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Isolation and Characterization of Rhizosphere Bacteria for the Biocontrol of *Phomopsis azadirachtae* Causing Die-back of Neem

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Abstract

In this current investigation, rhizosphere bacteria were isolated from plant's rhizosphere of *Andrographis paniculata.* The isolated bacteria were evaluated for antagonistic activity by dual culture method against *Phomopsis azadirachtae*, the fungus causing die-back of neem. One bacterium (C8) that exhibited excellent inhibition of pathogen was selected and characterized by biochemical tests and molecular methods. The ethyl acetate extract of this bacterium was screened against *P. azadirachtae* at different concentrations such as 2, 4, 6, 8, 10, 20, 40, 50, 60, 80 and 100 ppm by poisoned-food method. The isolated rhizobacterial strain significantly inhibited *P. azadirachtae* at 40 ppm concentration. This bacterium could be a potential candidate for the biocontrol of *P. azadirachtae* as well as for the integrated management strategies against this pathogen.

Keywords: *Phomopsis azadirachtae*; Die-back of neem; Rhizosphere bacteria; *Andrographis paniculata*; Biocontrol.

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1. Introduction

Neem (*Azadirachta indica*) is one of the most valuable trees in the arid and semi-arid tropics [1]. Neem has a wide range of utilization, and both wood along with non-wood products are employed in various ways. Neem products possess antiallergic, antibacterial, antidermatic, antifungal, anti-inflammatory, insecticidal, larvicidal, nematicidal, spermicidal, pesticidal properties along with several versatile biological properties [2,3]. The neem extracts, mainly leaf extract, have been employed in the synthesis of nanoparticles [4] with multiple bioactivities. However, neem is also susceptible to microbial illnesses and several bacteria along with fungi are known to infect neem [5].

The most dangerous neem pathogen at present is *Phomopsis azadirachtae* which causes die-back disease [6]. The application of fungicides like Bavistin may control the infection. Nevertheless, synthetic fungicides generally cause residual issues, the buildup of harmful contaminants in soil or subsurface water, and harm to the related soil microbiota [7,8]. Hence,

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it is not an ecofreindly approach. Therefore, it is crucial to manage disease utilizing an environmentally friendly alternative technique.

Utilizing microorganisms to biologically control plant diseases offers a potential substitute for reducing the usage of agrochemicals in agriculture [9,10]. Because of their capacity for colonization in root along with offensive mechanisms against pathogens through generation of allelochemicals that include lytic enzymes, siderophores, and volatile as well as diffusible antibiotics, rhizosphere-associated bacteria have garnered a lot of attention [11,12].

Native to India and Sri Lanka, *Andrographis paniculata* is an annual herbaceous plant of Acanthaceae family. It is well known for medicinal characteristics and is used traditionally for treatment of many illnesses, for example high blood pressure, ulcer, cancer, diabetes, skin diseases, dysentery, influenza, etc. [13].

In this present study, the rhizosphere soil of *Andrographis paniculata* was screened to isolate bacteria having the antagonistic properties. Attempts were made to identify the bacterium and for the *in vitro* management of *P. azadirachtae* through estimating ethyl acetate extract fraction of culture filtrate of isolated rhizosphere bacterium against pathogen. Reports on the isolation and screening of rhizosphere bacteria of *A. paniculata* for antimicrobial activity are very limited. Isolation of *Streptomyces* spp., from *A. paniculata* rhizosphere and its antibacterial activity is reported [14]. Isolation of *Bacillus subtilis* from rhizosphere of *A. paniculata* and its screening for pectinase production has been reported [15]. This is the first report of isolation and evaluation of *B. subtilis* from rhizosphere of *A. paniculata* against *P. azadirachtae.*

2. Materials and Methods

2.1. *Materials*

Every chemical employed in this investigation is of analytical grade and is employed straight away, without any further purification. Nutrient broth (NB), nutrient agar (NA), potato dextrose agar (PDA), had been acquired from Himedia, Mumbai, India and ethyl acetate was acquired from Merck, Bengaluru, India. Distilled water had been employed when necessary, during whole experiment. As stated in standard procedures, all of the reactions have been carried out in ambient conditions. Molecular identification work was carried out at GeneSpy Research Services, Mysuru, India. Primers were procured from Xenobiotech, Bengaluru, India; Taq DNA polymerase from Sigma Aldrich, Bengaluru, India and all other chemicals from Merck BioSciences, Bengaluru, India. BioRad thermal cycler was used for PCR.

