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Effect of the endophyte *Ceratobasidium stevensii* on 4-HBA degradation and watermelon seed germination

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A preliminary trial indicated that the replanting obstacle of watermelon could be mitigated by the addition of the endophytic fungus *Ceratobasidium stevensii* B6 to the soil. The mechanism by which this treatment reduced the obstacles to continuous cropping was not clear, although the phenolic acids secreted by watermelon are a known problem. Consequently, we investigated the degradation of phenolic acids by *C. stevensii*, using 4-hydroxybenzoic acid (4-HBA) as a model. When the concentration of 4-HBA rose to 500 mg/L, it completely inhibited the germination of watermelon seeds. We therefore investigated the optimal conditions for 4-HBA degradation. When active *C. stevensii* broth was inoculated into degradation culture broth at a density of 5% (v/v), the 4-HBA concentration decreased to a level below the level of detection after 6 days. *C. stevensii* could also degrade 4-HBA when it was used as a sole carbon source. Glucose, sucrose, starch, and fructose stimulated the degradation of 4-HBA. High concentrations of glucose enhanced 4-HBA degradation. The optimal conditions for the degradation of 4-HBA by *C. stevensii* were 28°C, initial pH 5.5, and a shaking speed of 150 rpm. The addition of 500 mg/kg of 4-HBA to dried soil lasted for 2 months, followed by addition of *C. stevensii* which reduced the concentration of 4-HBA in soil below the level of detection after 7 days. Watermelon seedlings cultured in treated soil had a significantly increased number of stems and greater leaf length compared to controls. Therefore, *C. stevensii* has great potential in effectively lessening the obstacles to watermelon continuous cropping caused by 4-HBA accumulation.

Key words: *Ceratobasidum stevensii*, 4-hydroxybenzoic acid, watermelon, continuous cropping obstacles, degradation.

INTRODUCTION

Watermelon (*Citrullus lanatus*) is widely cultivated in China for its short growth period, high yield, and high monetary return. The current planting area of this crop is approximately 1.81×10^6 ha (Ministry of Agriculture of the People's Republic of China, 2011). However, once watermelon has been planted for a year, the land

Table 1. Basic physical and chemical properties of soil.

Soil	pH (H_2O)	Organic matter (g/kg)	Total nitrogen (g/kg)	Available nitrogen (mg/kg)	Available phosphorus (mg/kg)	Available potassim (mg/kg)	Clay (<0.002 mm) (%)
Soil from Jiangxi (Agri-Udic Ferralosols)	4.45	11.8	0.56	62.71	7.20	168.20	39.35
Soil from Hunan (Haplic-Udic Ferralosols)	4.82	11.6	0.89	77.32	25.17	588.31	32.99
Soil from Nanjing (Ferri-Udic Argosols)	7.58	11.8	0.59	124.2	40.06	111.10	25.86

becomes unsuitable for planting ordinary watermelon seed under normal circumstances for 5-6 years (Wu, 2008). One of the most important causes of this obstacle to continuous cropping is the toxic effect of phenolic allelochemicals in the soil (Hao et al., 2010). 4-HBA is a common and typical phenolic allelochemical that is released into the environment by plants through foliar leaching and residue decomposition. Nie et al. (2007) reported that the addition of 4-HBA significantly impaired watermelon seed germination and seedling growth. Previous studies have shown that 4-HBA can reduce the ability of plant roots to absorb ions, induce depolarization of plant cell membranes, and lead to increased membrane permeability to cations and anions (Baziramakenga et al., 1995; Yu and Matsui, 1997). In addition, 4-HBA also affects the activity of plant defense enzymes and the synthesis of hormones and proteins (Wang et al., 2009). Therefore, the removal of this compound from soil is likely very important for crop growth.

Endophytic fungi live within the inner tissues of plants without causing visible symptoms of disease (Aly et al., 2010). In recent years, an increasing number of studies have shifted from examining the ecological functions of endophytic fungi *in vivo* toward *in vitro* experiments (Borges et al., 2009; Chen et al., 2013; Russell et al., 2011). The recently characterized novel fungal endophyte strain *C. stevensii* B6, which was isolated from the inner bark of the stem of *Bischofia polycarpa*, could decompose 89.51% of phenanthrene (a polycyclic aromatic hydrocarbon) within 10 days (Dai et al., 2010). Thus, it may be assumed that these endophytic fungi have the potential to degrade single cyclic molecules such as 4-HBA, which have simpler structures than PAH.

Therefore, the objectives of the present study were to investigate the ability of *C. stevensii* B6 to degrade 4-HBA as a sole carbon source *in vitro,* determine the optimal conditions of 4-HBA degradation, and study the effect of inoculation with *C. stevensii* B6 on the germination of watermelon seeds.

