

Article

Control Efficiency of Biochar Loaded with *Bacillus subtilis* Tpb55 against Tobacco Black Shank

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Abstract: Black shank caused by *Phytophthora nicotianae* has become a destructive soil-borne disease to different flue-cured tobacco cultivars in Southwest China. The use of biochar amendments for microorganism synergy is a promising effective strategy for *P. nicotianae* development control. In this study, biochar samples were prepared from tamarisk with different pyrolyzation temperatures (300–500 °C). The effect of pyrolytic temperatures on the bacteria immobilization efficiency of biochar was investigated. *B. subtilis* Tpb55 was successfully loaded on different biochars as biocontrol composites. The survival investigation of the inoculum suggested that biochar pyrolyzed at 300 °C (BC300), with a large pore opening diameter; a greater pore volume exhibited a better Tpb55 immobilization. A pot experiment indicated that Tpb55-loaded BC300 had a more pronounced decrease in the disease severity index of black shank disease and an increase in the soil pH, alkali-hydrolyzable nitrogen, soil-available phosphorus, and available potassium. BC300 inoculated with Tpb55 showed the highest control effect (79.60%) against tobacco black shank in the pot experiments, with the lowest copy number of *P. nicotianae* DNA. In conclusion, biochar-immobilized Tpb55 may provide a new strategy for preventing and controlling tobacco black shank.

Keywords: biological control; biochar; tobacco black shank; *Bacillus subtilis*



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1. Introduction

The black shank of tobacco caused by *Phytophthora nicotianae* is one of the devastating diseases of flue-cured tobacco (*Nicotiana tabacum*) in China. Currently, the general method to manage tobacco black shank is the application of fungicides such as metalaxyl and its active ingredient, mefenoxam [1,2]. However, the intense and long-term usage of fungicides might enhance the resistance of *P. nicotianae* to them. It also has a risk of increasing fungicide residues [1,3].

Using biological control agents (BCAs) is one of the most promising alternatives for controlling tobacco black shank [4–6]. Many bacterial and fungal species (such as *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Trichoderma harzianum*) exhibit the ability to suppress soil-borne fungal and oomycete diseases. In addition, it has been proved that an ammonium sulfate precipitation of a *Bacillus velezensis* suspension could promote the pH of soil and eliminate the disease severity of *P. nicotianae* [2]. Han et al. found that *B. subtilis* Tpb55 strains exhibited a strong antagonism against *P. nicotianae* by inhibiting its mycelia growth, colonizing in the tobacco rhizosphere [4]. However, merely applying a BCA to field soil may not achieve a desirable effect due to the variability in soil environmental factors. For instance, beneficial antagonists may not be able to thrive due to soil microfauna predation and competition from better-adapted native microflora [6,7].

To reduce the use of fungicides and achieve sustainable agriculture, biochar amendments that have been suggested to enhance soil physicochemical properties and fertility

have attracted tremendous attention [8]. As a microbe carrier and soil amendment in agricultural applications, biochar has great advantages due to its cost-effectiveness, biomass availability, and physical and chemical properties [9,10]. Moreover, the properties of a large specific surface area and high porosity indicate that it could provide microbes with a suitable habitat and protect them from predation and desiccation. Biochar could also meet the needs of carbon (C), energy, and mineral nutrients [11,12]. Therefore, biochar is considered to be a good carrier material for cell immobilization. Cells immobilized on biochar have been used to remove soil and water contaminants such as polycyclic aromatic hydrocarbons, pesticides, and heavy metals [13–15]. In addition, biochar has been proposed to be a management tool for suppressing soil-borne diseases, including fusarium crown and root rot of tomatoes, rice root-knot nematodes, cucumber damping-off, carrot root-lesion nematodes, and tobacco bacterial wilt [16–19]. It has been reported that biochar could inhibit *Phytophthora* spp. and improve the community of the fungal rhizosphere [20]. However, the application of antagonistic bacteria formulated with biochar against soil-borne pathogens has not been extensively studied. Previous research has predominantly focused on the impact of biochar characteristics on the disease inhibition capability of immobilized bacteria cells, with few studies focusing on the interaction of biochar and antagonistic microorganisms or the field control effect of biochar applications.

In this study, we first aimed to examine the cell visibility of strain Tpb55 immobilized with tamarisk at pyrolysis temperatures of 300 °C, 400 °C, and 500 °C for biochar. The physicochemical changes in the soil properties with the application of biochar as an inoculum carrier was determined. Finally, the control efficiency of Tpb55 formulated with biochar against *P. nicotianae* was evaluated and compared with a single Tpb55 application in pot experiments.

