Disease severity (\%)} = \sum \left(\frac{\text{disease rate} \times \text{number of plants with this rate}}{\text{total number of plants} \times \text{maximum value of disease scale}} \right) \times 100 \quad (1)$$

$$\text{Disease control efficiency (\%)} = (A - B) / B \times 100 \quad (2)$$

Where: A - the disease index of the treatment with inoculated pathogen; B - the disease index of each treatment.

Evaluation of biochar amendment against soybean root rot

The experiment was carried out to study the effect of various biochars on the severity of soybean root rot. Soil and sand were mixed (2:1 v/v) and autoclaved for 2 h. The soil-sand mixture was mixed thoroughly with different biochars (20 g kg⁻¹ dry weigh). Each soil-sand-biochar mixture was inoculated with sorghum inoculum of *F. oxysporum* (30 g kg⁻¹). The same amount of non-inoculated sorghum mixed with soil-sand mixture was used as blank control. All treatment pots were watered and equilibrated for 10 days before sowing. Soybean seeds were treated as mentioned above (six seedlings per pot). The pots were incubated at 25°C and 60% moisture content (Liu *et al.* 2019). Each of six treatments was replicated four times. Shoot and root height, length and dry weight were measured 30 days after seeding. The disease index and the disease control efficiency were obtained as specified above.

Evaluation of the effect of bio-control bacteria with biochar on soybean root rot

Based on the screening and evaluation of the effect of antagonistic bacteria and biochar on soybean root rot, the plant growth experiments with 7 treatments were set up. The experimental treatments were as follows: (1) sorghum inoculum of *F. oxysporum* with solid bio-control agent (*B. amyloliquefaciens* NH2 and *B. subtilis* DBK respective), (2) sorghum inoculum of *F. oxysporum* with solid bio-control agent (*B. amyloliquefaciens* NH2 and *B. subtilis* DBK respective) and bamboo biochar, (3) sorghum inoculum of *F. oxysporum*, (4) sorghum inoculum of *F. oxysporum* with carbendazim 80% WP and (5) non-inoculated sorghum as blank control. The application amounts of bio-control agent, biochar and sorghum inoculum of *F. oxysporum* are shown in Table 1. Plants were grown in pots as described above. After sowing, 100 mL of 80% carbendazim WP was added to the treatment #4. Each treatment was replicated three times. Five seedlings were retained in each pot. The disease index, the disease control efficiency and plant dry weight were obtained as detailed above.

Data Processing and Statistical Analysis

Data were tested for statistical significance using one-way analysis of variance (ANOVA). Mean comparisons were conducted using the least significant difference (LSD) test ($P \leq 0.05$) (SPSS version 19.0, SPSS Inc., Chicago, USA).

Results

Effect of biocontrol strains on soybean root rot

The disease severity index of soybean inoculated by *F. oxysporum* only was above grade 3 (the diseased area accounted for more than 50% of the total area), and the plant growth was poor (Fig. 1A and B). Compared with blank control, in *B. amyloliquefaciens* BQD1 treatment assays, the biomass dry weight of soybean was enhanced 17.61% in terms of promoting growth. The biomass dry weight treated with *B. amyloliquefaciens* NH2 was 1.2 times of that treated with pathogen in terms of disease prevention. The disease control efficiency of NH2 strain was 60.61%, which was significantly ($P \leq 0.05$) higher compared to the others. *Bacillus* not only controlled soybean root disease, but also promoted plant growth (Table 2).

Biological control of soybean root rot in biochar-amended soil

The plant height, root length and biomass of soybean treated with bamboo biochar were not significantly different from those of other biochar treatments, but were significantly higher than those of treatment with pathogen inoculation only (Fig. 2 and Table 3). The plant height, root length and biomass of soybean treated with bamboo biochar were 1.29 times, 1.78 times and 1.61 times higher than those of the control inoculated with pathogen. The disease severity index of each biochar treatment was significantly different from that of inoculated control. The disease control effect of bamboo biochar on soybean root rot was 77.41%. The results showed that biochar not only controlled soybean root rot disease, but also promoted plant growth (Fig. 3A and B).

Effect of biocontrol *Bacillus* and bamboo biochar on soybean root rot and growth

The shoot and root dry weight of soybean was significantly higher than that of the control when the NH2 and DBK strains were separately combined with bamboo biochar (Table 4). The disease control effect of NH2 strain combined with bamboo biochar on soybean root rot was 64.86%, which was equivalent to that of the chemical fungicide treatment. Moreover, the root system of soybean was well-developed, the plant growth was robust, and the root rot was not obvious (Fig. 4).