MATERIALS AND METHODS

Strain, materials and reagents

Ceratobasidium stevensii B6 was isolated from the stem endothelium of *Bischofia polycarpa* at Jiangsu Key Laboratory for Microbes and Functional Genomics (Dai et al., 2006). The endophyte was maintained on potato dextrose agar (PDA) slants at 4°C.

Soil samples were collected from layers 0 to 20 cm in depth from monocropped watermelon fields in Yongzhou, Hunan province (N26°45′, E111°53′); Yingtan, Jiangxi province (N28°04′, E117°09′); and Nanjing, Jiangsu province (N32°16′, E118°79′). Table 1 shows the physical and chemical properties of the soils. Soil pH was measured in a 1:2.5 (v/v) soil to water ratio using a DMP-2 mV/pH detector (Quark Ltd, Nanjing, China). Soil organic matter was analyzed by wet digestion with H_2SO_4–$K_2Cr_2O_7$, and total nitrogen was determined by the semi-micro Kjeldahl method. Available nitrogen in soil was determined using an alkaline hydrolysis method. The available phosphorous in soil was extracted with 0.05 M $NaHCO_3$, and the absorbance at 700 nm was detected on a spectrophotometer. The available potassium in soil was extracted with 1.0 mol/L ammonium acetate (NH_4OAc) and measured using emission flame spectrometry. Soil texture was determined by the pipette method (Lu, 2000).

4-hydroxybenzoic acid (4-HBA) was obtained from Sigma-Aldrich. Methanol, acetonitrile, and glacial acetic acid were all HPLC-grade. All other reagents used were of analytical grade. The variety of watermelon seed (*Citrullus lanatus*) was Black Beauty.

Effect of different 4-HBA concentrations on the germination of watermelon seeds

Five concentrations of 4-HBA, 0, 125, 250, 375, and 500 mg/L, were used in the present experiment. Watermelon seeds were sterilized for 10 min in 4% NaOCl and rinsed three times with sterile distilled water. For each treatment, twenty seeds were placed in Petri dishes containing filter paper, with 3 replicates. Seed germination was recorded after 3 days in the dark at 28°C.

Degradation of 4-HBA by B6

C. stevensii was first activated in potato dextrose broth (PDB: peeled potato extract 200 g/L, glucose 20 g/L, pH 7.0) in a shaker at 180 rpm and 28°C for five days. Five milliliters of the active

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fungal suspension (containing dry fungal hyphae at approximately 3.77 g/L) was then used to prepare fungal inoculum by culturing for 2 days at 28°C at 180 rpm in 100 mL of 4-HBA degradation medium (peeled potato extract 10 g/L, glucose 15 g/L, $NaNO_3$ 1.5 g/L, $K_2HPO_4 \cdot 3H_2O$ 1.3 g/L, KCl 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $FeSO_4 \cdot 7H_2O$ 0.02 g/L, 4-HBA 500 mg/L, pH 7.5), using 250 mL Erlenmeyer conical flasks.

Five milliliters of inoculum containing fungal mycelium at 0.57 g/L was added to the 4-HBA degradation test medium described above; kept at 28°C; and shaken at 180 rpm for 0, 1, 2, 3, 4, 5, 6, and 7 days. The fermentation broth was then filtered through filter paper. The fungal mycelia were extracted by stirring with 20 mL of methanol (25°C at 150 rpm) for 1 h and then filtered. The 4-HBA adsorbed on the mycelium was dissolved in methanol, and extracts were evaporated at 30°C to dryness and redissolved in 1 mL of water: methanol (80:20 v/v). The residue was filtered through a 0.45 µm membrane. The amount of 4-HBA adsorbed on the mycelium (w1) was determined by high-performance liquid chromatography (HPLC) (Reis et al., 2011). At the same time, 1 mL of broth (without fungal hyphae) was filtered through a 0.45 µm membrane, and the amount of 4-HBA remaining in the broth was determined (w2). The total amount of 4-HBA remained was calculated as w1 + w2. Another bottle of fermentation broth was also filtered, and the mycelium was washed 3 times with double-distilled water (ddH_2O). The mycelium was dried at 60°C to constant weight to measure the biomass of *C. stevensii* B6. Blank control cultures without the fungus and inactivated controls with inactivated B6 fungal mycelium were processed in an identical manner. Each treatment was repeated 3 times.

4-HBA degradation test

Several ecological factors were used to investigate the influence of culturing conditions on 4-HBA degradation. We used temperatures of 18, 23, 28 and 33°C. Shaking speeds were 120, 150, 180 and 210 rpm. The initial pH values of the culture solutions were adjusted to 4.5, 5.5, 6.5, 7.5 and 8.5 with HCl (0.2 M) or NaOH (0.2 M). Starch, fructose, glucose, cellulose, and sucrose were used to test different carbon sources. Glucose was utilized as the carbon source to explore the effects of different concentrations of carbon on the degradation of 4-HBA. Finally, inoculation quantities of 5, 10, 15, 20 and 25% (v/v) were applied to test the effect of inoculation concentration.