2. Materials and Methods

2.1. Biochar Production and Physicochemical Properties

Pulverized tamarisk (*Tamarix chinensis* Lour) stems were sieved with a 2 mm sieve. The biochar production was conducted by the slow pyrolysis of the tamarisk powder in a muffle furnace at 300 °C (TB300), 400 °C (TB400), and 500 °C (TB500) for 2 h under a N₂ flow of 500 mL·min^{−1} [21]. The biochar was milled, passed through a 75 µm sieve, and stored in sealed containers. The physical and chemical characteristics of the biochar were measured according to the method described by Sun et al. [22].

2.2. Bacteria Strain and Pathogen

B. subtilis Tpb55 (CGMCC No. 2853) and competent strain Tpb55 with a rhizosphere-stable pRP43-GFP plasmid [23] was obtained from the Key Laboratory of Tobacco Pest Monitoring Controlling & Integrated Management, Qingdao, China. *B. subtilis* Tpb55 cells were cultured in a liquid LB medium containing kanamycin at 28 °C overnight to promote the growth phase in a 100 mL conical flask. The cell pellets were then collected by centrifugation at 10,000 rpm at 4 °C for 10 min. The cells were washed twice with sterile sodium chloride (NaCl; 0.85%) and centrifuged at 4000 × g for 30 min at 4 °C. The collected pellets were resuspended in half the initial culture volume with 0.85% sterile NaCl. The number of colonies were counted by using hemocytometers. This cell suspension, with a final density of 7.96×10^{10} cells, was used for all treatments.

The highly virulent *P. nicotianae* strain JM1 from the Key Laboratory of Tobacco Pest Monitoring Controlling & Integrated Management was used. A *P. nicotianae* mycelial mat was grown on a potato dextrose agar (PDA) medium at 26 °C for 7 days in constant light [4]. Spores of *P. nicotianae* were collected by the addition of 10 mL of a 0.1% potassium nitrate solution per plate and filtrated through 4 layers of cheesecloth. The final concentration of the spore suspensions was adjusted to 1.0×10^8 spores mL^{−1} with a hemocytometer. The suspension was inoculated to a millet seed medium for five days before being mixed into the soil.

2.3. Tpb55–Biochar Composite Preparation

Approximately 0.25 g (dry weight) of biochar was blended into fresh LB (1:20, *w:v*) in 100 mL flasks. A total of 2.5 mL of the Tpb55 culture fluid was then added and incubated for 48 h in a rotary shaker at 170 rpm at 30 °C. The planktonic cells were removed by separating the mixture with a 75 mm sieve and then were rinsed three times with deionized water. Before the inoculation, the Tpb55 composite was collected and stored at 4 °C. All operations were performed under aseptic conditions.

After 48 h of culture on the biochar, the Tpb55 cells were reintroduced to fresh LB and incubated on a rotary shaker (30 °C; 150 rpm) for 20 days. On days 2, 4, 6, 8, 12, 14, 16, 18, and 20, three flasks were collected for each sampling point. The GFP copy numbers of strain Tpb55 were determined at each point. Environmental scanning electron microscope (ESEM) images were obtained using a Hitachi TM 1000 table-top microscope (Hitachi Co., Ltd., Tokyo, Japan). The biochar samples for imaging were flash-frozen in liquid nitrogen-chilled isopentane. A total of 10–15 electron micrographs were collected for each treatment. The Tpb55 pRP43-GFP genetic expression on the biochar pieces was imaged using confocal laser scanning microscopy (CLSM; Leica TCS SP8, Solms, Germany).

The accumulated biomass of strain Tpb55 on the tamarisk biochar and soil were evaluated by real-time PCR. An EasyPure Bacteria Genomic DNA Kit (MoBio Laboratories, Carlsbad, CA, USA) was used to extract the DNA from the bacterial inoculum in the biochar and a FastDNA SPIN Kit (MoBio Laboratories, Carlsbad, CA, USA) was used to extract the DNA from the soil samples following the manufacturers' instructions. MIQE (minimum information for the publication of quantitative real-time PCR experiments) standards were used to perform the quantitative PCR (qPCR) and data analysis [24]. The trends of the GFP copies in the biochar–Tpb55 agents and soil are shown in Figure 1a,b. The primers presented are listed in Table S1.