Table 1: Soil treatment and application rate of bio-control bacteria

Treatment	Application rate	
	Biological agents (mL kg ⁻¹ soil)	Inoculum (g kg ⁻¹ soil)
NH2 + pathogen	20	40
DBK + pathogen	20	40
NH2 + bamboo biochar + pathogen	20	40
DBK + bamboo biochar + pathogen	20	40
pathogen	-	40
Carbendazim 80% WP (500 fold solution)+pathogen	100	40
Blank control	-	-

NH2: *B. amyloliquefaciens*; DBK: *B. subtilis*

Table 2: Soybean growth and the efficacy of root rot control by bacterial bio-control strains

Isolate	Shoot dry weight (g plant ⁻¹)		Root dry weight (g plant ⁻¹)		Bio-control efficacy (%)
	Pathogen	No Pathogen	Pathogen	No Pathogen	
NH2 <i>B. amyloliquefaciens</i>	1.56 ± 0.24a	1.60 ± 0.13b	0.37 ± 0.05bc	0.34 ± 0.05b	60.61 ± 8.18a
YS7 <i>B. amyloliquefaciens</i>	1.38 ± 0.16b	1.30 ± 0.11e	0.45 ± 0.06a	0.33 ± 0.06bc	52.52 ± 2.31b
DBQ1 <i>B. amyloliquefaciens</i>	1.54 ± 0.24a	1.72 ± 0.15a	0.36 ± 0.04c	0.35 ± 0.07ab	53.52 ± 10.78b
DBK <i>B. subtilis</i>	1.31 ± 0.15c	1.45 ± 0.06c	0.39 ± 0.04b	0.34 ± 0.03b	51.52 ± 7.33b
JHK <i>B. subtilis</i>	1.13 ± 0.12f	1.27 ± 0.06e	0.27 ± 0.03d	0.31 ± 0.04c	33.33 ± 2.31e
N5B8 <i>B. subtilis</i>	1.21 ± 0.30e	1.48 ± 0.11c	0.24 ± 0.03e	0.31 ± 0.05c	42.42 ± 7.33d
FY <i>B. aryabhatai</i>	1.24 ± 0.16de	1.28 ± 0.07e	0.29 ± 0.01d	0.31 ± 0.03c	48.48 ± 11.16c
Blank control	-	1.39 ± 0.05d	-	0.37 ± 0.03a	-
Pathogen	1.28 ± 0.11cd	-	0.38 ± 0.03bc	-	-

Mean ± standard deviation

Values within the same column for each parameter followed by letters are significantly different ($P \leq 0.05$)

Table 3: The root rot disease of soybean with different biochar treatments

Treatments	Disease index (%)	Bio-control efficacy (%)
Rice husk biochar A	15.00 ± 2.89b	70.97 ± 8.97
Bamboo biochar B	11.67 ± 2.46b	77.41 ± 3.45
Rice husk biochar C	23.33 ± 4.62b	54.85 ± 2.77
Wheat straw biochar D	16.67 ± 2.89b	67.74 ± 5.48
Pathogen	51.67 ± 6.11a	—
Blank control	1.11 ± 1.92c	—

Mean ± standard deviation

Values within the same column for each parameter followed by letters are significantly different ($P \leq 0.05$)

Table 4: Efficacy of biochars with potential bio-control agents in promoting soybean plant growth and controlling disease in greenhouse

Treatment	Shoot dry weight (g)	Root dry weight (g)	Bio-control efficacy (%)
NH2 + pathogen	4.20 ± 0.36abc	1.32 ± 0.22ab	43.66 ± 14.00bc
DBK + pathogen	4.31 ± 0.85abc	1.11 ± 0.14bc	50.62 ± 10.54abc
NH2 + bamboo biochar B + pathogen	4.32 ± 0.20abc	1.24 ± 0.19ab	64.86 ± 3.64a
DBK + bamboo biochar B + pathogen	4.41 ± 0.46abc	1.34 ± 0.17ab	58.13 ± 5.47ab
pathogen	3.69 ± 0.07cde	1.12 ± 0.04bc	-
Carbendazim + pathogen	3.71 ± 0.26cde	1.30 ± 0.18ab	68.13 ± 5.47a

NH2: *B. amyloliquefaciens*; DBK: *B. subtilis*

Mean ± standard deviation

Values within the same column for each parameter followed by letters are significantly different ($P \leq 0.05$)

Discussion

In our paper, seven antagonistic *Bacillus* strains were used to evaluate the effect on soybean *F. oxysporum* root rot in pots. The result showed that, compared with the pathogen control, *B. amyloliquefaciens* NH2 strain showed its benefits both on promoting the growth of soybean and reducing the disease, which bio-control efficacy reached 60.61%. It is consistent with the previous studies results which proved the *Bacillus* as potent bio-control agents. The ability of bio-control agents to efficiently colonize surfaces

of plant roots is a prerequisite for phytoprotection (Etesami and Alikhani 2018). *Bacillus* is considered to be a potential bio-control agent because of its wide antibacterial spectrum and strong tolerance to experiment conditions (Kadaikunnan et al. 2015; Zouari et al. 2016).

