

Research Article

Evaluation of the Biocontrol Potential of Endophytic Fungus *Fusarium solani* CEF559 against *Verticillium dahliae* in Cotton Plant

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Received 2 September 2019; Revised 15 November 2019; Accepted 4 December 2019

Academic Editor: Atanas Atanassov

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Verticillium wilt of cotton, caused by the soilborne pathogen *Verticillium dahliae*, is one of the most serious diseases of cotton worldwide. Increased concerns about the side effects of synthetic pesticides have resulted in greater interest in developing biocontrol strategies against Verticillium wilt. We evaluated a *Fusarium solani* CEF559 isolate, obtained from the endosphere of healthy cotton plants, for its biocontrol potential against *V. dahliae* *in vitro* and *in vivo*. In addition to disease assessment, three key genes in the lignin metabolism pathway and four pathogenesis-related (PR) genes were monitored using qRT-PCR. In the laboratory tests, *F. solani* CEF559 inhibited *V. dahliae* colony growth by 75% and sporulation by nearly 80% and completely suppressed conidial production. However, volatile metabolites from CEF559 did not affect *V. dahliae* colony growth. In the greenhouse study, CEF559 significantly reduced wilt development, with a control efficacy greater than 60% when assessed 25 days postinoculation. In a field experiment, CEF559 reduced wilt development, with the efficacy ranging from 30.1% to 56.3%. PR genes and those key genes in the lignin metabolism pathway were transiently upregulated in the cotton roots pretreated with CEF559 when subsequently inoculated with *V. dahliae*, compared with those plants inoculated with *V. dahliae* only. Moreover, CEF559 inhibited *V. dahliae* colonisation of both the roots and hypocotyls. The present results suggest that this cotton endophytic fungal strain, *F. solani* CEF559, confers protection against *V. dahliae*.

1. Introduction

Verticillium dahliae Kleb. is a soilborne plant pathogenic fungus causing wilt disease on more than 200 plant species [1, 2], including cotton (*Gossypium hirsutum* L.). Yield losses in affected cotton plants were estimated to be around 80% in *V. dahliae*-infested soil [1]. The pathogen can survive in the soil as melanized microsclerotia without a host plant for at least 14 years [3]. Incidence of Verticillium wilt of cotton increases with increasing soil inoculum density [4]. Controlling Verticillium wilt in cotton is difficult because of the inaccessibility of *V. dahliae* propagules during the infection by usual control agents, long-term survival of pathogen

propagules in the field, and its broad host range [1, 2, 5]. Soil fumigation with pesticides has been an indispensable tool for controlling soilborne pathogens; however, several of these fumigants are already banned under the Montreal Protocol [6]. The remaining fumigants (e.g., chloropicrin and dazomet) are expensive and face an uncertain future due to legislations restricting their use.

Over the last three decades, many nonpesticide strategies have been evaluated and used to control Verticillium wilt, including crop rotation, biofumigation, soil amendment with green manures or organic materials, anaerobic soil disinfestation, and soil solarization [5, 6]. However, all these methods have their own specific limitations [5]. For

example, crop rotation with nonhosts of *V. dahliae* has not been adopted in the Xinjiang province (the main cotton production region) in China—due to the difficulties in changing cropping systems and saline-alkaline soils. The control efficacy achieved with biofumigation in naturally infested soil was variable and often low because of the low rate of releasing 2-propenyl isothiocyanate [7].

Using biological control agents (BCAs) to manage soilborne diseases is another alternative to chemical control [5]. Microbial-based products have been used commercially in agriculture for over 120 years, but only recently they have received increased attention due to their potential in providing environmentally safe disease control [8]. Biocontrol using antagonists to control Verticillium wilt has been practiced worldwide, such as the use of *Bacillus* spp. [9, 10], *Pseudomonas fluorescens* [9], *Streptomyces plicatus* [11], nonpathogenic *Fusarium oxysporum* [12], *Piriformospora indica* [13], *Trichoderma* spp. [14], *Purpureocillium lilacinum* [15], and *Paenibacillus alvei* [16]. However, examples of successful biocontrol products against soilborne diseases in commercial crop production under open field conditions are limited [17]. Although antagonistic interactions among BCAs are more likely to occur than synergistic interactions, the efficacy of combined use of BCAs is expected to be greater than individual components [18]. Therefore, applying multiple BCAs in the same niche with multiple functional attributes may increase biocontrol efficacies over the use of single biocontrol products.

Plant endophytes inhabit living tissue of plants and do not cause any apparent or detectable symptoms in the host. They include fungi, bacteria, and actinomycetes [19]. Host plants may sometimes benefit from coexisting endophytes that provide resistance to biotic and abiotic stresses [20, 21]. For example, some endophytes are able to inhibit pathogen growth through antibiosis, competition, and parasitism [22] or by induced systemic resistance [23]. We isolated 642 endophytic fungal strains from healthy cotton plants [24] and demonstrated that *Penicillium simplicissimum*, *Lep-tosphaeria* sp., *Talaromyces flavus*, and *Acremonium* sp. can reduce wilt development in cotton [25].

Nonpathogenic *Fusarium* spp. are gaining interest in agriculture as BCAs to control soilborne plant pathogens [12,26–30]. However, there is little information about using *Fusarium* spp. as a BCA against cotton Verticillium wilt. Thus, we carried out investigations on both *in vitro* and *in vivo* biocontrol potential of a cotton endophyte isolate of *F. solani* (CEF559) against cotton wilt. Furthermore, we studied the expression of pathogenesis-related genes and key genes in the lignin metabolism pathway in cotton against *V. dahliae* following inoculation with *F. solani* CEF559.

2. Materials and Methods

2.1. Fungal Isolates. A *Fusarium solani* strain, CEF559, was an endophyte, isolated from a healthy cotton plant and identified by the rDNA ITS sequences [24] and complementary elongation factor 1- α gene (GenBank accession number: KU382502). A highly aggressive defoliating isolate

of *V. dahliae* Vd080 isolated from a cotton plant was used in this study [31].

2.1.1. Preparation of Fungal Liquid Culture. Both *V. dahliae* Vd080 and *F. solani* CEF559 were cultured separately in liquid Czapek-Dox medium for 6 days at 25°C in a shaker at 150 rpm. Conidia were harvested from the liquid medium and resuspended to approximately 1×10^7 spores·mL⁻¹ in sterile distilled water for further experimentation.

2.1.2. Preparation of Fungal Solid Culture. An 800 mL Claisen flask containing broken maize-sand medium (vol/vol = 1:1) was steam-sterilized at 121 kPa for 45 min and then inoculated with 10 mL spore suspension of *F. solani* CEF559. The flask was then incubated at 25°C for 7 days and shaken vigorously by hand for 2 min every day. The dried maize-sand culture was grounded to particles of 1~2 mm in size and stored at 4°C until further use.

2.1.3. Preparation of Cell-Free Culture Filtrate

F. solani CEF559 agar-mycelium disks (5 mm diameter), taken from the edge of an actively growing fungal colony, were inoculated to a 500 mL Erlenmeyer flask containing 300 mL liquid Czapek-Dox medium, and the culture was maintained at 25°C on a shaker incubator at 150 rpm for 5 days. The crude culture filtrate was filtered with three layers of filter paper, and the filtrate was filter-sterilized through a 0.2 μ m millipore filter [32].

2.2. Evaluation of the Antagonistic Activity of *F. solani* CEF559

2.2.1. Dual Culture Assay. Antifungal assay was performed in 9 cm Petri dishes, each containing 20 mL PDA medium. A 5 mm diameter Vd080 mycelial disk was placed at the center of a PDA plate, and four 5 mm diameter mycelial disks of *F. solani* CEF559 were placed next to the Vd080 mycelial disk (south, west, east, and north, 25 mm from the Vd080 mycelial disk). The plate was then immediately sealed with plastic film and incubated in the dark at 25°C for 14 days. *V. dahliae* colony diameter was measured in two directions daily. A plate inoculated with *V. dahliae* alone served as the control. There were three replicate plates, and the experiment was repeated twice. The percentage of growth inhibition (IR) 6 days after inoculation was calculated as follows: IR (%) = [(control colony diameter – treatment colony diameter)/control colony diameter] \times 100.