Simulation of conditions under which *C. stevensii* B6 degrades 4-HBA in soil

Soil samples were dried in an electric dryer at 40°C for 12 h and then passed through a 0.85 mm sieve. 4-HBA, dissolved in water, was added to the dried soil (500 mg/kg). This mixture was placed in pots in the botanical garden at Nanjing Normal University and aged for 2 months (Hu et al., 2011). This period is long enough to allow natural equilibration of the various sorptions in the soil. During the equilibration, soil water was maintained 80% maximum water holding capacity by adding water and weighing the pots on a weekly basis. After undergoing fermentation for 7 days, broth containing *C. stevensii* B6 mycelium was passed through 4 layers of gauze. Mycelia were washed 3 times with ddH_2O. Wet fungal mycelia (20.0 g, equivalent to 0.7 g dry fungal weight) were suspended in 20 mL ddH_2O and mixed with 200 g of dry soil containing residual 4-HBA. The treated soil samples from Jiangxi, Hunan, and Jiangsu were identified as J-B6, H-B6, and Z-B6, respectively. Twenty grams of wet inactivated mycelia (121°C, 20 min) and 20 ml of ddH_2O were mixed with 200 g of dried soil as the inactive control (MB6) and blank control (CK), respectively. Therefore, nine treatments were designed: J-CK, J-MB6, J-B6, H-CK, H-MB6, H-B6, Z-CK, Z-MB6, and Z-B6. Mycelia and soil mixtures were put into 250 ml Erlenmeyer flasks. Each treatment was repeated 3 times. Soils were kept in an incubator in the dark at 33°C, and ddH_2O was added to keep the soil moist (Zhang et al., 2010).

Twenty-five fresh soil samples were taken from each flask to determine the amount of residual 4-HBA using HPLC at 0, 1, 2, 3, 4, 5, 6 and 7 d. Soil phenolic compounds were extracted from soils based on the method described by Macias et al. (2004) and determined by HPLC.

Determination of the amount of residual 4-HBA using HPLC

The amount of residual 4-HBA was analyzed using high-performance liquid chromatography (HPLC) (Agilent 1100). The chromatography conditions were as follows: ultraviolet detector, 260 nm; flow rate, 1 mL/min; mobile phase, acetonitrile:1.3% glacial acetic acid solution (17:83). The chromatography column was a Venusil MP C18 with an internal diameter of 4.6 mm and length of 250 mm (Agela Technologies Inc.). The injection volume was 20 µL. The column temperature was 35°C. Samples were filtered through a 0.45 µm membrane prior to HPLC analysis and were diluted to concentrations proper for interpolation within their corresponding standard curves. The levels of 4-HBA were determined using the corresponding peak area. 4-HBA in culture solution and soil extracts was identified by its retention time and co-injection tests with 4-HBA standard compounds. The recovery rate of 4-HBA was 97.6% in pure solution.

Seedling growth test

Watermelon seeds were germinated in ddH_2O for 48 h. Uniform germinated seeds were transferred to soil to simulate the conditions of *C. stevensii* B6 degradation of 4-HBA in soil over 7 days. Soil from Hunan with no added 4-HBA or *C. stevensii* B6 was used as a control (CK) (the background value of 4-HBA was 2.53 ± 0.01 mg/kg). The lengths of leaves and roots were measured after one month's growth in an artificial climate chamber (temperature 24 ± 1°C, light intensity 2000–2500 LX, illumination time 16 h/day).

Statistical analysis

Data were analyzed using Microsoft Excel. Values are the means of 3 replicates (mean ± SD) for each treatment. One-way ANOVA was used to detect significant differences between treatments, and multiple comparisons were performed using Duncan's multiple range tests with SPSS version 13.0 (Chicago, IL, USA). Differences at P=0.05 were considered significant.

RESULTS

With increased concentration of 4-HBA, the germination rate of watermelon seeds decreased significantly (Figure 1). When the concentration of 4-HBA reached 500 mg/L, the seed germination of watermelon was 100% inhibited. Therefore, the initial concentration of 4-HBA in the liquid media was set to 500 mg/L.