2.2. *Isolation of rhizosphere bacteria from soil*

Rhizosphere soil samples of *Andrographis paniculata* were collected. After weighing a 1.0g soil sample, it had been subsequently serially diluted up to a 10^{-9} dilution. 0.1 mL of inoculum from last three dilutions i.e., 10^{-7}, 10^{-8}, 10^{-9} was spread inoculated on solidified nutrient agar plates aseptically using a sterile spreader. The plates were then incubated in

an inverted position at 37 °C for 24-48 h. After incubation, the distinct appearing cultures were pure cultured on to nutrient agar plates by streaking and maintained at 4 °C until use.

2.3. *Screening of rhizosphere bacteria against* Phomopsis azadirachtae *by dual-culture method*

After sterilizing the Petriplates, 20 mL of sterile PDA media was added, and the plates had been left to harden. *P. azadirachtae*'s 5.0 mm mycelial disc was positioned in middle of the plate, as well as bacterial inoculum had been streaked in a circle around fungal disc. All 10 bacterial isolates underwent equal process. The control group consisted of PDA plates that had been just inoculated with the fungal disc. For 7-10 days, every inoculated petriplates underwent incubation at 37 °C. After incubation, plates were examined for bacterial antifungal activity (a decline in fungal mycelial growth relative to the control plate). Strain chosen for additional research was one that demonstrated the strongest suppression of fungal growth.

2.4. *Characterization of rhizosphere bacterium showing significant antagonism against* Phomopsis azadirachtae

In addition to staining, identification of bacteria mainly depends on the biochemical tests. To characterize the selected bacterial strain, catalase test, IMViC test, gelatin hydrolysis test, starch hydrolysis, and urease test had been executed in accordance with standard manual [16]. Furthermore, molecular characterisation had been performed on selected strain of bacteria.

2.5. *Molecular characterization*

2.5.1. *Extraction of DNA*

Bacterium were grown in nutrient broth medium for overnight at 37 °C. After centrifuging culture for 10 min at 6000Xg, pellet obtained was resuspended in 200 µL of lysis buffer (100 mmol/L Tris-HCl, pH 8.0; 100 mmol/L EDTA; 0.75 mol/L sucrose; 10U lysostaphin; 10 mg/mL lysosyme) as well as 30 min incubation was done at 37 °C. After adding 20 µL of Proteinase K solution (20 mg/mL) along with 1% SDS, mixture had been again incubated for 2 h at 37 °C. Resulting lysates have been separated utilizing a 25:24:1 phenol-chloroform-isoamyl alcohol solvent mixture, they were precipitated employing 0.9 volume of isopropanol as well as 1/10 volume of 3 mol/L sodium acetate solution. Isolated DNA was then washed utilizing ethanol, then dissolved in 50 µL water. The purity as well as DNA concentration was established at 260 nm/280 nm using spectrophotometer [17].

2.5.2. *Amplification of gyrase gene*

Gyrase B gene amplification was carried out using GyrB-F: (ATG GAA CAG CAA AAT AAT TAC G) and GyrB-R: (TAT CCA AAT TCTTTACATATA TCG G) primers

according to the procedure prescribed by Yamamoto and Harayama [18] with slight modifications. The 25 µL reaction mixture confined: 4 µL of DNA template, 5 µL of dNTPs, 0.5 µL of taq polymerase, 1 µL of Primer, 2.5 µL of 10X PCR buffer, 2 µL of $MgCl_2$. The circumstances for PCR amplification were initial denaturation step for 5 min; then 30 cycles of 1 min 94 °C, 1 min 60 °C and 2 min 72 °C, then final extension step for 5 min. 5 µL water without bacterial DNA served as negative control.

2.5.3. *Phylogenetic tree construction*

To create the phylogenetic tree, the sequence had been compared to the strains from GenBank that shared the most similarities (accession number in parenthesis). The Kimura 2 model was utilized to build the tree employing the bootstrap approach. MEGA2 software was employed to create the neighbour-joining tree along with sub-tree. Bootstrap support level from 1,000 repeats has been displayed in numbers. With branch lengths in same units as evolutionary distances employed for calculating phylogenetic tree, tree is depicted to scale. Only in pairwise sequencing comparisons, all places with alignment gaps as well as missing data removed [19].