2.2.2. Nonvolatile Metabolite Inhibitory Assay. Production of nonvolatile metabolites was estimated by placing a 5 mm CEF559 mycelial disc centrally on two layers of sterilized cellophane covering PDA medium. The plates were incubated for 6 days at 25°C before the two layers of cellophane, CEF559 cultures were removed, and a 5 mm disc of *V. dahliae* Vd080 was placed at the center. The control plates were only inoculated with *V. dahliae* Vd080. The dishes were incubated for a further 12 days. The percentage of growth

inhibition after inoculated with *V. dahliae* Vd080 was calculated as described above. There were three replicate plates, and the experiment was repeated twice.

2.2.3. Volatile Metabolite Inhibitory Bioassay. Inhibition of *V. dahliae* mycelial growth by volatiles of CEF559 was tested using the double-dish method [33]. A mycelial agar plug of CEF559 was placed onto PDA in a Petri dish and incubated at 25°C for 7 days. Another fresh PDA Petri dish containing a mycelial agar plug (5 mm diameter) of *V. dahliae* Vd080 was placed inversely over the Petri dish containing the 7-day-old culture of CEF559; this double-dish set was immediately sealed with parafilm. In the control treatment, a PDA Petri dish inoculated with *V. dahliae* Vd080 was placed inversely over another Petri dish containing PDA but without CEF559 to make a double-dish set. There were three double-dish sets serving as three replicates. Diameter of the *V. dahliae* Vd080 colony in each double-dish set was measured 7 days after incubation at 25°C, and the percentage of growth inhibition was calculated using the method as described above. The experiment was performed three times.

2.2.4. Antifungal Activity of *F. solani* CEF559 Filtrate on Sporulation and Spore Germination of *V. dahliae*. Cell-free culture filtrate of CEF559 (20 mL) was added to a 50 mL sterile Erlenmeyer flask, and then 100 µL spore suspension (1×10^7 spores·mL⁻¹) of Vd080 cultured in liquid Czapek-Dox medium was added. The same volumes of liquid Czapek-Dox medium and spore suspension of Vd080 were added to another flask as the control. The flasks were shaken at 150 rpm for 4 days at room temperature (25°C). Spore concentrations were estimated with a hemocytometer on days 4 and 6. This test was repeated twice.

2.2.5. Antifungal Activity of *F. solani* CEF559 Metabolites on Spore Germination of *V. dahliae*

V. dahliae Vd080 spores were harvested 5 days post-inoculation (dpi) in liquid Czapek-Dox medium. Conidia were purified using a previously published method [24]. Spore concentration was adjusted to 2×10^3 spores·mL⁻¹; 100 µL of the spore suspension was evenly spread onto a PDA plate. In order to determine the effect of high temperature on the activity of *F. solani* CEF559 metabolites, a PDA plate with metabolites of *F. solani* CEF559 was autoclaved at 121°C for 20 min and then inoculated with *V. dahliae* Vd080. The control plates were only inoculated with *V. dahliae* Vd080. Then, plates were maintained at 25°C and germination was assessed under a microscope 3 dpi. Germination inhibition was calculated as follows: GIR = (germination in control – germination in the CEF559 treatment)/germination in control × 100%. There were three replicate plates, and the experiment was repeated twice.

2.2.6. Antifungal Activity of Crude Protein of CEF559 on *V. dahliae*. The crude protein was precipitated from cell-free culture broth supernatant with 60% saturated ammonium

sulphate (w/v) that was stirred overnight at 4°C to allow protein precipitation. The precipitated proteins were pelleted by centrifugation at 6000 rpm for 30 min at 4°C, dissolved in a 1/10 (v/v) phosphate buffer (0.2 M, pH 7.0), and dialysed for 12 h to remove ammonium sulphate. Some of the crude proteins were dissolved in phosphate buffer (0.2 M, pH 7.0), and their antifungal activity was tested against *V. dahliae*. The PDA plate was spread inoculated with *V. dahliae* Vd080 spore suspension, and then two wells were drilled symmetrically. The phosphate buffer saline (PBS) containing proteins (2 mg·mL⁻¹) was added to one well, and the PBS-treated hole as the control. The plates were then incubated at 25°C for 3 days. There were three replicate plates, and the experiment was repeated twice.

2.3. Suppressive Effect of CEF559 on *V. dahliae* in Greenhouse. The efficacy of CEF559 spore suppression against *V. dahliae* was studied in a greenhouse. Plants were inoculated with CEF559 via either liquid or solid culture inoculation.

2.3.1. Liquid Culture Inoculation. Seeds of cv. Jimian11 (highly susceptible to *V. dahliae*) were surface-disinfected in 1% NaClO solution for 5 min and rinsed three times with sterile distilled water and then dried and sown in paper pots (6 cm in diameter and 10 cm in height, made of newspaper) filled with autoclaved substrate (vermiculite/sand [V: V] = 3:2) [25]. The pots were placed on a plastic tray (35 × 45 cm). Two weeks after sowing, each pot was thinned to leave five plants. The roots of cotton plants were inoculated with *F. solani* CEF559 (1×10^7 spores·mL⁻¹, 50 mL/pot) after the first true leaves presented. Four days after inoculation with CEF559, the plants were inoculated with spore suspension of *V. dahliae* strain Vd080 (1×10^7 spores·mL⁻¹, 10 mL/pot). The same volume of liquid Czapek-Dox medium instead of CEF559 spore suspension was used in the control treatment.

2.3.2. Solid Culture Inoculation. The solid culture medium of CEF559 (1×10^8 spores·g⁻¹) was mixed with the sterile vermiculite and sand (V: V = 3:2) in a ratio of 2:98 (V: V) [25]. Cotton seedlings with one true leaf were inoculated with *V. dahliae* Vd080 spore suspension (1×10^7 spores·mL⁻¹) following a published method [34].

There were six pots for each treatment, each with 3~5 seedlings. Seedlings from both the liquid and solid culture inoculation experiments were maintained in a greenhouse under a 12 h/12 h day/night cycle at 25~28°C. Disease severity of each seedling was recorded 21 days dpi on the following five categories: 0 = healthy, no symptoms on leaves; 1 = one or two cotyledon leaves showing symptoms; 2 = a single true leaf showing symptoms; 3 = more than two leaves showing symptoms; and 4 = plant dead. An overall disease index for each treatment was estimated as follows: disease index = $[(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4)/4n] \times 100$, where n_0 – n_4 were the numbers of plants with each of the corresponding disease

scores, and n was the total number of plants assessed for each treatment.

The disease control efficacy was then estimated as follows: efficacy (%) = [disease index_(control) - disease index_(treatment)] / disease index_(control) × 100. Each experiment was repeated twice.

2.4. Effect of *F. solani* CEF559 on Cotton Emergence and Biomass under Greenhouse Conditions. The effect of *F. solani* CEF559 on cotton emergence and biomass was evaluated in a greenhouse. Paper pots were inoculated with CEF559 solid culture as described above, and the control was mock-inoculated with maize-sand medium. After sterilization for 5 min in a 1% NaClO solution, seeds of cotton cv. Jimian11 were rinsed three times in sterile distilled water and air-dried in a flow cabinet. For each treatment, there were six pots, each with five seedlings. Five days after sowing, the numbers of cotton seedlings in each pot were counted and thereafter every 2 days until no more emergence. Plant height, root length, and fresh weight were measured 20 days after sowing. The assay was repeated twice.

2.5. Quantification of *V. dahliae* in Cotton Roots and Hypocotyls. To quantify pathogen colonisation level in roots and hypocotyls following CEF559 treatments, *V. dahliae* biomass in cotton plants was estimated by quantitative PCR (qPCR). Ten (cv. Jimian11) seedlings were harvested 21 dpi with *V. dahliae* Vd080 for each of the following three treatments of liquid culture inoculation: (i) root inoculated with 50 mL CEF559 cell-free culture and inoculated with 10 mL spore suspensions of Vd080 4 days later; (ii) root inoculated with Vd080 and inoculated with CEF559 4 days later; and (iii) root inoculated with Vd080 alone. DNA was extracted from roots and hypocotyls of all 11 plants using the CTAB method and quantified by NanoDrop 2000. For each sample, 200 ng of DNA was used in the qPCR with the primer pair Vd-F (CCGCCGGTCCATCAGTCTCTCTGTTTATAC)/Vd-R (CGCCTGCGGGACTCCGATGCGAGCTGTAAC) [35]. The cotton *ubiquitin* gene was used for normalization with the primer ubiquitin-F/R (Table 1). The experiment was repeated twice.