As shown in Figure 2, CK and sterilized *C. stevensii* B6 could hardly utilize 4-HBA, while residual 4-HBA could hardly be detected in active B6 culture solution after 6 days (Figure 3). We therefore chose to detect 4-HBA

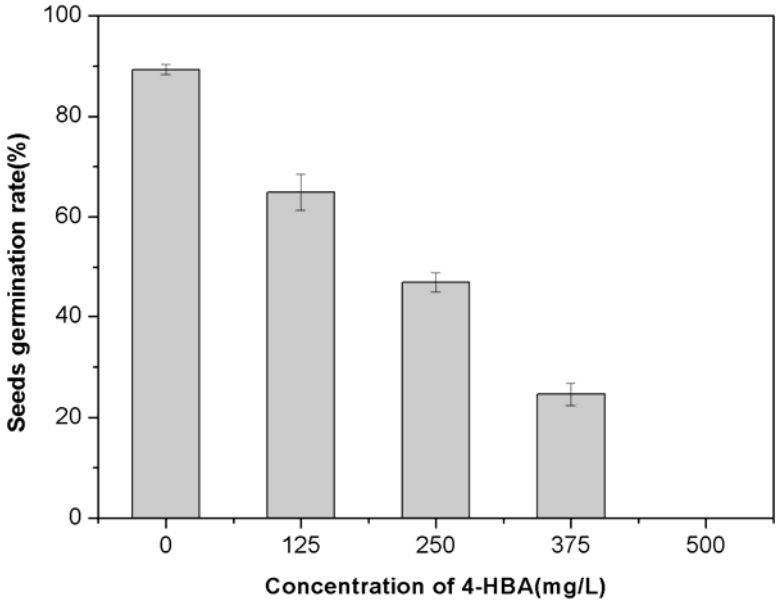

Figure 1. Effect of different 4-HBA concentrations on the germination of watermelon seeds. Each value is the mean ±SD of three replicates.

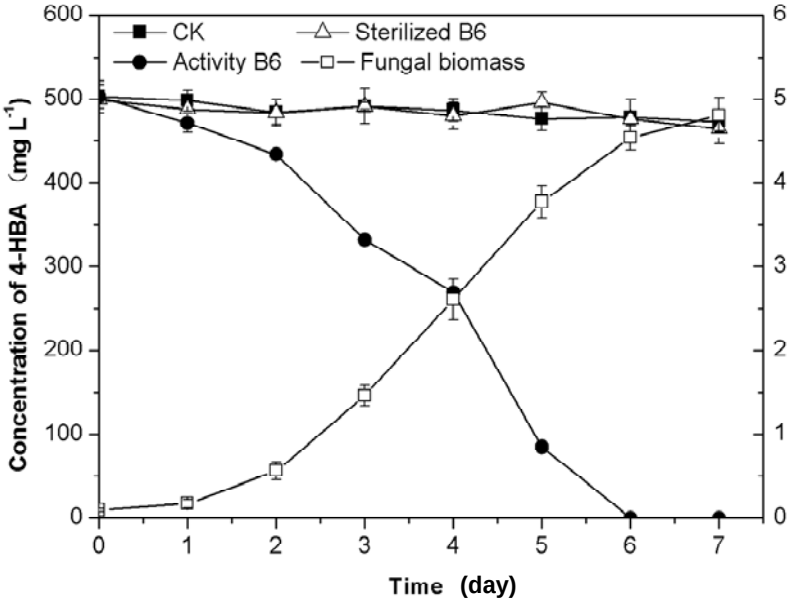

Figure 2. Effect of time on the degradation of 4-HBA. The initial concentration of 4-HBA was 500 mg/L. CK: five milliliters of sterile water was added to 100 mL of culture solution. Sterilized B6: five milliliters of inactive seed culture solution (121°C, 20 min) was added to 100 mL of culture solution. B6: five milliliters of active seed culture solution was added to 100 mL of culture solution. Each value is the mean ± SD of three replicates.

within one week in subsequent 4-HBA degradation experiments.

4-HBA was decomposed by B6 in all temperature tests, and its degradation efficiency increased as the temperature increased from 18 to 28°C. The degradation of 4-HBA was slower at 33°C than that at other temperatures, but the concentration of 4-HBA was still below the level of detection (1 mg/L) on the fifth day (Figure 4a). Initial pH significantly affected the degradation of 4-HBA. Degradation efficiency increased