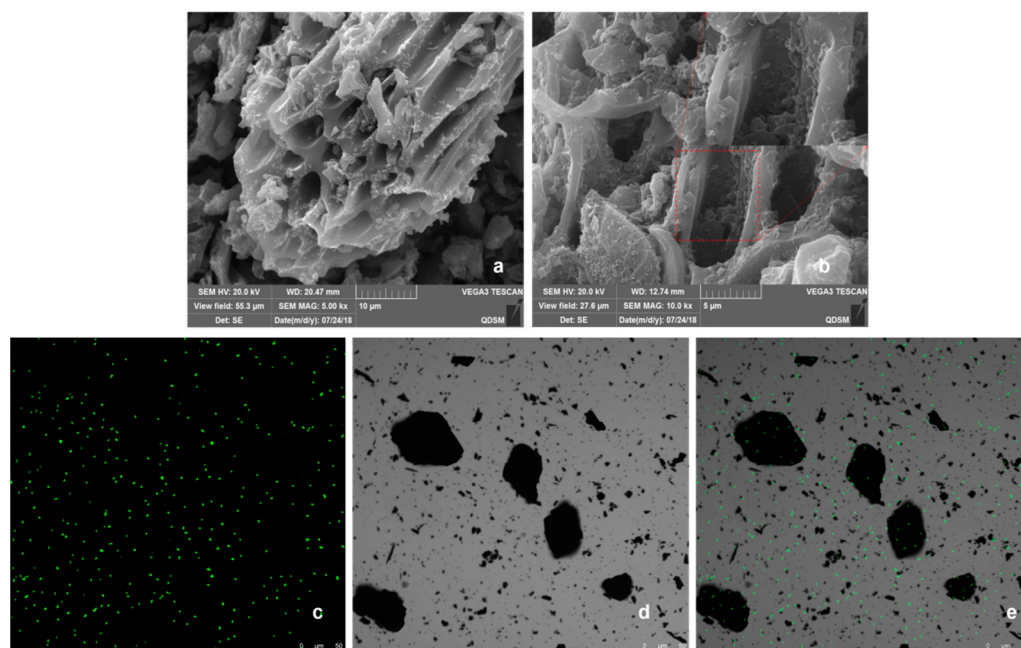


Figure 1. ESEM images of biochar treated with (a) 0.85% NaCl, (b) Tpb55-inoculated biochar, and fluorescent microscopy images of (c) Tpb55–pRP43 on biochar particle GFP excitation, (d) bright-field, and (e) an overlay of the bright-field and GFP image.

2.4. Biofilm Matrix Component Quantification and Shelf-Life Determination

The biofilm was harvested and suspended in 1 mL ultrapure water through ultrasonic oscillation. The supernatant solution was acquired from the centrifuged biofilm suspension (10,000 rpm; 2 min) and then used to quantify the total protein and polysaccharide content in the EPS through a Bradford assay [25] and the phenol–sulfuric acid method [26],

respectively. The amounts of polysaccharides and proteins were both determined directly through a standard curve (Figure 2).

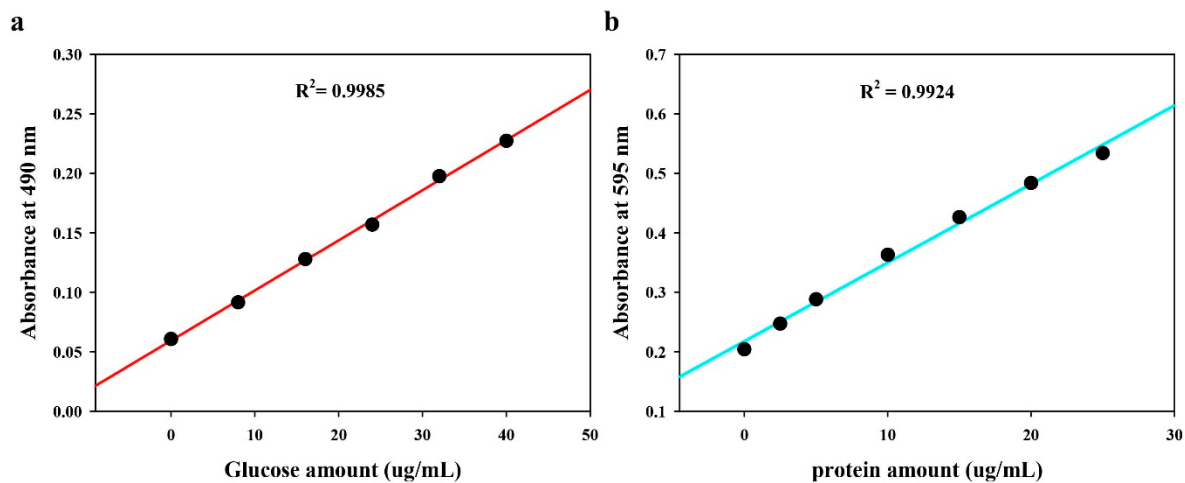


Figure 2. Standard curves for quantification analysis of polysaccharide (a,b) protein.

The shelf-life of the inoculant was determined according to Siddiq et al. [27], with a few minor revisions. The survival of Tpb55 in different biochar composites was assayed over three months at 20 °C with different time intervals (0, 20, 30, 40, 50, 60, 70, 80, 90, and 100 days). For the measurement of viable bacterial densities, the samples were suspended in 2 mL tubes containing 1 mL PBS (0.1 M) and 3 zirconium beads using a Mixer Mill MM400 (Retsch, Haan, Germany) with a frequency of 30 beats per second. The PBS suspensions were serially diluted (1:10) in PBS on NA plates in triplicate. After incubation for 24 h at 28 °C, the colony counts were conducted.