2.6. Control Efficacy of CEF559 against Verticillium Wilt in Field. Field experiments were conducted to evaluate the efficacy of CEF559 against Verticillium wilt. Two fields with a history of severe wilt infestation, located in Anyang (36°05'19.46"N, 114°30'47.21"E) and Akesu (41°10'04.29"N, 80°32'31.92"E), China, were used. Both fields had more than 10 years of continuous cotton crops. There were two treatments: with or without (control) CEF559 inoculation. Solid cultures of CEF559 (1×10^8 spores·g⁻¹) were scattered into seed furrows at the rate of 20 g per meter of the furrow; the sterile maize-sand medium was used as control. Surface-sterilized cotton seeds (cv. Jimian11) were sown in the field. A completely randomized design was used with three replications per treatment. Each replicate plot had 6 rows of 8 m in length, containing about 200 cotton plants. Wilt severity

TABLE 1: Oligonucleotide primers used in this study for reverse transcription quantitative PCR of defense genes and pathogenesis-related or reference genes from cotton.

Gene name	Primer sequence (5' to 3')
<i>β-1,3-Glucanase</i>	F: CACAGGTGCTGAAGTTGGT R: CGATGGAGGGAAAGATGA
<i>Basic chitinase</i>	F: CTTAGCCCAAACCTCCCA R: TACATTGAGTCCACCGAGAC
<i>Acidic chitinase</i>	F: GCTTTGATGGTTGTGCTCA R: CCACCCACCTGTAGTTTCA
<i>4CL</i>	F: ATTCAAAAGGGAGATGCC R: GAGAAGGGCAAAGCAACA
<i>PAL</i>	F: TGGTGGCTGAGTTTAGGAAA R: TGAGTGAGGCAATGTGTGA
<i>PPO</i>	F: ATATCCTTGTCTGTCTGCTA R: CTCCTTCTACCGTCTCTTC
<i>POD</i>	F: CCGCATAACCATCACAAAG R: ACTTCTACACCTTCAACA
<i>Ubiquitin</i>	F: GAGTCTTCGGACACCATTG R: CTTGACCTTCTCTCTTGTGC

of each plant was recorded 60, 80, and 100 days after sowing on a scale of 0 to 4: 0 = no symptoms, 1 = ≤33%, 2 = >33% and ≤66%, 3 = >66% and ≤99%, and 4 = 100% leaves with wilt symptoms. Disease index for each plot was then calculated as described above.

2.7. Expression Analysis of Pathogenesis-Related (PR) Genes and Key Genes in the Lignin Metabolism Pathway using qRT-PCR. The relative transcript levels of PR genes and key genes in the lignin metabolism pathway were determined with a quantitative reverse transcription PCR (qRT-PCR) method. Cotton seedlings were first inoculated with CEF559 (1×10^7 spores·mL⁻¹, 50 mL per paper pot) at the one true leaf stage and then with *V. dahliae* Vd080 (1×10^7 spores·mL⁻¹, 10 mL per pot) 4 days later. For each sampling time point (1, 2, 3, 4, and 7 dpi with *V. dahliae* Vd080), root samples of each biological replicate and ten pooled cotton root samples were obtained for each treatment. Frozen samples were ground to a fine powder in liquid nitrogen using a pestle and mortar. Total RNA was isolated with Plant RNA Kit (Tiangen, Beijing, China), and cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's procedure. The qRT-PCR was performed to quantify the transcript levels of the following genes: three key genes in the lignin metabolism pathway (peroxidase, *POD*; phenylalanine ammonia lyase, *PAL*; and polyphenol oxidase, *PPO*) and four pathogenesis-related (PR) genes (4-coumarate: CoA ligase, *4CL*; *basic chitinase*; *acidic chitinase*; and *β-1,3-glucanase*) with specific primers (Table 1); the cotton *ubiquitin* gene was used for normalization. Cycle thresholds were determined in three biological replicates per sample using LightCycler 480 system (Roche Diagnostics, Mannheim, Germany) and the SYBR Green Premix Ex Taq TMII (Takara, Beijing, China) as the reporter dye. Amplification conditions consisted of denaturation for 10 min at 95°C followed by 40 cycles of 30 s

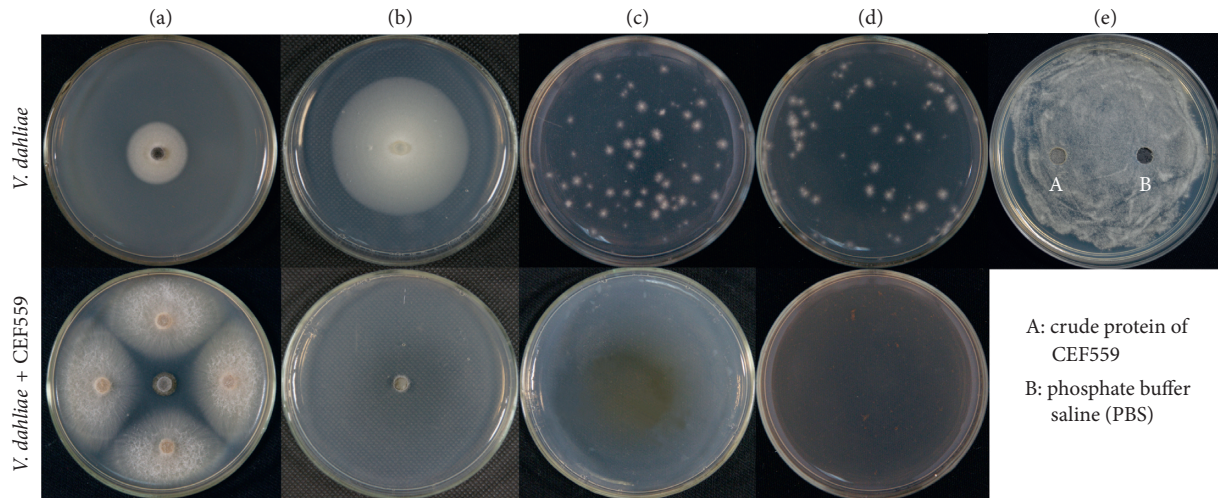


FIGURE 1: Growth inhibition of *Verticillium dahliae* by *Fusarium solani* CEF559: antifungal activity of *F. solani* CEF559 against *V. dahliae* in the dual culture test after incubation for 6 days (a); morphology of *V. dahliae* Vd080 incubated alone (top) and dual cultured with CEF559 on PDA media (bottom) for 14 days (b); inhibition of *V. dahliae* germination in the unsterilized (c) and sterilized (d) metabolites of CEF559; antifungal activity of crude protein *F. solani* CEF559 towards *V. dahliae* after incubation for 3 days (e).

at 95°C, 30 s at 60°C, and 30 s at 72°C. A final extension step was performed for 10 min at 72°C, followed by a melting curve program at 60°C to 95°C with an increase step of 0.5°C. Gene expression data were normalized using *ubiquitin*.

2.8. *Statistical Analysis.* Comparisons of treatment means for each experiment were conducted based on analysis of variance (ANOVA) and the SPSS 20.0 software; means were separated by Tukey’s honestly significant difference (Tukey’s HSD) test.

3. Results

3.1. *In Vitro Antifungal Activity of the Endophytic Fungus CEF559 against V. dahliae.* The dual culture tests showed that CEF559 inhibited mycelial growth of *V. dahliae* (Figure 1(a)), with an average inhibition of 75%. Microsclerotia were produced on the edge of the dual culture colony, but not in *V. dahliae* only (Figure 1(a)). Nonvolatile metabolites of CEF559 also completely inhibited mycelial growth of *V. dahliae* (Figure 1(b)). However, *F. solani* CEF559 volatile compounds did not inhibit *V. dahliae* mycelial development (data not shown). CEF559 metabolites completely inhibited conidial germination of *V. dahliae* irrespective of whether the metabolites were autoclaved or not (Figures 1(c) and 1(d)). The crude protein of CEF559 did not show any antifungal activity towards *V. dahliae* (Figure 1(e)).