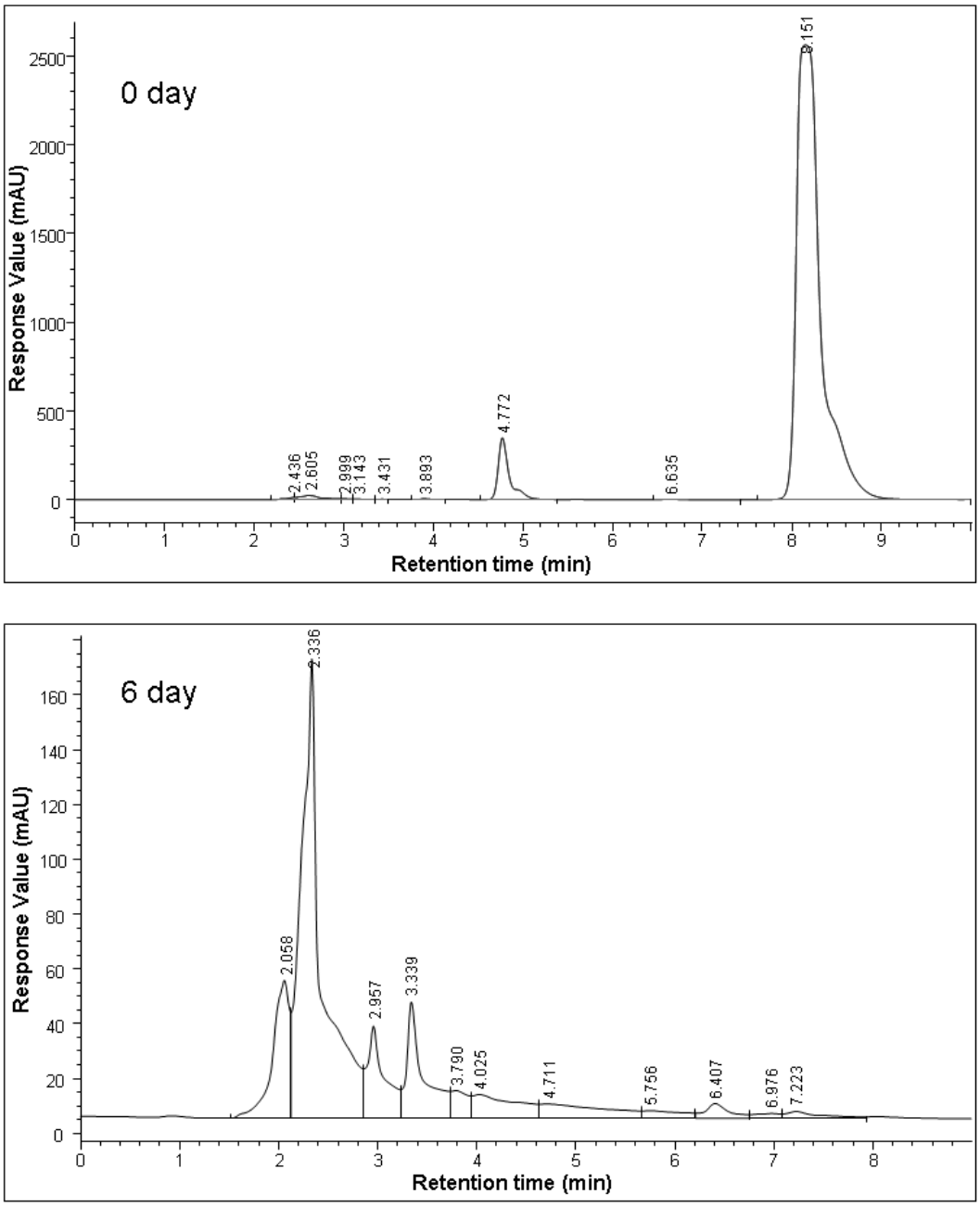

Figure 3. HPLC chromatogram of 4-HBA degradation. The retention time of 4-HBA was 8.151 min. Samples for each culture solution were collected 0, 1, 2, 3, 4, 5, 6, and 7 days after inoculation. Chromatograms at 0 and 6 days are presented here.

as the initial pH decreased from 8.5 to 5.5 (Figure 4b). pH below 5.5 was found to adversely affect degradation. When the shaking speed was 150 rpm, the degradation efficiency was higher than at 120, 180, or 210 rpm (Figure 4c). *C. stevensii* B6 could not utilize cellulose, and 4-HBA reductions over the first 3 days may have resulted from the residual glucose in seed culture solution. Other carbon sources, and glucose in particular, were suitable for 4-HBA degradation. With glucose as a carbon source, the mass of residual 4-HBA was only 153.58 mg, which was the fastest degradation among all treatments over 5 days (Figure 4d). The degradation efficiency depended on glucose concentration. A higher glucose concentration corresponded to faster decomposition of 4-HBA (Figure 4e). The inoculation quantity was an important factor in the degradation of 4-HBA. 4-HBA decomposed best when the inoculation density was 20 or 25% (v/v). When the inoculum was