2.5. Analysis of the Soil Physicochemical Properties

The soil samples were air-dried and ground (<2 mm) after the pathogen inoculation for 20 days to determine the soil properties and available nutrients. The pH of the soil was measured at a soil-to-water ratio of 1:2.5 (*w:v*). The ammonium acetate method was used to calculate the cation exchange capacity (CEC) [28]. Sodium bicarbonate was used to extract the available phosphorus (AP), which was then estimated using the molybdenum blue method [29]. The amount of available potassium (AK) was determined using a flame photometer, after extracting it with neutral normal ammonium acetate. A dry combustion C/N element analyzer was used to determine the soil organic carbon (SOC) and total nitrogen (N) (VarioMax CNS, Germany) [30]. The soil conductivity was determined with a soil conductivity real-time detector [28]. Alkali-hydrolyzable nitrogen (AN) was determined following the alkaline hydrolysis diffusion method [31]. The metal ions were determined by using an elemental analyzer.

2.6. Plant Maintenance

The susceptible tobacco seeds (Zhongchuan 208) were surface-sterilized with 10% NaClO for 5 min and then immediately rinsed with double-distilled water three times. The sterilized seeds were planted in a tray containing potting mixture with or without biochar (0, 0.5, 1, and 3%; *w:w*) in a greenhouse free of pests and diseases at 26 ± 1 °C under sprinkler irrigation. The biochar was added to a mixture of peat and tuff (7:3, *v:v*; Shacham Givat Ada, Israel) in each pot (3 L; diameter, 16 cm; 5 pots per treatment) after the germination of healthy and consistent tobacco seedlings (six-day-old seedlings) (0, 0.5, 1, and 3%; *w:w*). To keep the transplanted seedlings healthy, they were kept in a pest- and disease-free greenhouse at a constant temperature of 26 ± 1 °C and irrigated twice daily with drip irrigation (5:3:8 NPK fertilizers; 120, 30, and 150 mg L⁻¹ of total N, P, and K, respectively; EC 2.2 dS m⁻¹), allowing 25–50% drainage.

2.7. Pathogen Inoculation and Disease Evaluation

In a pot experiment, the impact of the biochar, strain Tpb55, and their combination on tobacco black shank was evaluated. Eight experimental treatments were performed as follows: (i) no treatment with biochar or strain Tpb55 (CK); (ii) treatment with strain Tpb55; (iii) treatment with BC300; (iv) treatment with BC400; (v) treatment with BC500; (vi) treatment with BC300 + strain Tpb55 (BT300); (vii) treatment with BC400 + strain Tpb55 (BT400); (viii) treatment with BC500 + strain Tpb55 (BT500). Five-day-old *P. nicotianae*-infested millet seeds were mixed at a concentration of 0.20% (*w:w*) with the rhizosphere soil collected from a tobacco field in Zunyi, Guizhou Province, China (107°31′48.63″ E, 28°08′1.81″ N). The rhizosphere soil was immediately transported to the laboratory, sieved, and used in the treatments. After being mixed for 24 h, the potted soils were amended with biochar (BC, 1% *w/w*), free cells of strain Tpb55 (1%, *w/w*), and immobilized bacterial cells (TB, 1% *w/w*) as the treatments, respectively. The tobacco seedlings were transplanted into pots (12 cm top diameter and 10 cm base diameter, 10 cm height) filled with the composition at the five-leaf stage.

The plants were incubated in a growth chamber (28 °C, 80% relative humidity, and 12 h/12 h light/dark cycles). The disease severity was recorded using a 0–9 index after the pathogen inoculation for 20 days. The severity of the disease was given a score, and the Han et al. method was used to calculate the disease index [4]. The experiment was repeated three times; each treatment consisted of 25 tobacco seedlings. Following the data collection, the disease severity and control effectiveness were computed as follows:

$$\text{Disease index} = (\Sigma (\text{number of diseased plants} \times \text{disease grade}) / (\text{total number of plants} \times \text{the highest disease grade})) \times 100.$$

$$\text{Control effect (\%)} = ((\text{infected control disease index} - \text{treatment disease index}) / \text{infected control disease index}) \times 100\%.$$

2.8. Statistical Analysis

Microsoft Excel 2018 and OriginPro 2018C were used for the data processing and to draw the charts, respectively. The data were analyzed by a one-way analysis of variance (ANOVA). The Student–Newman–Keuls method or the Kruskal–Wallis test was used for the pairwise comparisons among the sample means. The differences were considered to be statistically significant at $p \leq 0.05$. The data presented were the mean $\pm$ SEM ($n = 3$). The mean values of the viable number of rhizobia per g of moist inoculant were then calculated for the different times and plotted on a logarithmic scale. A one-way ANOVA was used to analyze the effect of the carrier on the bacterial survival for each strain at each sampling date, and the Tukey test was used for post hoc comparisons of the means. Levene’s test was used to check for the homogeneity of the variance required by the ANOVA. Moreover, in order to ascertain if there were differences in the overall survival behavior between the two strains, a Student’s *t*-test was carried out to compare the mean survival data of the two strains on all the sampling dates and in all the carriers.