3.2. *Effects of F. solani CEF559 on V. dahliae Sporulation.* Four days after *V. dahliae* Vd080 conidia were cultured with the cell-free culture filtrate of CEF559, the average conidial concentration was 4.4×10^6 spores·mL⁻¹, much less ($P < 0.01$) than the control 2.0×10^7 spores·mL⁻¹. On day 6,

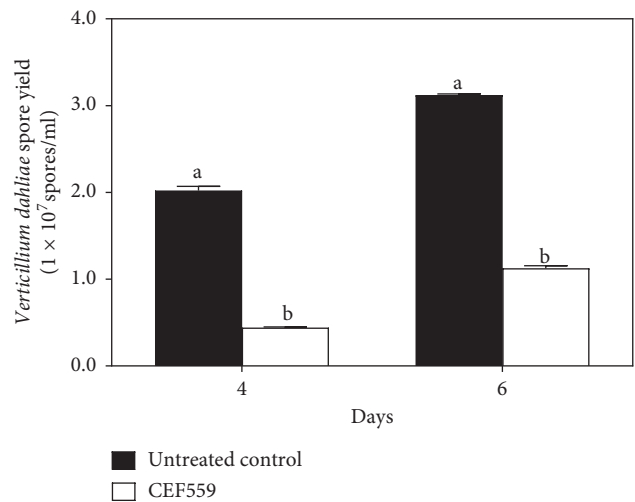


FIGURE 2: Effect of *Fusarium solani* CEF559 metabolites on sporulation of *Verticillium dahliae*. Means ± standard errors ($n = 3$) labeled with different letters indicate significant difference ($P < 0.05$) according to Tukey’s honestly significant difference (Tukey’s HSD) test.

the corresponding values were 1.1×10^7 and 3.1×10^7 spores·mL⁻¹ (Figure 2).

3.3. *Suppression of Cotton Verticillium Wilt in Greenhouse.* In the liquid culture inoculation study, cotton plants treated with CEF559 4 days before inoculation with *V. dahliae* reduced ($P < 0.05$) wilt development when assessed 21 dpi (Figure 3(a)): disease index of 8.0 for the treated, compared to 38.8 for the control (Figure 3(b)). Inoculating with CEF559 after *V. dahliae* inoculation failed to reduce wilt development (Figure 3(b)). Similarly, in the solid culture inoculation, cotton plants inoculated with *F. solani* CEF559 4 days before inoculation of *V. dahliae* Vd080 reduced

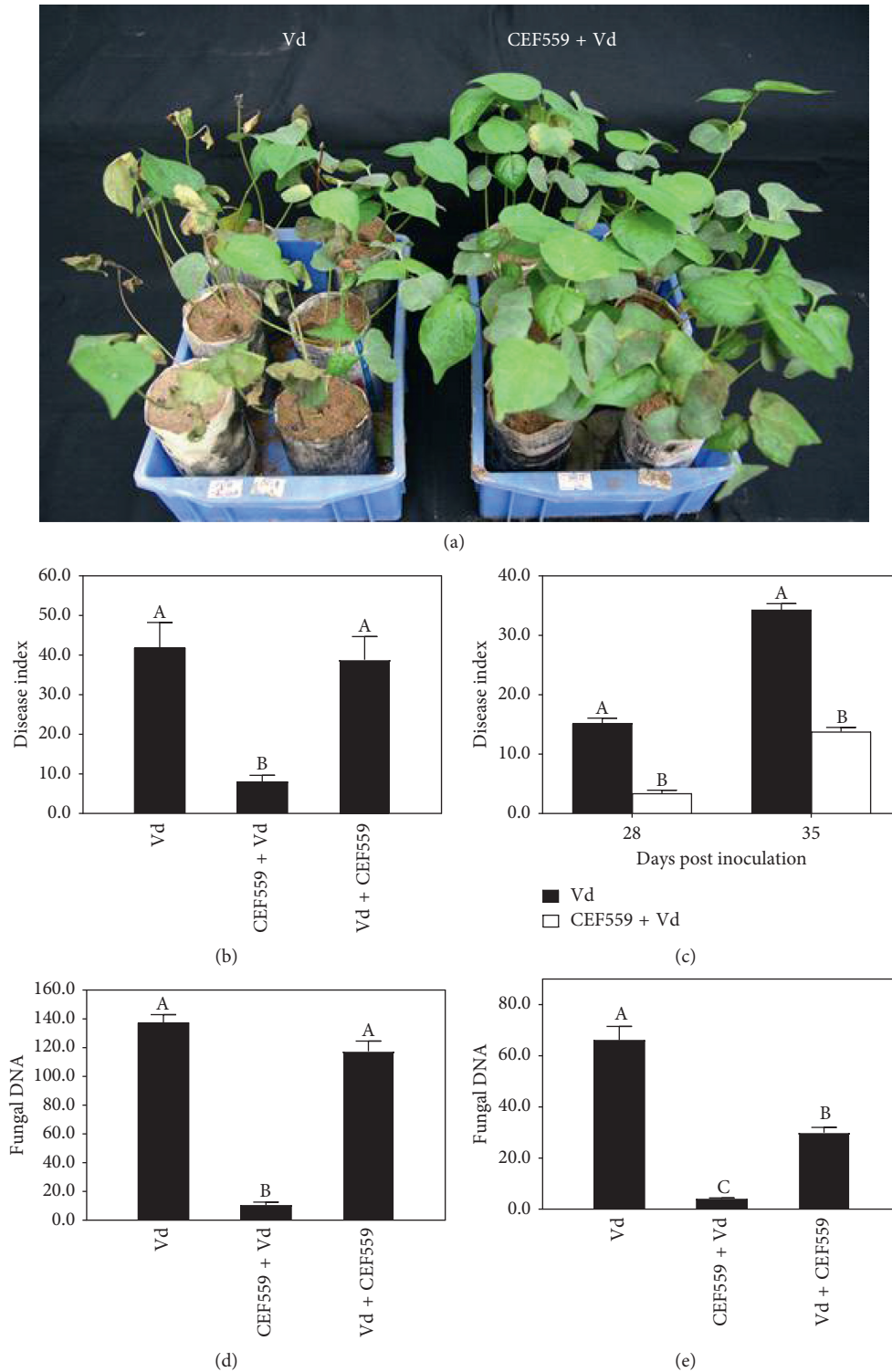


FIGURE 3: *Fusarium solani* CEF559 protects cotton plants from *Verticillium dahliae* infection. (a) Cotton plants of cv. Jimian11 were inoculated with *F. solani* CEF559 4 days before inoculation with *V. dahliae* strain Vd080 (Vd); photographed 21 dpi with *V. dahliae*. (b) Wilt index of cotton plants inoculated with *V. dahliae* Vd080 (Vd), inoculated with *F. solani* CEF559 liquid culture 4 days before inoculation with *V. dahliae* (CEF559 + Vd), and with *V. dahliae* 4 days before inoculation with *F. solani* CEF559 liquid culture (Vd + CEF559). (c) Wilt index of cotton plants inoculated with *F. solani* CEF559 solid culture 4 days before inoculation with *V. dahliae*. Quantified *Verticillium dahliae* DNA in roots (d) and hypocotyls (e) of cotton plants for three treatments as in (b). Data are means of three replicate experiments in greenhouse assay. Vertical bars represent standard errors of the means. Different letters indicate significant differences based on Tukey's HSD test (SPSS, v 20.0) at $P = 0.05$.

TABLE 2: Effect of *Fusarium solani* CEF559 on cotton emergence and biomass.

Treatment	Emergence rate (%)	Plant height (cm)	Root length (cm)	Fresh weight (g)
CK	85.67a	10.2 ± 1.26a	9.6 ± 0.65a	0.98 ± 0.11a
CEF559	83.33a	9.9 ± 0.652a	9.5 ± 0.791a	1.12 ± 0.114a

Data (mean ± SE) in the table are the averages of three biological experiments. Data followed by different letters indicate statistically significant differences at the level of $P < 0.05$ according to Tukey's HSD.

($P < 0.05$) wilt development: the control efficacy was 77.6% and 59.9% when assessed 28 and 35 dpi of Vd080, respectively (Figure 3(c)). The qPCR results on *V. dahliae* biomass in the roots showed the same pattern as for the wilt index (Figures 3(b) and 3(d)). However, for *V. dahliae* biomass in the hypocotyls, CEF559 treatment after inoculation of *V. dahliae* also led to a significant reduction in *V. dahliae* biomass (Figure 3(e)).