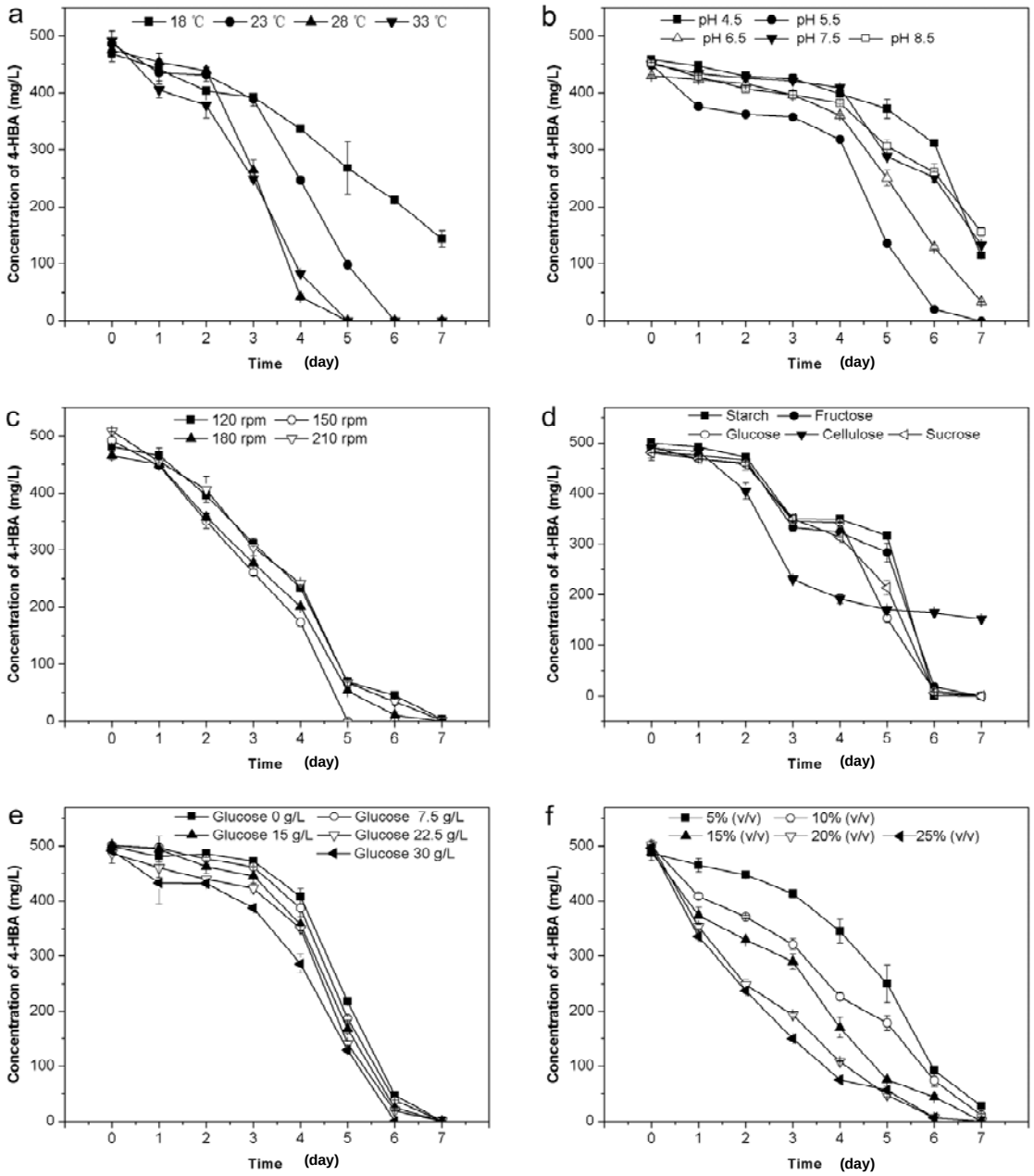

Figure 4 Effect of single factors on the degradation of 4-HBA. The initial concentration of 4-HBA was 500 mg/L. (a) Temperature; (b) Initial pH; (c) Shaking speed; (d) Carbon source; (e) Concentration of glucose; (f) Inoculation amount. Each value is the mean ± SD of three replicates.

below 15%, the degradation rate was relatively slow for the first 6 days; however, by the 7th day, the differences between treatments were small (Figure 4f).

The 4-HBA content in the soil decreased from 500 to 4-5 mg/kg in dried soil after it was aged for 2 months. This decrease may have been due to soil adsorption and transformation by indigenous microorganisms after 2 months in the natural environment (Hu et al., 2011). The 4-HBA levels in the control (CK) and MB6 were barely reduced in 3 soil tests (Figure 5a-c). However, 4-HBA could not be detected (≤ 1 mg/kg dry soil) following *C. stevensii* B6 treatment over 7 days.