2.6. *Isolation of ethyl acetate fraction from bacterial culture filtrate*

Process outlined by Girish *et al*. [20] has been employed to extract antifungal ethyl acetate fraction from bacterial culture filtrate. In a 500 mL Erlenmeyer flask, 100 mL of nutrient broth had been introduced with a loop of rhizosphere bacterium cultured for 24 h. 10 flasks were inoculated overall. For 72 h, every flask had been incubated at 37 °C. Then, employing centrifugation (9000 g for 10 min at 4 °C) cells had been extracted. After collecting supernatant, culture filtrate had been filtered utilizing a 0.45 µm membrane filter (Sartorius, Gottingen, Germany), diluted with sterile distilled water to a volume of 1.5 L, and then kept at 4 °C. A flash evaporator had been employed to concentrate culture filtrates to 10 % of their initial volume at 50 °C for extraction, as well as 1.0 N HCl had been utilized to bring pH of 150 mL bacterial culture filtrates down to 3.6. Then, employing an identical volume of ethyl acetate, the culture filtrates have been extracted 3 times. Brownish, semi-solid crude extract had been obtained by pooling and evaporating organic extracts of culture filtrates at room temperature, while discarding aqueous fraction.

2.7. *Bioassay of antifungal activity of ethyl acetate extract*

Bacterial ethyl acetate fraction stock solution (1000 ppm) had been made through dissolving the separated material in the sterile distilled water that contained 0.1 percent Tween-20 (1.0 mg/mL). Control solution contained sterile distilled water with 0.1 percent Tween-20 in it [20].

The poison-food technique was employed to evaluate bacterial culture's ethyl acetate fraction against pathogen. Ethyl acetate fraction’s stock solution had been incorporated separately into sterile PDA to gain various concentrations of 2, 4, 6, 8 and 10 ppm in the

initial screening and then to gain various concentrations of 20, 40, 50, 60, 80 and 100 ppm in further screening. As a control, PDA had been modified with either 10 ppm or else 100 ppm of control solution. About 20 ml of all treated PDA were transferred onto individual 9.0 mm diameter Petri plates, let to solidify, then inoculated with a 5 mm mycelial-agar disc that had been removed from edge of mycelial mat of a *P. azadirachtae* culture that had been cultured for 7 days. For 10 days, inoculated Petri dishes were cultured at room temperature with 12 h photoperiod. Experiment was conducted twice, with 3 replications for each treatment. It had been noted how much ethyl acetate fraction would be necessary to completely halt mycelial development. Average colony diameter was calculated. To evaluate fungitoxicity, colony diameter was contrasted with the control. After 15 days of incubation, the number of pycnidia had been determined.

3. Results and Discussion

3.1. *Isolation of rhizosphere bacteria and selection of a bacterial strain*

Ten different bacterial species were isolated from the rhizosphere soil. For the ease of experiment, the bacterial species were named as isolates C1 to C10. One bacterial strain (C8) that showed the best inhibition activity in the dual culture method (Fig. 1) was selected.

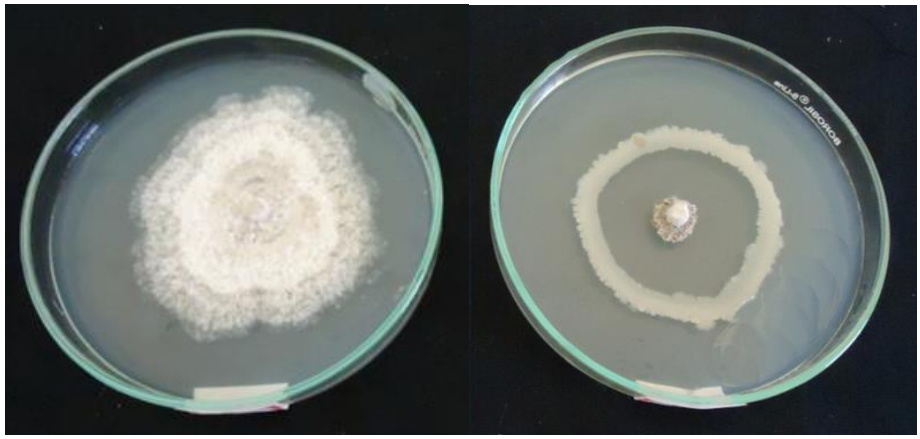

Fig. 1. (A) Control plate; (B) Bacterium C8 showing good inhibition of *Phomopsis azadirachtae* in dual culture method.