3.4. Effect of *F. solani* CEF559 Inoculation on Cotton Seed Emergence and Seedling Development

F. solani CEF559 did not affect the growth of cotton plants (Table 2). The proportion of cotton seed emergence 5 days after sowing did not differ among treatments: 83.3% (CEF559) and 85.7% for the control. Similarly, no significant differences were observed between the two treatments in plant height, root length, and fresh weight when assessed 20 days after sowing, compared to the control (Table 2).

3.5. Field Suppression of Cotton Verticillium Wilt. Wilt index increased over time at both sites (Figure 4). For the CEF559 treatment, the average disease index ranged from 6.6 to 15.5 and from 5.8 to 16.8 for Anyang and Akesu sites within 100 days after sowing, respectively, compared to the corresponding values for the control from 14.0 to 22.1 and from 13.1 to 38.4 (Figure 4). CEF559 treatment led to significant ($P < 0.05$) reductions in wilt index at both sites, with the control efficacy ranging from 30.1% to 56.3% (Figure 4).

3.6. Expression of Key Genes in the Lignin Metabolism Pathway and PR Genes. Although gene expression patterns varied greatly with time, compared with the untreated plants, the three key genes in the lignin metabolism pathway were all upregulated ($P < 0.01$) in the roots of those plants treated with CEF559 2 dpi compared with those plants inoculated with *V. dahliae* only (Figure 5). The expression of *POD* and *PPO* genes reached the peak 2 dpi, with the respective 2.0- and 1.3-fold increases over the control (Figures 5(b) and 5(c)). The expression of *PAL* reached the peak 4 dpi, ca. 2.2-fold increases over the control (Figure 5(a)). However, the gene expression level of *PAL* and *POD* genes 1 dpi and *PPO* 4 dpi showed a downregulation than the control (Figure 5).

As for the key genes in the lignin metabolism pathway genes, CEF559 also induced PR gene expression in the treated roots. Except for *4CL*, the transcription level of *basic chitinase*, *acidic chitinase*, and β -1,3-*glucanase* were all upregulated on 3 dpi, compared with those plants inoculated with *V. dahliae* only, with increases of 4.9-fold, 5.0-fold, and

5.5-fold, respectively (Figure 6). The expression of *4CL* in treated plants was approximately 9.9-fold of the control 4 dpi (Figure 6(a)).

4. Discussion

Biological control of plant pathogens is a safer alternative to chemical treatments. In our previous study [24], 642 endophytic fungi were obtained from Verticillium wilt-resistant cotton varieties, and 80 of these endophytes were evaluated for their *in vitro* inhibition activity against *V. dahliae* isolate Vd080. Thirty-nine of these 80 isolates exhibited varying degrees of inhibition against *V. dahliae*. Of these 39 isolates, CEF818, CEF714, CEF642, and CEF193 were further tested for their biocontrol against cotton wilt in greenhouse and field trials [25]. In the present study, CEF559 strain, acquired from further screening for the strain pool excluding the tested 80 strains, can protect cotton plants against Verticillium wilt.

Over the last two decades, nonpathogenic *Fusarium* sp. isolates have been shown to have a varying degree of biocontrol potential against Verticillium wilt in several crop plants [12,16,27–29,36–39] although *F. oxysporum* and *F. solani* are also soilborne pathogens of some crop plants. For example, *F. oxysporum* strain F2 significantly reduced *V. dahliae* disease development in eggplants [16, 28, 29, 36]. *F. oxysporum* strain By125 and *F. oxysporum* strain CanR-46 achieved 69% and 92% control against cotton wilt in greenhouse, respectively [30, 37]. The present study suggested a new *Fusarium* strain (*F. solani* CEF559) could protect cotton plants against wilt with greater than 60% control in greenhouse studies, similar to the control level (50%) achieved by another *F. solani* strain (Bx215) in a greenhouse experiment [37]. Even in fields that were previously infested heavily with Verticillium wilt, this new isolate significantly reduced wilt development. For a given endophyte, biocontrol efficacy may depend on specific abiotic and biotic conditions [38]. Thus, it is not surprising that control efficacy in the present study varied between the two field sites.

Understanding the mechanisms involved in the antagonistic effects against plant pathogens is important for selecting effective and sustainable biocontrol strategies. In the present study, a dual culture of *V. dahliae* and *F. solani* CEF559 showed a decrease in the growth rate of the pathogen. Therefore, CEF559 could be acting at least partially through antagonistic interactions against *V. dahliae*. Similar results were also obtained from *F. oxysporum* strain F2, which has been reported to inhibit *V. dahliae* in eggplant by competition [29]; indeed, many nonpathogenic *Fusarium* strains can consume carbon

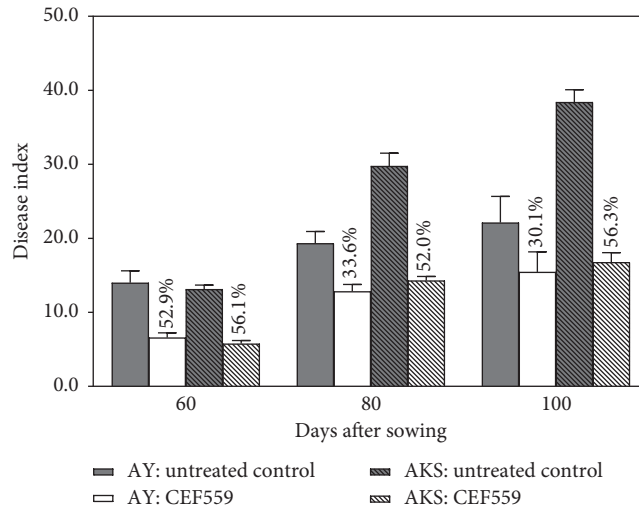


FIGURE 4: *Fusarium solani* CEF559 protects cotton plants from *Verticillium dahliae* infection at field sites in Anyang (AY) and Akesu (AKS) with a history of severe cotton wilt. Data are means of three replicate experiments in field assay. Figures above the bars are the average efficacy for the CEF559 treatment compared with the same site and date of investigation.

