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Antifungal Activity of Borrelidin Produced by a *Streptomyces* Strain Isolated from Soybean

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ABSTRACT: In this study, an endophytic *Streptomyces* sp. neau-D50 with strong antifungal activity against *Phytophthora sojae* was isolated from healthy soybean root, using an in vitro screening technique. A bioactivity-guided approach was then employed to isolate and determine the chemical identity of bioactive constituents with antifungal activity from strain neau-D50. The structure of the antifungal metabolite was elucidated as borrelidin on the basis of spectral analysis. To our knowledge, this is the first report that borrelidin has strong antifungal activity against dominant race 1 of *P. sojae* with EC_{50} and EC_{95} of 0.0056 and 0.026 mg/L, respectively. The values were respectively 62.5- and 262.3-fold lower than those of the commercial fungicide metalaxyl, which has been used to treat soybean seed for the control of *P. sojae*. The in situ bioassays demonstrated that borrelidin at 10 mg/L reduced *P. sojae* race 1 lesions on soybean seedlings by 94.72% without affecting root growth. Thus, borrelidin might be a promising candidate for new antifungal agents against *P. sojae*.

KEYWORDS: *antifungal activity, borrelidin, Phytophthora sojae, Streptomyces sp. neau-D50*

INTRODUCTION

Phytophthora root and stem rot, caused by *Phytophthora sojae* Kaufmann & Gerdemann, is a destructive disease affecting soybean production throughout the world. The disease was first reported to be associated with soybean [*Glycine max* (L.) Merr] in 1958 by Kaufmann and Gerdemann.[1] In China, this disease was observed in 1991 by Shen and Su[2] in Heilongjiang Province, which is the largest soybean-producing area in China. Since then, the disease has become widespread in Heilongjiang, and the annual infected crop area is estimated at over 150 000 ha.[3,4] To date, 14 races of *P. sojae* and a number of pathotypes of *P. sojae* have been reported in China, among which race 1 is the dominant race in Heilongjiang Province.[4−7]

Race-specific resistance of soybean cultivars has been the most important method to control *P. sojae*. However, race-specific resistance can promote the buildup of new pathogen races and result in the failure of resistant soybean cultivars.[8−13] Therefore, it is necessary to develop alternative or complementary control methods. The fungicide metalaxyl has been used to supplement soybean genetic resistance against root diseases. Although the seed-applied formulation of metalaxyl, Apron (Gustafson Inc., Plano, TX), has offered short-term protection against infection of seeds and seedlings by *P. sojae*, research has indicated that metalaxyl has reduced effectiveness in some cases due to resistance evolved by *Phytophthora* root rot strains.[14,15] Thus, development of new antifungal agents of natural origin would benefit the control of this disease.

In an effort to discover antifungal compounds with novel chemical structures or modes of action against *P. sojae*, a potential endophytic antagonist, *Streptomyces* sp. neau-D50, effective against the growth of *P. sojae*, was isolated from healthy soybean root by an in vitro screening technique, and the methanolic extracts from the mycelium were tested for their antifungal activities. An antifungal compound was subsequently isolated and identified as borrelidin. Borrelidin, an unusual polyketide with a trans and a cis double bond, has been reported to possess many biological activities, including antibacterial activity,[16] antiviral activity,[17] antimalarial activity,[18] insecticidal and herbicidal activity,[19] and antiangiogenesis activity.[20] However, borrelidin has not found application as a medicine due to its high toxicity, which was confirmed by an experiment with nude mice in which the maximum tolerated dose (MTD) was in the range of 5−15 mg/kg.[21] In 2001, the antifungal activity of borrelidin against phytopathogenic fungi in vitro was first disclosed by a patent,[22] which prompted us to evaluate the antifungal activity of borrelidin against *P. sojae* in vitro. A surprising result was then obtained that borrelidin was more specific to *P. sojae* than to other reported fungi. Thus, we further assessed the in situ antifungal activity and the phytotoxicity by applying borrelidin onto the germinated soybean seeds. The results suggesting that borrelidin might have the potential to be further developed as a novel agricultural antifungal agent against *P. sojae*.