3.2. *Characterization of rhizosphere bacterium showing significant antagonism against* **Phomopsis azadirachtae**

Results of IMViC test, starch hydrolysis, catalase test, urease test, as well as gelatin hydrolysis test have been represented in the Table 1. However, the results of biochemical tests were not very conclusive for the precise identification of the isolated bacterium up to species level.

Table 1. Biochemical characterization of rhizosphere bacteria.

Tests	C8
Gram's Staining	Purple cells
Shape	Rod shaped
Catalase test	--
Indole	+
Methyl Red	--
Voges Proskauer	+
Citrate utilization	+
Triple sugar iron agar	+
Gelatin hydrolysis	+
Starch hydrolysis	+
Casein hydrolysis	--

3.3. *Molecular characterization*

3.3.1. *DNA isolation and quantification*

From C8 culture, DNA had been extracted. OD 260/280 value was 1.53 as well as 97.2 µg/mL was the DNA concentration.

3.3.2. *PCR amplification and sequencing*

Amplification with GyrB-F: (ATG GAA CAG CAA AAT AAT TAC G) and GyrB-R: (TAT CCA AAT TCT TTA CAT ATA TCG G) resulted in 1.5 kb amplicon (Fig. 2). Table 2 displays sequence data for same.

3.3.3. *Phylogenetic tree and identification of bacterium*

The culture C8 had been determined to be identical to *Bacillus* sp. on the basis of findings of experiment. NCBI blast search indicated that C8 gyrase B partial sequence had 99 percent homology with bacteria *Bacillus subtilis* (Fig. 3). Owing to this the isolated bacterium was given the name *Bacillus subtilis* C8.

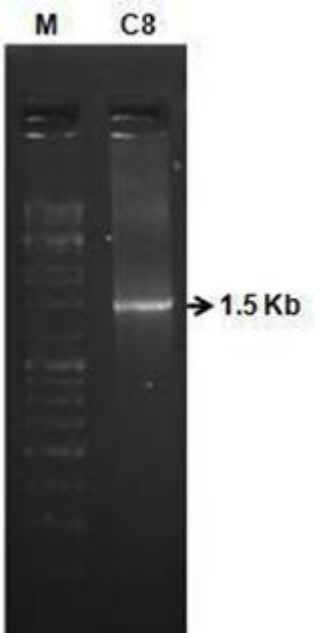

Fig. 2. Amplification of rhizosphere isolate C8 using the GyrB-Fand GyrB-R primers yielding 1.5 kb band. Lane M: Marker DNA ladder.

Table 2. The sequence data of gyrase B of bacterium C8 (partial).

>C8 gryrase B partial sequence

GGTGTAGGTGCGTCGGTCGTAAACGCACTATCAACAGAGCTTGATGTGACGGTTCACCGT
GACGGTAAAATTCACCGCCAAACCTATAAACGCGGAGTTCCGGTTACAGACCTTGAAATC
ATTGGCGAAACGGATCATACAGGAACGACGACACATTTTGTCCCGGACCCTGAAATTTTC
TCAGAAACAACCGAGTATGATTATGATCTGCTTGCCAACCGCGTGCGTGAATTAGCCTTT
TTAACAAAGGGCGTAAACATCACGATTGAAGATAAACGTGAAGGACAAGAGCGCAAAAAT
GAATACCATTACGAAGGCGGAATTAAAAGTTATGTAGAGTATTTAAACCGCTCTAAAGAG
GTTGTCCATGAAGAGCCGATTTACATTGAAGGCGAAAAGGACGGCATTACGGTTGAAGTG