The inhibition of stems and leaf elongation by 4-HBA was reversed by *C. stevensii* B6 treatments. The stem lengths in H-B6, J-B6, and Z-B6 increased by 62.6, 23.7 and 66.2%, respectively, compared with the control. The leaf lengths in H-B6, J-B6, and Z-B6 increased by 30.0, 10.0, and 50.0%, respectively, compared with the control. Compared with CK, H-B6 significantly reversed the 4-HBA inhibition of watermelon growth, and root, stem, and

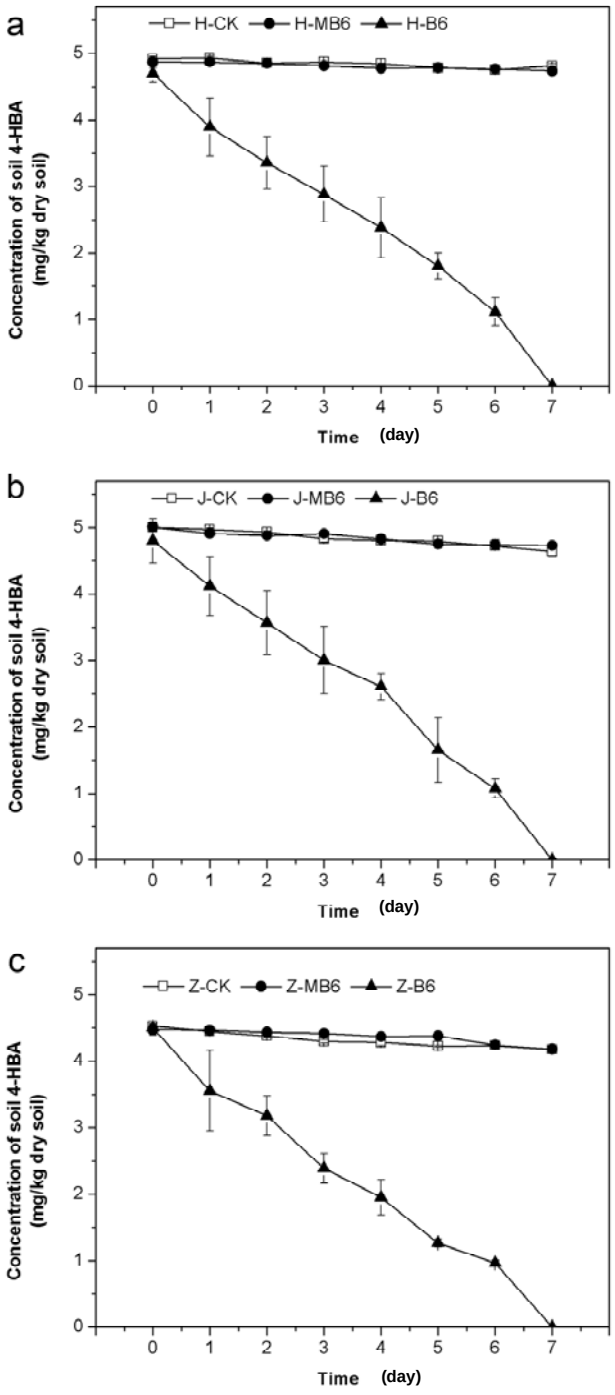

Figure 5. Effect of soil type on the degradation of 4-HBA. Each soil sample contained 500 mg/kg of 4-HBA. (a) Soil from Hunan Province; (b) Soil from Jiangxi Province; (c) Soil from Jiangsu Province. CK: control; MB6: inactivated B6; B6: activated B6.

leaf growth increased by 19.2, 62.6, and 30.0%, respectively. With the addition of sterilized B6 mycelium, the growth states were the same as in the controls (Figure 6).

DISCUSSION

Phenolic acids are typical self-toxic allelochemicals secreted by plant roots. 4-HBA is a phytotoxic allelochemical in plant root exudates that accumulates in the cytosol and cell walls of plants and in root exudates and plant residues released into soils (Sircar et al., 2007; Chen et al., 2008; Sircar and Mitra, 2008). Some phenolic acids used as carbon sources are able to stimulate the production of spores in *F. oxysporum* (Wu et al., 2009). We found that addition of *C. stevensii* B6 to the soil decreased the number of *F. oxysporum* colony forming units (CFUs), and the DNA of B6 could be detected in soil after 4 weeks (Xiao et al., 2012 a,b). Disease caused by *F. oxysporum* was the main factor leading to cropping obstacles (Wu et al., 2008; Wu et al., 2010 a,b). We observed in this study that C. *stevensii* B6 effectively degraded 4-HBA and mitigated its toxicity to watermelon. This degradation is the probable mechanism by which *C. stevensii* lessens the obstacles to continuous cropping.

In all three soils, *C. stevensii* B6 decomposed nearly 100% of the available 4-HBA within 7 days, much more efficiently than *Trichoderma harzianum,* which was shown to result in only 88% degradation within 8 days (Chen et al., 2011), suggesting that the endophytic fungus *C. stevensii* B6 could adapt to the soil environment and promote the degradation of 4-HBA. The concentration of 4-HBA in the control soil also decreased, indicating that other microbes in soil had the potential to utilize 4-HBA. Middelhoven et al. (1992) and Zhang et al. (2010) have screened *Pseudomonas* sp. and ascomycetous yeast strains from soil that effectively degrades phenolic compounds. However, in our study, the indigenous soil microorganisms did not completely degrade 4-HBA after 2 months, and the residual concentration of 4-HBA in the non-bioaugmented soil microcosms remained relatively high.