MATERIALS AND METHODS

Chemicals. Metalaxyl 95% technical material (TC) was obtained from Jiangsu Lufeng Biology Medicine Industry Co., Ltd., Yancheng, China. Metalaxyl 25% a.i. wettable powder (WP) was purchased from Hebei Shuangji Chemical Co., Ltd., Hebei, China. All other chemicals used were of analytical grade and were purchased from Nanjing Chemical Reagent Co., Ltd., Nanjing, China. Methanol (MeOH) of liquid chromatographic grade was obtained from Merck, Inc.

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Biological Material. *P. sojae* race 1, 3, 4, 5, 9, 13, 44, and 54 were kindly provided by Soybean Research Institute of Northeast Agricultural University (Harbin, China); *Phytophthora capsici* was kindly provided by the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences (Beijing, China). All nine isolates were maintained on carrot agar medium (CA: carrot, 200 g; agar, 15 g; distilled water, 1 L) in the dark at 28 °C. *P. sojae* race 1 has been deposited in the China General Microbiological Culture Collection Center (CGMCC 3.14914). Soybean seeds (Hefeng-25) were kindly provided by Soybean Research Institute of Northeast Agricultural University (Harbin, China).

Isolation and Identification of Strain Neau-D50. Strain neau-D50 was isolated from healthy soybean root collected from Harbin, Heilongjiang province, north China (45°45′N, 126°41′E), by using moist incubation and desiccation (MI&D) method.[23] After 21 days of aerobic incubation at 28 °C, colonies were transferred and purified on oatmeal agar (International *Streptomyces* Project medium no. 3, ISP3) and maintained as glycerol suspensions (20%, v/v) at −80 °C. Genomic DNA of strain neau-D50 was isolated by the standard procedures[24] and PCR amplification of 16S rDNA gene was performed as previously described.[25] The amplified DNA was sequenced and compared with those of the other strains from the GenBank database. Clustal X 1.83 software was used to determine the matching alignment of multiple sequences, and Mega 4.0 was employed to calculate the evolutionary distance. The matrix distance through sequence data was calculated according to the "Kimura two-parameter" technique.[26] The phylogenetic tree was then constructed with the neighbor-joining method of Mega 4.0 software.[27] The topology structure's stability of the phylogenetic tree was appraised by using a bootstrap value with 1000 repeats.[28]

Activity Evaluation of Culture Extracts of Neau-D50 against *P. sojae*. Strain neau-D50 was cultured on ISP3 medium slants. The spore suspension was prepared from the agar slants incubated at 28 °C for 7 days. Then a total of 9 mL of sterile water was added to the slant medium. The spores were scraped with a sterile glass rod. The spore suspension was filtered through four layers of cheese cloth to remove the mycelia. The concentration of spores was determined by means of a hemocytometer and adjusted to 1×10^6 spores/mL with sterile distilled water. One milliliter of the suspension was transferred to a 250-mL Erlenmeyer flask containing 50 mL of medium consisting 0.4% glucose, 1% maltodextrin, 0.4% yeast extract, 0.2% $CaCO_3$, pH 7.2−7.4. The culture was incubated at 28 °C for 48 h on a rotary shaker at 250 rpm. Then 4 mL of the culture was transferred into a 250-mL Erlenmeyer flask containing 50 mL of medium consisting of glucose (1%), soluble amylum (4%), yeast extract (0.5%), soybean powder (2.5%), peptone (0.5%), $CaCO_3$ (0.2%), $MgSO_4{\cdot}7H_2O$ (0.8%), $FeSO_4{\cdot}7H_2O$ (0.6%), $ZnSO_4{\cdot}7H_2O$ (0.2%), $MnSO_4{\cdot}H_2O$ (0.2%), $CoCl_2{\cdot}6H_2O$ (0.05%), $Na_2MoO_4{\cdot}2H_2O$ (0.2%), pH 7.0. After 5 days of cultivation, a total of 50 mL of fermentation broth was filtered and the mycelial cake was extracted for about 10 h with 50 mL of methanol. The methanol extract was then used for antifungal activity screening. The inhibitory effect of the extract obtained from the isolate was tested using the paper disk (diameter, 7 mm) assay method.[29] Five-millimeter disks of the target fungi strains, taken from the fresh margin of the mycelium grown on CA medium, were placed in the center of the agar plates. A 25 μL volume of the methanol extract suspension was pipetted onto each disk and incubated at 28 °C, and the diameters of the inhibition zones were measured after 7 days. A 25 μL volume of methanol was used as the control.