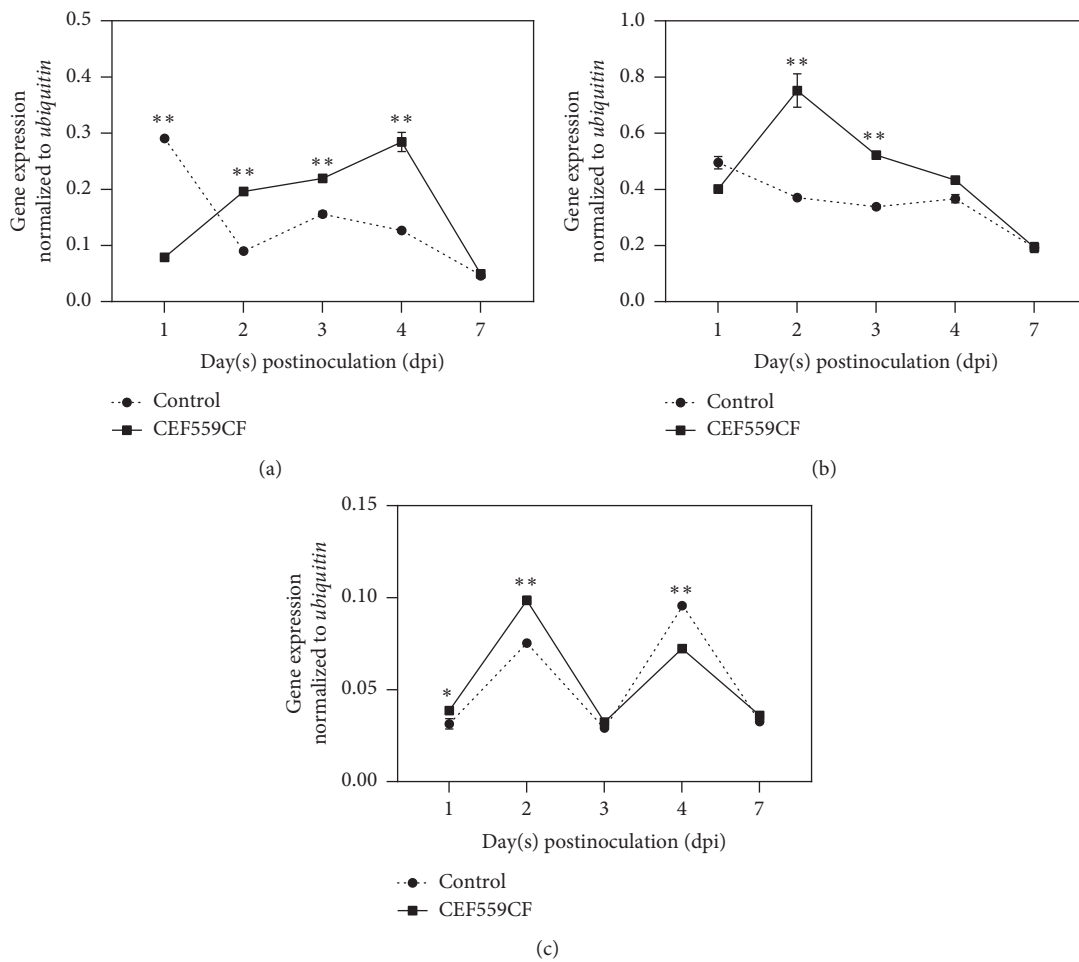


FIGURE 5: Effect of *Fusarium solani* CEF559 on the relative expression levels of three key genes in the lignin metabolism pathway in cotton roots after inoculation with *Verticillium dahliae*. Data are means of three replicate experiments in greenhouse assay. The bars represent the average induction (\pm SE) of gene transcripts normalized to the *ubiquitin* gene for three replicates. The vertical bars represent the standard error of the means. Comparisons of gene expression among treatments were conducted using a Tukey's HSD test (SPSS, v 20.0). Asterisks indicate statistically significant differences compared with control roots (double asterisks, $P < 0.01$; single asterisk, $P < 0.05$): (a) *PAL*; (b) *POD*; (c) *PPO*.

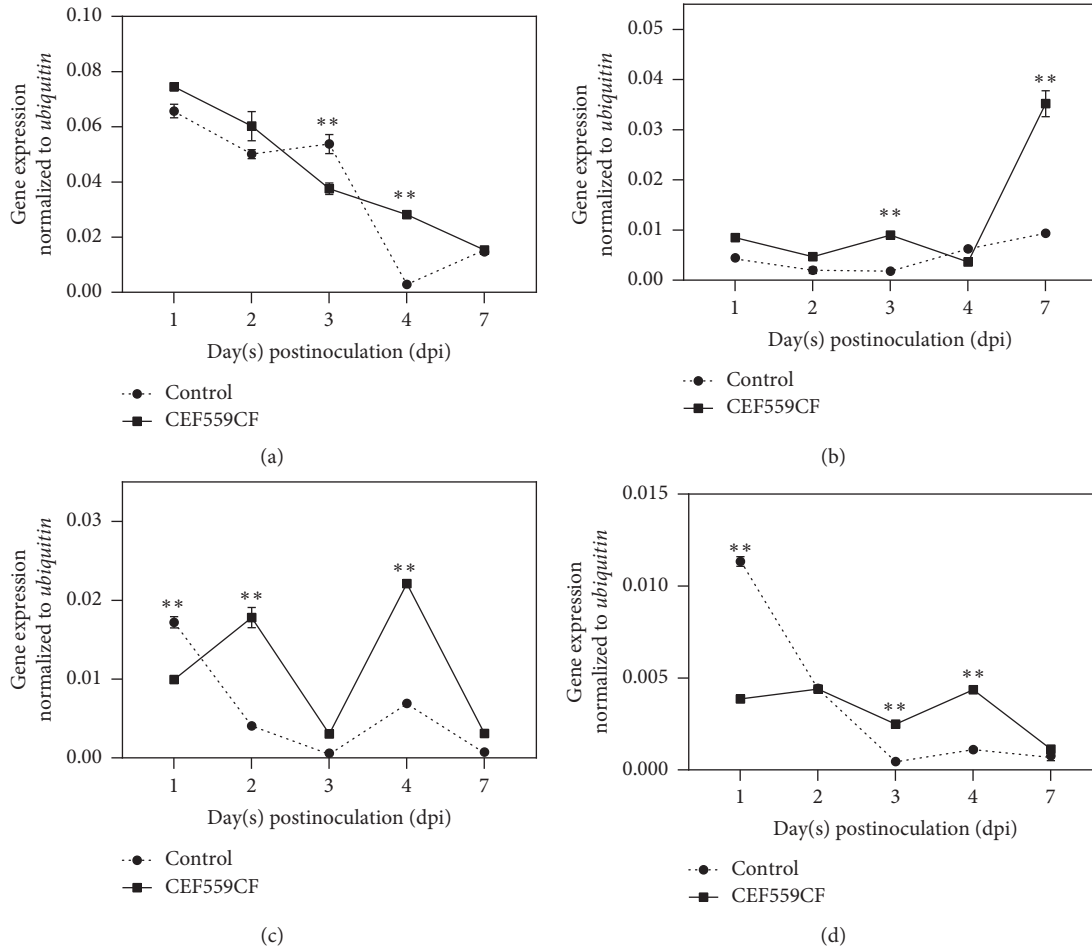


FIGURE 6: Effect of *Fusarium solani* CEF559 on relative expression levels of four pathogenesis-related (PR) genes in cotton roots after inoculation with *Verticillium dahliae* using quantitative reverse transcription PCR (qRT-PCR). Data are means of three replicate experiments in greenhouse assay. Vertical bars represent standard errors of means. Comparisons of gene expression among treatments were conducted using a Tukey's HSD test (SPSS, v 20.0). Asterisks indicate statistically significant differences compared with control roots (double asterisks, $P < 0.01$; single asterisk, $P < 0.05$): (a) 4CL. (b) Basic chitinase. (c) Acidic chitinase. (d) β -1,3-Glucanase.

sources more efficiently than pathogens [39]. Beneficial fungi can deprive pathogens of space and nutrients by colonizing the shared ecological niche [40]. CEF559 was isolated from cotton plants and, therefore, it probably can colonize cotton plants efficiently. Treating cotton roots with CEF559 reduced *Verticillium* wilt; in addition, the growth rate of CEF559 is greater than *V. dahliae*. Many biocontrol strains produce diverse biologically active secondary metabolites, which can inhibit pathogen mycelial growth and spore germination [41]. Various isolates of nonpathogenic *Fusarium* sp. have different competitive abilities against pathogens, and their indirect effects may vary. Nonvolatile metabolites of CEF559 also inhibited mycelial growth of *V. dahliae*, whilst volatile compounds did not inhibit *V. dahliae* mycelial development. Moreover, CEF559 metabolites completely inhibited conidial germination of *V. dahliae* irrespective of whether the metabolites were autoclaved or not. On the other hand, the crude protein produced by CEF559 did not show suppressive effect on the growth of *V. dahliae*. Therefore, we may conclude that the inhibitory activity of CEF559 is mainly

due to the low molecular nonvolatile compounds instead of proteins and direct mycoparasitism.

Induced resistance is an important biocontrol mechanism [42]. Phenylpropanoid metabolic pathway in plants plays an important role in the plant defense response, and the enzymes of PAL, PPO, and POD were involved in the synthesis of lignin through this pathway [43]. Increased PPO, PAL, and POD activity was correlated with disease resistance in plants [44]. PPO and PAL activities are also important in plant disease resistance because they help to avoid oxidative damages [45]. In addition, the enhanced expression of defense-related enzyme genes contributes to the activation of defense system [46]. The level of enzyme activity may be related to the level of gene expression. For example, *Sporidiobolus pararoseus* strain Y16 treatment induced expression of these genes and increased enzyme activities, indicating a positive relationship of the gene expression level with enzyme activity [47]. Compared with those plants inoculated with *V. dahliae* only, PAL, POD, and PPO in root tissues were all significantly upregulated after CEF559 treatment, indicating that

locally induced defense through the increased expression of these key genes in the lignin metabolism pathway is an important mechanism of CEF559 against *V. dahliae*. In addition, many plant resistance-related genes help to limit the growth and expansion of pathogens [48, 49]. In the present study, CEF559 induced a moderate increase of pathogenesis-related gene transcripts, including *basic chitinase*, *acidic chitinase*, *4CL*, and β -1,3-*glucanase*. Similarly, *F. oxysporum* isolate Fo47 can induce defense responses in several crops [26, 27]. *F. oxysporum* strain F2 induced the expression of defense-related genes *PR1* and *PR4* in eggplant, which was positively correlated with BCA population sizes in the rhizospheres [16]. Induced resistance in cotton by CEF559 in relation to innate resistance requires further research. Furthermore, additional research is needed to ascertain whether the host responses induced by CEF559 are conditional on the presence of *V. dahliae*.