3. Results

3.1. Immobilization and Survival of Tpb55 on Biochar

The Tpb55 inoculation of the biochar was visually confirmed. The Tpb55-inoculated biochar ESEM micrographs revealed that the bacteria were mostly attached to the tubular structure and lateral folds of the biochar, with only a few attached on the surface (Figure 1a,b). The CLSM images revealed the presence of viable GFP-expressing cells adhered to the biochar (Figure 1c–e), which confirmed the inoculation of the live Tpb55–pRP43 cells. Thus, ESEM imaging and qPCR revealed the efficient and durable immobilization of Tpb55 cells on biochar in LB.

The physicochemical characteristics of the biochars prepared from the tamarisk (*T. chinensis* Lour) stems are shown in Table 1. The results indicated that the biochar contained a series of nutrients that could enrich the soil.

Table 1. Physicochemical characteristics of biochars from tamarisk (*T. chinensis* Lour) stems.

Characteristics	BC300	BC400	BC500
WHC ^a (%)	73.51	71.80	78.61
pH	7.11	8.50	10.11
EC ^b (mS/cm)	1.48	2.03	8.67
BET Surface Area (m ² g ⁻¹)	0.21	0.71	1.77
Pore Opening Diameter (nm)	5.3	12.88	14.48
Total Pore Volume (cm ³ g ⁻¹)	0.00076	0.0023	0.0011
C (wt %)	53.55	59.38	61.77
H (wt %)	4.17	2.29	1.28
N (wt %)	1.09	2.31	2.22
S (wt %)	0.57	1.58	2.36
C/H	15.24	25.94	45.42
C/N	58.13	25.69	27.88
Ash Content (wt %)	6.21	18.55	21.44
Ca (wt %)	0.59	1.07	3.82
Mg (wt %)	4.32	4.12	5.30
K (wt %)	1.49	1.80	3.54
Na (wt %)	0.57	0.56	1.04

^a WHC: water-holding capacity; ^b EC: electrical conductivity.

The stability and viability of the Tpb55 cells in BC500 (500 °C) were higher when compared with BC300 (300 °C) and BC400 (400 °C) over a period of 100 days (Table 2). The highest CFU g⁻¹ was obtained in BC500 (12.17 ± 0.16) on day 30 at 20 °C. At 20 °C, the CFU g⁻¹ was found to rapidly increase and then sharply plummeted whereas at 10 °C, the increase was slower but stable; even after 60 to 90 days, a higher CFU was noted. Overall, the maximum bacterial populations were observed in BC500, which indicated that a higher temperature biochar provided the most effective habitat for Tpb55 cells over a period of more than 3 months.

Table 2. Shelf-life of Tpb55 on biochar under various storage temperatures (initial concentration of 10⁹ CFU g⁻¹).

Storage Temperature (°C)	Carrier Material	(Log CFU g ⁻¹ Carrier Material)							
		Days after Inoculation (DAI)							
		0	15	30	45	60	75	90	100
10	BC300	9.46 ± 0.21	9.97 ± 0.09	10.19 ± 0.24	9.96 ± 0.98	9.79 ± 0.46	8.92 ± 0.47	8.69 ± 0.62	8.26 ± 0.43
	BC400	9.47 ± 0.14	9.99 ± 0.11	10.15 ± 0.35	10.02 ± 1.02	10.00 ± 0.37	9.76 ± 0.70	9.53 ± 0.79	9.42 ± 0.29
	BC500	9.47 ± 0.10	10.15 ± 0.14	11.27 ± 0.26	11.00 ± 0.73	10.98 ± 0.45	10.23 ± 0.65	10.09 ± 0.35	9.99 ± 0.36
	CK	9.47 ± 0.14	9.91 ± 0.09	10.02 ± 0.15	9.91 ± 0.79	9.53 ± 0.66	8.62 ± 0.25	8.35 ± 0.46	8.11 ± 0.57
25	BC300	9.47 ± 0.11	10.92 ± 0.04	10.81 ± 0.29	10.65 ± 0.01	9.95 ± 1.29	9.12 ± 0.17	8.74 ± 0.08	7.92 ± 0.43
	BC400	9.45 ± 0.20	10.66 ± 0.08	10.95 ± 0.43	10.46 ± 0.10	9.98 ± 0.32	9.55 ± 0.33	9.12 ± 0.46	8.18 ± 0.29
	BC500	9.46 ± 0.15	11.49 ± 0.06	12.17 ± 0.16	11.31 ± 0.56	10.34 ± 0.45	10.15 ± 0.17	9.99 ± 0.24	8.92 ± 0.35
	CK	9.46 ± 0.08	10.92 ± 0.04	10.81 ± 0.29	10.65 ± 0.01	9.95 ± 1.29	9.12 ± 0.17	8.74 ± 0.08	7.92 ± 0.43