GCTTTGCAATACAATGACAGCTACACAAGCAACATTTACTCGTTTACAAACAACATTAAC
ACGTACGAAGGCGGTACCCATGAAGCTGGCTTCAAAACGGGCCTGACTCGTGTTATCAAC
GATTACGCCCGACAAAAGGGCTTATTAAGAAAATGATCCAACCTAAGCGGAGATGACGTA
GGGAAGGGCTGACAGCGATTATTTCAATCAAACACCCTGATCCGCAGTTTGAGGGCCAAA
CGAAAACAAAGCTGGGCAACTCAGAAGCACGGACGATCACCGATACGTTATTTTCTACGG
CGATGGAAACATTTATGCTGGAAAATCCAGATGCAGCGAAAAAAATTGTCGATAAAGGCT
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GTGCTTTGGAAATTTCAAACCTGCCCG

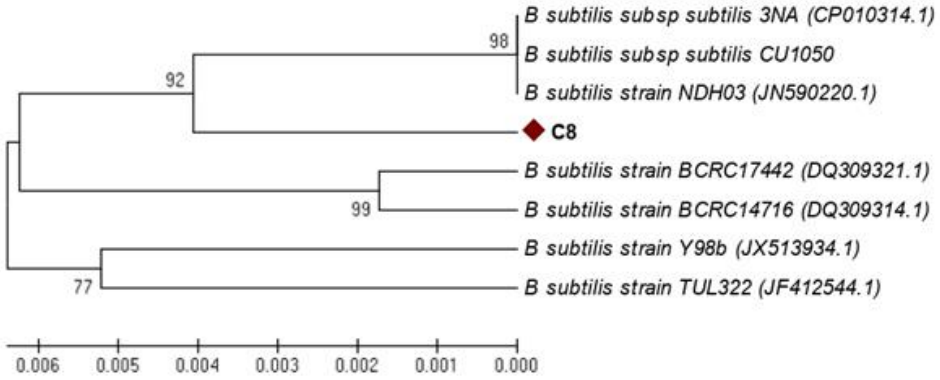

Fig. 3. Phylogenetic tree and identification of bacteria (Bacterium C8 showed 99 % homology with *Bacillus subtilis*).

3.4. *Effect of ethyl acetate fraction of rhizosphere bacterial culture filtrate on growth of* **Phomopsis azadirachtae**

The amount of ethyl acetate fraction obtained from the culture filtrate of antagonistic bacteria *B. subtilis* C8 was 85.4 mg. *B. subtilis* C8 was incapable of entirely inhibiting *P. azadirachtae* growth at 2, 4, 6, 8 and 10 ppm concentrations of the ethyl acetate fraction (Fig. 4, Table 3). At 40 ppm concentration, however, *B. subtilis* C8 ethyl acetate fraction showed entire suppression of pathogen's mycelial development. At 10 ppm and 20 ppm concentrations, through mycelial growth was observed, pycnidial formation was completely inhibited (Fig. 5, Table 3).

In the present study, among the ten bacterial isolates obtained from the rhizosphere of *Andrographis paniculata*, one bacterium named as C8 suppressed the growth of *Phomopsis azadirachtae* more effectively in dual culture approach. Dual culture has been frequently utilized to test bacteria for antifungal activity [21,22]. This bacterial isolate was characterized on the basis of biochemical tests. Isolate C8 was Gram positive rod. It was not possible to precisely identify the bacteria, based on the results of biochemical tests conducted. Therefore, molecular characterization of bacteria was done by amplification of gyrase B gene. Phylogenetic analysis along with NCBI blast search revealed that the C8 gryrase B partial sequence shares 99 % homology with *B. subtilis*. Members of the *B. subtilis* group may be effectively identified and subjected to taxonomic investigation utilizing *gyrB* gene as an alternative target [23].

At concentration of 40 ppm, bacterial (*B. subtilis* C8) ethyl acetate fractions completely inhibited *P. azadirachtae* growth. The bacterial extract also showed significant reduction in the pycnidial number at even lesser concentrations of 20 ppm and 10 ppm. This is in accordance with Singh *et al.* [24] report wherein ethyl acetate portion of *Leptoxyphium axillatum* substantially inhibits both saprophytic as well as plant pathogenic fungi. There are many reports on isolation of antagonistic bacteria from rhizosphere, characterizing them and employing them for the biocontrol of plant pathogens [25,26].