Creating conditions appropriate for the growth of *C. stevensii* in agricultural settings is very important. The degradation rate is likely to accelerate as temperatures rise. The maintenance of soil pH at approximately 5.5 would be suitable for the growth of *C. stevensii*, similar to the ideal pH for the rot fungus *Phanerochaete chrysosporium* (Bhattacharya et al., 2013). Therefore, if B6 were applied to acidic soil in subtropical China, the use of an alkaline fertilizer would be beneficial to adjust soil pH. In addition, as this is an aerobic fungus, moderate tillage during watermelon planting is also advisable to maintain soil oxygen. *C. stevensii* would be able to take advantage of starch, fructose, glucose, and sucrose as carbon sources. Watermelon root exudates are rich in carbohydrates (Hao et al., 2010) and conducive to the survival of *C. stevensii* in the soil. *C. stevensii* colonization of soil may be used to resolve 4-HBA. Increasing soil organic matter by the application of organic fertilizers would enhance the degradation of 4–HBA by *C. stevensii*. Inoculum size is best maintained at

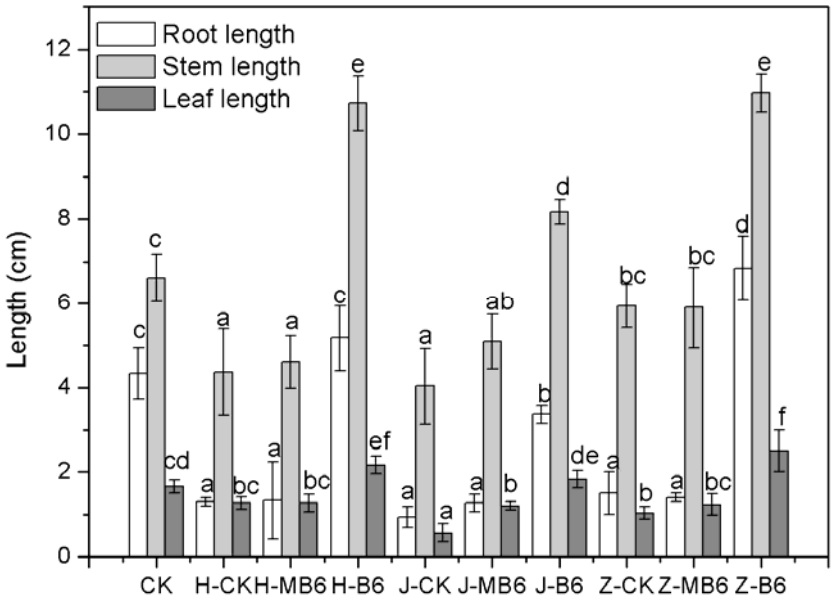

Figure 6. Effect of *C. stevensii* B6 on seedling growth inhibition by 4-HBA. The different letters represent the significance between pairs of mean values at P≤0.05 according to a Duncan test.

15% (v/v, volume of inoculation seed liquid to volume of the soil). In terms of cost, it should be noted that a 5% inoculum was sufficient after one week.

Following placement in the natural environment for 2 months, the original 4-HBA content in the soil decreased from 500 mg/kg to 4.92 mg/kg. This decrease may have been due to soil adsorption and transformation by indigenous microorganisms (Middelhoven et al., 1992; Zhang et al., 2010). Even after monocropping peanuts for 15 years, the 4-HBA content in the soil was only 2.76 mg/kg (Li et al., 2010). When the 4-HBA concentration reaches 4.92 mg/kg, the allelopathic effects on crop growth are likely to be very serious (Li et al., 2010). In nature, phenolic acids usually mix together, and the toxicity of the mixed chemicals is more significant than that of single chemicals such as 4-HBA (Li et al., 2010). In addition, although the level of phenolic acids in soils is not very high, it may be high in partial rhizosphere soil and may thus be toxic for plant growth. The degradation of 4-HBA from 4.92 mg/kg to zero is of key importance to agriculture.

In our experiment, the dry mycelial biomass applied to the soil reached 3.55 g/kg dry soil. In the field conditions, watermelons are planted in holes, and each hole contains approximately 15 kg of soil. If the watermelon line spacing is 2 m and the plant spacing is 1 m, the amount of dry mycelium needed is approximately 266 kg/ha. In the future, strategies to improve the adaptation of *C. stevensii* to soil conditions should be considered to further improve degradation efficiency and reduce microbial dosage. In addition, the efficiency of a *C. stevensii* symbiotic host in facilitating the degradation of 4-HBA warrants future investigation.

Conflict of interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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