3.2. Quantification of the Biofilm Matrix Components

These results demonstrated that biochar increased the concentrations of exoproteins and exopolysaccharides with variety. Compared with the control (CK), the polysaccharides in the biofilm matrix of Tpb55 increased by 1.54% and by 0.71% in TB300 and TB400 after 72 h of incubation, respectively (Figure 3a) whereas the polysaccharides had the highest concentration in TB500 (Figure 3a). The proteins increased by 3.47%, 2.71%, and 1.82% after 72 h of incubation in TB300, TB400, and TB500, respectively (Figure 3b).

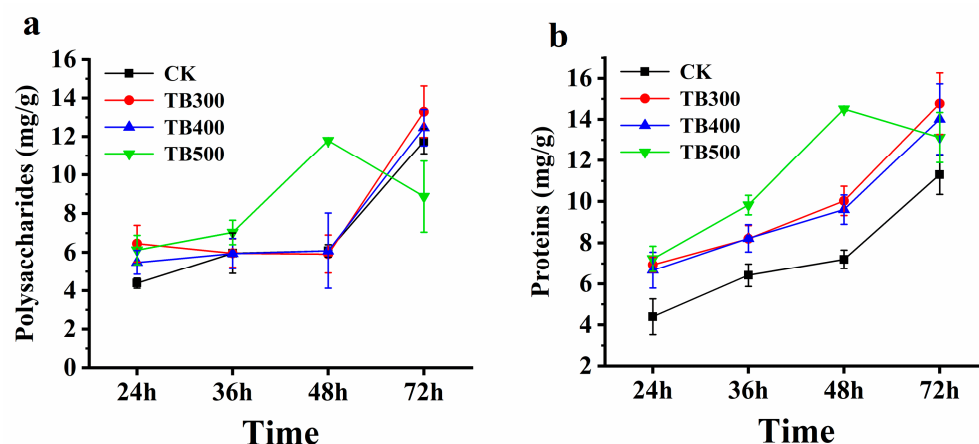


Figure 3. Effects of biochar on the content of (a) polysaccharides and (b) proteins of EPS in Tpb55.

3.3. Soil Physical and Chemical Properties under the Treatments

The soil bulk density was remarkably higher in the CK than in either the single biochar or inoculated biochar treatment groups (Figure 4a). The density was lowered by 13.80%, 12.50%, and 11.81% in BC300, BC400, and BC500, respectively. The density increased by 8.33%, 10.41%, and 9.02% in TB300, TB400, and TB500, respectively, when compared with the CK. The water-holding capacity (WHC) increased after the biochar addition and the highest WHC was found in TB300 (21.92% higher than the CK) (Figure 4b). The biochar resulted in a high specific surface area and water adsorption capacity [32]. Tpb55 alone did not improve the soil pH; TB300 had the highest pH (Figure 4c). TB500 increased the cation exchange capacity (CEC) of the soil by 16.37% compared with the CK, but the Tpb55 culture fluid showed no difference (Figure 4d). The available nutrients in the soil were promoted in the biochar alone and the composite microbial inoculum with Tpb55, according to the results of the determination of alkali-hydrolyzable nitrogen, available phosphorus, and available potassium content (Table 3).

Table 3. Effects of different treatments on the available nutrients of soil.

Treatment	CK	Tpb55	BC300	BC400	BC500	TB300	TB400	TB500
AP (mg/kg)	36.65 ± 2.47 d	53.01 ± 0.02 b	38.06 ± 0.19 d	49.86 ± 2.68 bc	60.27 ± 1.74 a	45.04 ± 0.43 c	47.21 ± 0.32 c	43.52 ± 0.23 c
AK (mg/kg)	349.50 ± 11.12 d	351.77 ± 1.15 d	659.56 ± 23.64 b	714.16 ± 23.08 a	734.14 ± 7.16 a	506.13 ± 15.68 c	498.65 ± 16.58 c	519.24 ± 18.65 c
AN (mg/kg)	380.53 ± 20.70 c	545.62 ± 10.14 a	350.20 ± 4.00 c	333.41 ± 5.26 c	441.83 ± 9.12 b	593.67 ± 29.34 a	539.76 ± 23.49 a	576.39 ± 34.29 a
Total nitrogen (g/kg)	0.56 ± 0.015 c	0.71 ± 0.022 b	0.75 ± 0.009 b	0.73 ± 0.012 b	0.74 ± 0.028 b	0.89 ± 0.015 a	0.87 ± 0.015 a	0.85 ± 0.015 a
SOC (g/kg)	4.56 ± 0.59 c	5.20 ± 0.47 c	11.04 ± 0.41 a	10.80 ± 0.26 a	10.36 ± 0.79 a	9.87 ± 1.47 b	9.46 ± 0.26 b	9.22 ± 0.79 b

Available phosphorus, available potassium, and alkali-hydrolyzable nitrogen are indicated by AP, AK, and AN, respectively.