The inherent biological complexity of the soil system has served as a challenge to the success of biocontrol of soilborne diseases. The rapid decline in the density of biological organisms typically encountered after introduction to the soil due to competitive inactions [50]. There has been an increasing interest in using the introduction of multiple BCAs to control soilborne disease with the component BCAs possessing different biocontrol mechanisms and ecological requirements [17, 18]. This could improve the survival of beneficial organisms in the temporally and spatially fluctuating rhizosphere environment and ensure that at least one of the beneficial microbes can survive. For example, a previous study has shown that the survival of introduced diverse probiotic consortia increased with increasing diversity, and high probiotic diversity could reduce pathogen density in the rhizosphere and decrease the disease incidence due to both intensified resource competition and interference with the pathogen [51]. Several cotton endophytic fungi isolates (including *F. solani* CEF559) showed biocontrol efficacy against cotton wilt [24, 25], and further research is needed to study their combined use to form a synthetic microbial consortium against cotton *Verticillium* wilt.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

Feng Wei and Yun Zhang are co-first authors.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

This work was supported by the National Key Research and Development Program of China (2017YFD0201900) and National Natural Science Foundation of China (31901938).

Special thanks are due to Professor Xiangming Xu of NIAB East Malling Research for revising this manuscript.

References

- [1] G. F. Pegg and B. L. Brady, "Control," in *Verticillium Wilts*, vol. 151, pp. 109-110, CABI, Wallingford, UK, 2002.
- [2] S. J. Klosterman, Z. K. Atallah, G. E. Vallad, and K. V. Subbarao, "Diversity, pathogenicity, and management of *Verticillium* species," *Annual Review of Phytopathology*, vol. 47, no. 1, pp. 39-62, 2009.
- [3] S. Wilhelm, "Longevity of the verticillium wilt fungus in the laboratory and field," *Phytopathology*, vol. 45, no. 3, pp. 180-181, 1955.
- [4] F. Wei, R. Fan, H. Dong et al., "Threshold microsclerotial inoculum for cotton verticillium wilt determined through wet-sieving and real-time quantitative PCR," *Phytopathology*, vol. 105, no. 2, pp. 220-229, 2015.
- [5] S. Deketelaere, L. Tyvaert, S. França, and M. Höfte, "Desirable traits of a good biocontrol agent against verticillium wilt," *Frontiers in Microbiology*, vol. 8, 2017.
- [6] F. N. Martin, "Development of alternative strategies for management of soil borne pathogens currently controlled with methyl bromide," *Annual Review of Phytopathology*, vol. 41, no. 1, pp. 325-350, 2003.
- [7] C. Neubauer, B. Heitmann, and C. Müller, "Biofumigation potential of *Brassicaceae* cultivars to *Verticillium dahliae*," *European Journal of Plant Pathology*, vol. 140, no. 2, pp. 341-352, 2014.
- [8] J. J. Parnell, R. Berka, H. A. Young et al., "From the lab to the farm: an industrial perspective of plant beneficial microorganisms," *Frontiers in Plant Science*, vol. 7, pp. 1-12, 2016.
- [9] A. K. Uppal, A. El Hadrami, L. R. Adam, M. Tenuta, and F. Daayf, "Biological control of potato verticillium wilt under controlled and field conditions using selected bacterial antagonists and plant extracts," *Biological Control*, vol. 44, no. 1, pp. 90-100, 2008.
- [10] J.-G. Li, Z.-Q. Jiang, L.-P. Xu, F.-F. Sun, and J.-H. Guo, "Characterization of chitinase secreted by *Bacillus cereus* strain CH₂ and evaluation of its efficacy against verticillium wilt of eggplant," *BioControl*, vol. 53, no. 6, pp. 931-944, 2008.
- [11] S. Aghighi, G. H. Shahidi Bonjar, and I. Saadoun, "First report of antifungal properties of a new strain of *Streptomyces plicatus* (strain101) against four Iranian phytopathogenic isolates of *Verticillium dahliae*, a new horizon in biocontrol agents," *Biotechnology*, vol. 3, no. 1, pp. 90-97, 2004.
- [12] J. Veloso and J. Diaz, "*Fusarium oxysporum* Fo47 confers protection to pepper plants against *Verticillium dahliae* and *Phytophthora capsici*, and induces the expression of defense genes," *Plant Pathology*, vol. 61, no. 2, pp. 281-288, 2012.
- [13] C. Sun, Y. Shao, K. Vahabi et al., "The beneficial fungus *Piriformospora indica* protects *Arabidopsis* from *Verticillium dahliae* infection by downregulation plant defense responses," *BMC Plant Biology*, vol. 14, no. 1, Article ID 268, 2014.
- [14] I. Carrero-Carrón, J. L. Trapero-Casas, C. Olivares-García, E. Monte, R. Hermosa, and R. M. Jiménez-Díaz, "*Trichoderma asperellum* is effective for biocontrol of verticillium wilt in olive caused by the defoliating pathotype of *Verticillium dahliae*," *Crop Protection*, vol. 88, pp. 45-52, 2016.
- [15] X. J. Lan, J. Zhang, Z. F. Zong, Q. Ma, and Y. Wang, "Evaluation of the biocontrol potential of *Purpureocillium lilacinum* QLP12 against *Verticillium dahliae* in eggplant," *BioMed Research International*, vol. 2017, Article ID 4101357, 8 pages, 2017.