Current investigation offers a foundation for creating an environmentally responsible and successful management plan to combat *P. azadirachtae*. The ability of ethyl acetate fraction from *A. paniculata* rhizosphere bacterium (*Bacillus subtilis* C8) to effectively control neem die-back at low concentrations implies that it may be utilized as a safe substitute for chemical fungicides. For successful implementation of the following mechanism for the environmentally friendly and efficient control of *P. azadirachtae*, more research is necessary.

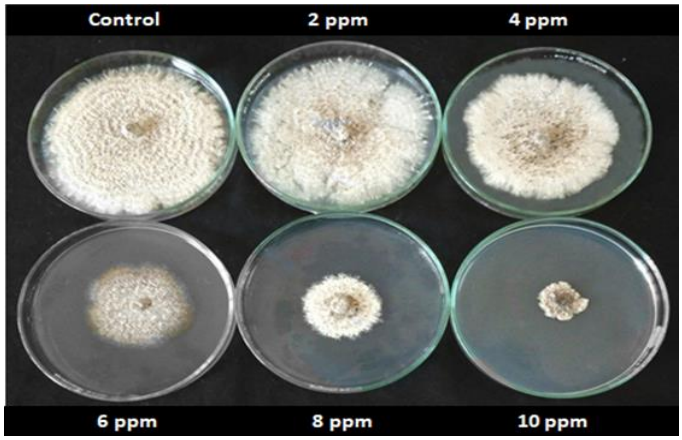

Fig. 4. Effect of ethyl acetate fraction of *Bacillus subtilis* C8 against *Phomopsis azadirachtae*.

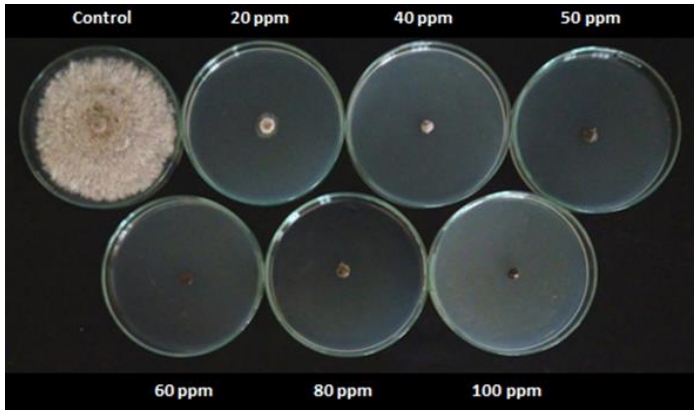

Fig. 5. Effect of ethyl acetate fraction of *Bacillus subtilis* C8 against *Phomopsis azadirachtae*.

Table 3. Effect of ethyl acetate fraction of *Bacillus subtilis* C8 on growth of *Phomopsis azadirachtae*.

Concentrations	Diameter of the mycelial mat (mm)	Growth Inhibition (%)	Number of Pycnidia
Control	82.33±0.56	0	224±1.18
2 ppm	77.0±0.37	6.46±0.45	150±1.34
4 ppm	70.0±0.58	14.98±0.70	75±1.10
6 ppm	44.33±0.49	46.15±0.60	49±1.29
8 ppm	31.66±0.61	61.54±0.75	24±0.73
10 ppm	18.66±0.49	77.33±0.60	0
20 ppm	13.33±0.42	83.81±0.51	0
40 ppm	0	100	0
50 ppm	0	100	0
60 ppm	0	100	0
80 ppm	0	100	0
100 ppm	0	100	0

Values are of two experiments, each with three replicates ± SE.

4. Conclusion

Phomopsis azadirachtae's incited die-back disease represents a serious threat to neem trees. Development of eco-friendly mechanisms for control of this disease in neem plant has been crucial. In current investigation, *in vitro* evaluation of rhizosphere bacteria of *A. paniculata* plant against the *P. azadirachtae* has shown effective results. The ethyl acetate fraction of this bacterial isolate (*Bacillus subtilis* C8) culture filtrate has completely suppressed the growth of *P. azadirachtae.* Thus, this could serve as an effective, eco-friendly method of controlling the pathogen, as the *Bacillus subtilis* C8 strain was obtained from the natural ecosystem. It would be beneficial if this technique could be developed as both an integrated disease control strategy as well as a biopesticide.

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