3.4. Control Efficacy of the Biochar—Tpb55 Agent on Tobacco Black Shank

The *P. nicotianae*-treated plants in the CK group (disease index 54.32) showed symptoms seven days after the inoculation. TB300 exhibited the highest control efficacy (79.60%) against tobacco black shank among the three biochar immobilization treatments (TB400, 65.66%; TB500, 68.09%). The control efficiency of the Tpb55 treatment was 36.36% whereas that of the different biochar alone of the BC300, BC400, and BC500 treatments was 22.72%, 18.19%, and 10.14%, respectively (Figure 5).

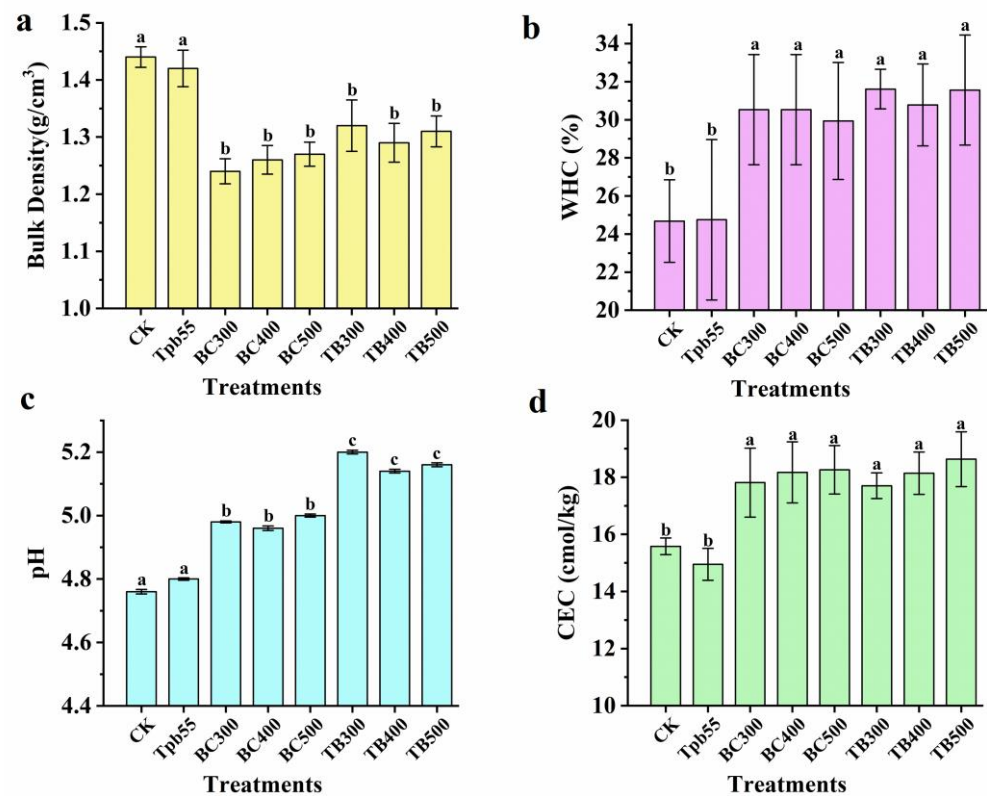


Figure 4. Influences of different treatments of biochar and Tpb55 on (a) bulk density, (b) water-holding capacity (WHC), (c) pH, and (d) cation exchange capacity (CEC) of soil; bars with the different letters show significant differences (LTK method, $p < 0.05$).