Four types of biochar were tested in our research in sterilized soil inoculated with *F. oxysporum*. The result showed that the soybean dry weight was higher in the soil-biochar treatment than in the pathogen treatment at the time of investigation. Among them, values of soybean treated by bamboo biochar were superior to others, and the

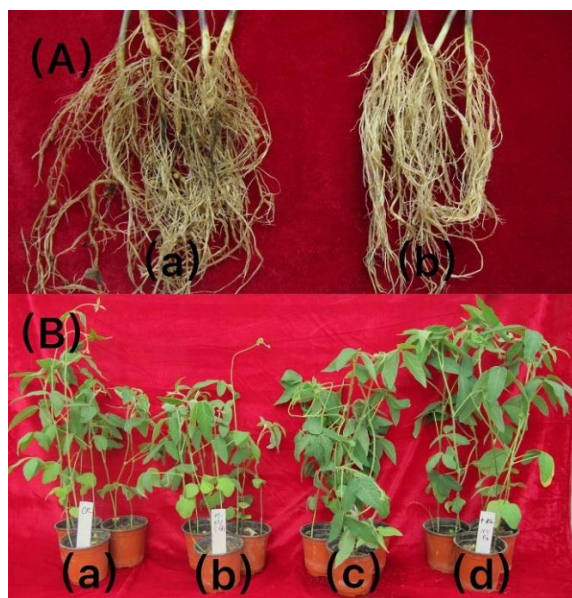


Fig. 1: Efficacy of bio-control agents on soybean root rot and plant growth
(A)-(a) treated by *F. oxysporum*; (A)-(b) treated by *F. oxysporum* with NH2 strain;
(B)-(a) blank control; (B)-(b) treated by *F. oxysporum*; (B)-(c) treated by *F. oxysporum* with NH2 strain; (B)-(d) treated by *F. oxysporum* with N5B8 strain

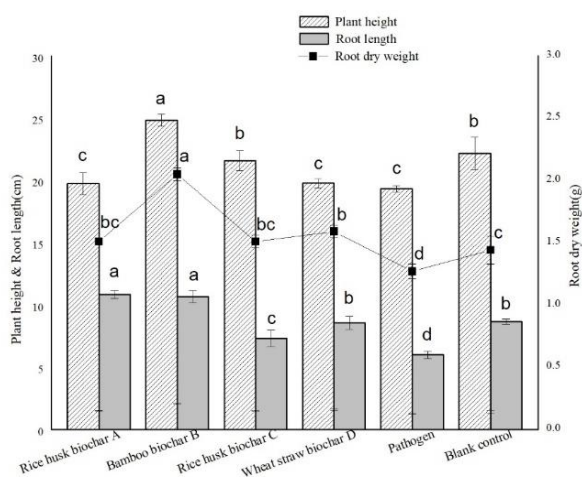


Fig. 2: The growth of soybean plants with different biochar treatments

disease control effect of bamboo biochar treatment was 77.41%. For soil-borne root diseases, it is conceivable that biochars diminish some compounds in the soil solution that might otherwise facilitate the capacity of pathogens to detect and infect roots (Lehmann *et al.* 2011). Moreover, bamboo biochar significantly reduced the incidence and severity of soybean root rot caused by *F. oxysporum*. The mechanisms that facilitate biochar-stimulated plant protection are still the subject of intensive research (Kolton *et al.* 2017). The relative abundance of *Fusarium* spp. in soil decreased with an increase in biochar content, indicating that biochar could reduce the spread of soil-borne plant

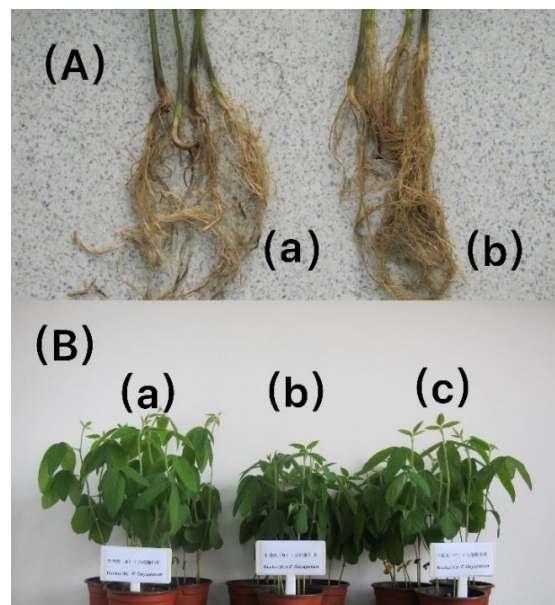


Fig. 3: Effects of biochar on soybean root rot and plant growth
(A)-(a) treated by *F. oxysporum*; (A)-(b) treated by *F. oxysporum* with biochar B; (B)-(a) treated by *F. oxysporum* with biochar B; (B)-(b) treated by *F. oxysporum* with biochar D; (B)-(c) treated by *F. oxysporum* with biochar C