- [16] D. J. Angelopoulou, E. J. Naska, E. J. Paplomatas, and S. E. Tjamos, "Biological control agents (BCAs) of verticillium wilt: influence of application rates and delivery method on plant protection, triggering of host defence mechanisms and rhizosphere populations of BCAs," *Plant Pathology*, vol. 63, no. 5, pp. 1062–1069, 2015.
- [17] M. Mazzola and S. Freilich, "Prospects for biological soilborne disease control: application of indigenous versus synthetic microbiomes," *Phytopathology*, vol. 107, no. 3, pp. 256–263, 2017.
- [18] X.-M. Xu, P. Jeffries, M. Pautasso, and M. J. Jeger, "Combined use of biocontrol agents to manage plant diseases in theory and practice," *Phytopathology*, vol. 101, no. 9, pp. 1024–1031, 2011.
- [19] D. Wilson, "Endophyte: the evolution of a term, and clarification of its use and definition," *Oikos*, vol. 73, no. 2, pp. 274–276, 1995.
- [20] R. N. Kharwar, V. C. Verma, G. Strobel, and D. Ezra, "The endophytic fungal complex of *Catharanthus roseus* (L.) G. Don," *Current Science*, vol. 95, no. 2, pp. 228–233, 2008.
- [21] S. K. Gond, V. C. Verma, A. Mishra, A. Kumar, and R. N. Kharwar, "Role of fungal endophytes in plant protection," in *Management of Fungal Plant Pathogens*, A. Arya and A. E. Perelló, Eds., pp. 183–197, CAB International, Wallingford, UK, 2010.
- [22] B. A. Bailey, H. Bae, M. D. Strem et al., "Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four *Trichoderma* species," *Planta*, vol. 224, no. 6, pp. 1449–1464, 2006.
- [23] S. A. Bonos, M. M. Wilson, W. A. Meyer, and C. R. Funk, "Suppression of red thread in fine fescues through endophyte-mediated resistance," *Applied Turfgrass Science*, vol. 2, no. 1, 2005.
- [24] Z.-F. Li, L.-F. Wang, Z.-L. Feng, L.-H. Zhao, Y.-Q. Shi, and H.-Q. Zhu, "Diversity of endophytic fungi from different *Verticillium*-wilt-resistant *Gossypium hirsutum* and evaluation of antifungal activity against *Verticillium dahliae* *in vitro*," *Journal of Microbiology and Biotechnology*, vol. 24, no. 9, pp. 1149–1161, 2014.
- [25] Y. Yuan, H. J. Feng, L. F. Wang et al., "Potential of endophytic fungi isolated from cotton roots for biological control against verticillium wilt disease," *PLoS One*, vol. 12, no. 1, Article ID e0170557, 2017.
- [26] N. Benhamou and C. Garand, "Cytological analysis of defense-related mechanisms induced in pea root tissues in response to colonization by nonpathogenic *Fusarium oxysporum* Fo47," *Phytopathology*, vol. 91, no. 8, pp. 730–740, 2001.
- [27] J.-G. Fuchs, Y. Moëgne-Loccoz, and G. Défago, "Non-pathogenic *Fusarium oxysporum* strain Fo47 induces resistance to fusarium wilt in tomato," *Plant Disease*, vol. 81, no. 5, pp. 492–496, 1997.
- [28] D. Gizi, I. A. Stringlis, S. E. Tjamos, and E. J. Paplomatas, "Seedling vaccination by stem injecting a conidial suspension of F2, a non-pathogenic *Fusarium oxysporum* strain, suppresses verticillium wilt of eggplant," *Biological Control*, vol. 58, no. 3, pp. 387–392, 2011.
- [29] I. S. Pantelides, S. E. Tjamos, I. A. Stringlis, I. Chatzipavlidis, and E. J. Paplomatas, "Mode of action of a non-pathogenic *Fusarium oxysporum* strain against *Verticillium dahliae* using Real Time QPCR analysis and biomarker transformation," *Biological Control*, vol. 50, no. 1, pp. 30–36, 2009.
- [30] Q. Zhang, L. Yang, J. Zhang et al., "Production of anti-fungal volatiles by non-pathogenic *Fusarium oxysporum* and its efficacy in suppression of verticillium wilt of cotton," *Plant and Soil*, vol. 392, no. 1-2, pp. 101–114, 2015.
- [31] Y.-L. Zhang, Z.-F. Li, Z.-L. Feng et al., "Isolation and functional analysis of the pathogenicity-related gene *VdPR₃* from *Verticillium dahliae* on cotton," *Current Genetics*, vol. 61, no. 4, pp. 555–566, 2015.
- [32] M. M. Elsharkawy, M. Shimizu, H. Takahashi, and M. Hyakumachi, "Induction of systemic resistance against *Cucumber mosaic virus* by *Penicillium simplicissimum* GP17-2 in *Arabidopsis* and tobacco," *Plant Pathology*, vol. 61, no. 5, pp. 964–976, 2012.
- [33] Q. Zhang, J. Zhang, L. Yang et al., "Diversity and biocontrol potential of endophytic fungi in *Brassica napus*," *Biological Control*, vol. 72, no. 9, pp. 98–108, 2014.
- [34] L. Naraghi, A. Heydari, S. Rezaee, M. Razavi, and H. Jahanifar, "Study on antagonistic effects of *Talaromyces flavus* on *Verticillium albo-atrum*, the causal agent of potato wilt disease," *Crop Protection*, vol. 29, no. 7, pp. 658–662, 2010.
- [35] A. K. Tzima, E. J. Paplomatas, P. Rauyaree, M. D. Ospina-Giraldo, and S. Kang, "*VdSNFI*, the sucrose nonfermenting protein kinase gene of *Verticillium dahliae*, is required for virulence and expression of genes involved in cell-wall degradation," *Molecular Plant-Microbe Interactions*, vol. 24, no. 1, pp. 129–142, 2011.
- [36] I. Malandraki, S. E. Tjamos, I. S. Pantelides, and E. J. Paplomatas, "Thermal inactivation of compost suppressiveness implicates possible biological factors in disease management," *Biological Control*, vol. 44, no. 2, pp. 180–187, 2008.
- [37] Y. Zheng, Q.-Y. Xue, L.-L. Xu et al., "A screening strategy of fungal biocontrol agents towards verticillium wilt of cotton," *Biological Control*, vol. 56, no. 3, pp. 209–216, 2011.
- [38] P. E. Busby, M. Ridout, and G. Newcombe, "Fungal endophytes: modifiers of plant disease," *Plant Molecular Biology*, vol. 90, no. 6, pp. 645–655, 2016.
- [39] Y. Couteaudier and C. Alabouvette, "Quantitative comparison of *Fusarium oxysporum* competitiveness in relation to carbon utilization," *FEMS Microbiology Letters*, vol. 74, no. 4, pp. 261–267, 1990.
- [40] M. Ghorbanpour, M. Omidvari, P. Abbaszadeh-Dahaji, R. Omidvar, and K. Kariman, "Mechanisms underlying the protective effects of beneficial fungi against plant diseases," *Biological Control*, vol. 117, pp. 147–157, 2018.
- [41] O. Tyc, C. Song, J. S. Dickschat, M. Vos, and P. Garbeva, "The ecological role of volatile and soluble secondary metabolites produced by soil bacteria," *Trends in Microbiology*, vol. 25, no. 4, pp. 280–292, 2017.
- [42] C. M. J. Pieterse, C. Zamioudis, R. L. Berendsen, D. M. Weller, S. C. M. Van Wees, and P. A. H. M. Bakker, "Induced systemic resistance by beneficial microbes," *Annual Review of Phytopathology*, vol. 52, no. 1, pp. 347–375, 2014.
- [43] L. Xu, L. Zhu, L. Tu et al., "Lignin metabolism has a central role in the resistance of cotton to the wilt fungus *Verticillium dahliae* as revealed by RNA-Seq-dependent transcriptional analysis and histochemistry," *Journal of Experimental Botany*, vol. 62, no. 15, pp. 5607–5621, 2011.
- [44] P. Yingsanga, V. Srilaong, S. Kanlayanarat, S. Noichinda, and w. B. McGlasson, "Relationship between browning and related enzymes (PAL, PPO and POD) in rambutan fruit (*Nephelium lappaceum* Linn.) cvs. Rongrien and See-Chompoo," *Postharvest Biology and Technology*, vol. 50, no. 2-3, pp. 164–168, 2008.
- [45] F. Ortega-García and J. Peragón, "The response of phenylalanine ammonia-lyase, polyphenol oxidase and phenols to

- cold stress in the olive tree (*Olea europaea* L. cv. Picual),” *Journal of the Science of Food and Agriculture*, vol. 89, no. 9, pp. 1565–1573, 2009.
- [46] J. Lin, D. Gong, S. Zhu, L. Zhang, and L. Zhang, “Expression of PPO and POD genes and contents of polyphenolic compounds in harvested mango fruits in relation to Benzothiadiazole-induced defense against anthracnose,” *Scientia Horticulturae*, vol. 130, no. 1, pp. 85–89, 2011.
- [47] Q. Li, C. Li, P. Li et al., “The biocontrol effect of *Sporidiobolus pararoseus* Y16 against postharvest diseases in table grapes caused by *Aspergillus niger* and the possible mechanisms involved,” *Biological Control*, vol. 113, pp. 18–25, 2017.
- [48] S. Chen, H. Dong, Y. Fan, W. Li, and Y. Cohen, “Dry mycelium of *Penicillium chrysogenum* induces expression of pathogenesis-related protein genes and resistance against wilt diseases in Bt transgenic cotton,” *Biological Control*, vol. 39, no. 3, pp. 460–464, 2006.
- [49] X.-J. Zhou, S. Lu, Y.-H. Xu, J.-W. Wang, and X.-Y. Chen, “A cotton cDNA (*GaPR-10*) encoding a pathogenesis-related 10 protein with in vitro ribonuclease activity,” *Plant Science*, vol. 162, no. 4, pp. 629–636, 2002.
- [50] V. Nihorimbere, M. Ongena, M. Smargiassi, and P. Thonart, “Beneficial effect of the rhizosphere microbial community for plant growth and health,” vol. 15, no. 2, pp. 327–337, 2011.
- [51] J. Hu, Z. Wei, V. P. P. Friman et al., “Probiotic diversity enhances rhizosphere microbiome function and plant disease suppression,” *mBio*, vol. 7, no. 6, pp. 1–8, 2016.