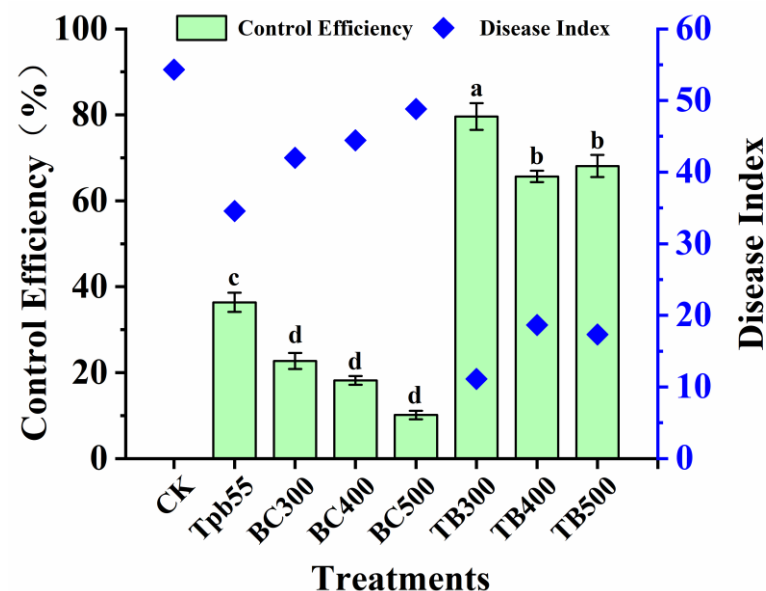


Figure 5. Disease severity index on tobacco black shank and the control efficiency of different treatments; bars with the same letters show no significant differences (LTK method, $p < 0.05$).

4. Discussion

The soil amendment with Tpb55 and biochar demonstrated its control effect against *P. nicotianae*, which supported the similar impact of previous studies [33]. The results of the ESEM images showed that the pore volume (Figure 2b) and the pore opening diameter of BC300 were larger than that of BC400 and BC500 (Table 1). BC300 could absorb more

Tpb55 than BC400 and BC500. Other studies have also found that the microbial adsorption capacity of biochar increased depending on specific biochar properties, including the surface area, hydrophobicity, and macropores [7,20,34]. Studies have also suggested that biochar provides a shelter for microbes [35]. The physical properties of biochar (such as its surface area, pore opening diameter, and water-filled pore space) may play a significant role in protecting pre-immobilized colonies from predation, and are closely associated with the development of inoculation after incorporation into soil [21]. The ESEM images and shelf-life of the Tpb55 cells (Figure 1 and Table 2) highlighted that biochar provided a suitable habitat for microbial colonization and enhanced the stability and viability of the Tpb55 cells [36–41]. Chathurika et al. and Rodriguez-Vila et al. suggested that biochar could supply soil microbes with the nutrients it contains [42,43]. The physicochemical characteristics of biochars demonstrated that H and C/N in BC300 significantly increased compared with BC400 and BC500 (nearly two-fold).

Previous studies have demonstrated two stages in the microbe immobilization on biochar [39]: the first is absorbing microbes onto the biochar and forming a biofilm, and the second is accumulating a biomass on the biochar. The second stage is associated with the specific intrinsic properties of biochar, which significantly varies with the preparation temperature and feedstock [43]. The results showed that the EPS production was enhanced, which could reinforce the adhesion between biochar and Tpb55.

Furthermore, microbes might play an initial role in the cell immobilization of biochar. One of the important features is the surface hydrophobicity (CSH) of cells; hydrophobic bacteria preferably attach to abiotic/hydrophobic surfaces [33]. Hydrophobic areas with the correct pore size served as centers for the clustering of *M. gilvum* cells and biofilms; a large surface area and pore volume supported successful cell immobilization. [44,45].

Previous studies have found that biochar has a positive effect against oomycete pathogens in the *Phytophthora* family [20,46], but reports on the synergy between biochar and antagonist bacteria are limited. According to our pot experiment, TB300 had the best control efficiency among all the treatments. Moreover, the combinations of tamarisk biochar and Tpb55 had a higher antagonism compared with applying biochar or Tpb55 alone. This suggested that biochar could enhance the antagonism of biocontrol bacterial agents and decrease the disease severity. Studies have demonstrated that the mechanisms by which biochar protects plants are varied, including increasing the diversity of soil microbes, providing nutrients, and inducing systemic plant defense mechanisms [47,48]. In addition, biochar promotes plants to recruit beneficial bacteria to the rhizosphere and thus develop a disease-suppressive rhizosphere microbiome [49–51]. The competition between biological control agents and soil-borne pathogens could be affected by biochar, and biochar might induce plant defenses [52].

To control tobacco black shank, we searched for an accessible and affordable biochar to load the biocontrol bacteria with a high efficiency. This study confirmed that biochar-immobilized antagonist microbes could be a promising biocontrol agent for tobacco black shank as well as improving acid soil properties. In the future, biochar loaded with biocontrol bacteria could be made into a carbon-based biocontrol bacteria fertilizer, which will surely have broad commercial market prospects. Biochar promotes plants to recruit beneficial bacteria to the rhizosphere and thus develop a disease-suppressive rhizosphere microbiome. Therefore, future studies analyzing the composition of tobacco root exudates may improve our understanding of the detailed role of the plant in the biochar-induced building up of soil suppressiveness against *P. nicotianae*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pr10122663/s1>, Table S1: Primers specifically designed for qRT-PCR assays.

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