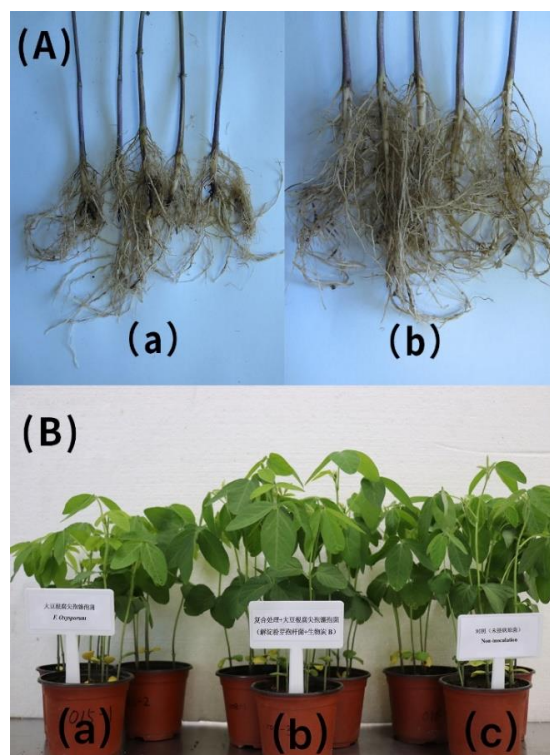


Fig. 4: Efficacy of biochar and antagonistic strains on soybean root rot and plant growth
(A)-(a) treated by *F. oxysporum*; (A)-(b) treated by *F. oxysporum* with NH2 strain and biochar B; (B)-(a) treated by *F. oxysporum*; (B)-(b) treated by *F. oxysporum* with NH2 strain and biochar B; (B)-(c) blank control

pathogens and inhibit the occurrence of plant diseases (Yao *et al.* 2017).

In our study, *B. amyloliquefaciens* NH2 and *B. subtilis* DBK were used in combination with bamboo biochar. Compared with the control inoculated with the pathogen, plant dry weight was increased by individual bio-control strains and biochar or the combination of a bio-control strain and biochar. The bio-control efficacy of soybean root rot was up to 64.86% in combination of NH2 strain and biochar treatment, which was significantly higher than that of strain or biochar applied separately. Soybean root rot is caused by multiple pathogens. The occurrence of soybean root rot is closely related to the changes in soil microbial community in the soybean rhizosphere. Therefore, it is difficult to achieve the positive effect with a single bio-control agent in practice. Previous studies have shown that the compound bio-control agents that use the synergistic advantages and complementary effects of different functional bio-control strains can achieve the reasonable bio-control effect of soil-borne diseases in crops (Palyzová et al. 2019), indicating the synergism between bio-control *Bacillus* and biochar.

We used sterilized soil in pot experiments. Compared with the non-sterilized soil mixed with antagonistic bacterial strains mixed with bamboo biochar, the soil microbial system might be relatively simple. It may be the reason why the control effect of different biochar on root rot was different from that of bio-control agent combined with bamboo biochar. In our study, bio-control bacteria and biochar were used to assess the prevention and control of soybean soil-borne root rot. However, further research is needed to clarify the main mechanism and the relevant factors governing biochar control of soil-borne diseases in order to maximize a contribution of each factor in the prevention and control of soil-borne diseases.

Conclusion

Two main outcomes were documented in our research. First, the combination of *B. amyloliquefaciens* NH2 strain and 1% bamboo biochar (w/w) can promote the growth and biomass of soybean. Secondly, the disease control effect of combined treatment on soybean *F. oxysporum* root rot was 64.86%, which was significantly higher than that of single *Bacillus* strain or biochar, clearly indicating a synergistic effect.

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Author Contributions

FY, CLL and XML planned the experiments, LL, XFJ,

YL and BHS investigated and interpreted the results, SW, ML and CX statistically analyzed the data and made original illustrations, FY and XML made the write up.

Conflicts of Interest

All authors declare no conflicts of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper.

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