Isolation and Characterization of the Antifungal Compound. The antifungal compound was isolated from the ethanol extract using an in vitro antifungal activity-guided method. A total of 3 L of fermentation broth was filtered. The resulting cake was washed with water, and both filtrate and wash were discarded. The washed cake was extracted twice for about 24 h with EtOH (1 L). The EtOH extract was diluted with water to about 30% EtOH and subjected to a HP-20 resin column eluting with 30%, 40%, 50%, 60%, 70%, and 80% EtOH (each concentration eluted 2 bed volumes). The eluents eluting with 70% and 80% EtOH were pooled and concentrated in vacuo at 50 °C to give a mixture. The mixture was subjected to chromatography on a silica gel column (200−300 mesh, Qingdao Marine Chemical Factory, Qingdao, China) and successively eluted with a stepwise gradient of petroleum ether/acetone (100:0−90:10, v/v) to obtain five fractions (fractions I−V). The bioactive fraction IV was then subjected to Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and eluted with EtOH and detected by TLC to obtain five fractions (A1−A5). The active fraction A1 was further separated by semipreparative HPLC (Agilent 1100, Zorbax SB-C18, 5 μm, 250 × 9.4 mm i.d.; 1.5 mL/min; 254 nm; Agilent, Palo Alto, CA) eluting with CH_3OH/H_2O (80:20, v/v), and a potent active principle (t_R 12.6 min, 170 mg) was finally isolated.

Spectrometry. The UV spectrum was obtained on a Varian CARY 300 BIO spectrophotometer. The IR spectrum was recorded on a Nicolet Magna FT-IR 750 spectrometer (ν_{max} in cm^{-1}). ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured with a Bruker DRX-400 spectrometer (Bruker, 268 Karlsruhe, Germany). Chemical shifts are reported as parts per million (δ), using the residual $CHCl_3$ (δ_H 7.26, δ_C 77.0) as an internal standard, and coupling constant (J) is reported in hertz. ^1H and ^{13}C NMR assignments were supported by the HMBC experiment. The ESI-MS and HRESI-MS spectra were taken on a Q-TOF Micro LC−MS−MS mass spectrometer (Agilent, Palo Alto, CA).

Structural Elucidation of Compound 1. Borrelidin (Figure 1), $C_{28}H_{43}O_6N$, light-yellow crystals. UV (EtOH) λ_{max} nm (log ε): 257 (4.25). IR (KBr) ν_{max} cm^{-1}: 3443, 2958, 2213, 1723, 1638, 1460, 1379, 1253, 1175, 1034, 972. ^1H NMR ($CDCl_3$): δ 6.83 (1H, d, J = 11.2 Hz, H-6), 6.39 (1H, dd, J = 14.5, 11.2 Hz, H-5), 6.21 (1H, m, H-4), 4.98 (1H, dt, J = 10.7, 3.2 Hz, H-2), 4.12 (1H, d, J = 9.6 Hz, H-8), 3.87 (1H, br d, J = 9.7 Hz, H-16), 2.71 (2H, m, H-2′), 2.60 (1H, m, H-3a), 2.57 (1H, m, H-3b), 2.49 (1H, m, H-1′), 2.41 (1H, dd, J = 15.8, 9.9 Hz, H-17a), 2.32 (1H, d, J = 15.8 Hz, H-17b), 2.03 (1H, m, H-5′a), 1.98 (1H, m, H-3′a), 1.92 (1H, m, H-5′b), 1.88 (1H, m, H-9), 1.82 (2H, m, H-4′), 1.68 (1H, m, H-15), 1.63 (1H, m, H-11), 1.58 (1H, m, H-13), 1.38 (1H, s, H-3′b), 1.22 (1H, m H-14a), 1.11 (1H, m, H-12a), 1.05 (1H, m, H-10a), 1.05 (3H, d, J = 6.4 Hz, H-19), 0.98 (1H, m, H-12b), 0.94 (1H, m, H-14b), 0.84 (3H, d, J = 6.4 Hz, H-20), 0.83 (3H, d, J = 6.7 Hz, H-21), 0.80 (3H, d, J = 6.2 Hz, H-22), 0.73 (1H, m, H-10b). ^{13}C NMR ($CDCl_3$): δ 180.1 (s, COOH), 172.2 (s, C-18), 144.0 (d, C-6), 138.5 (d, C-4), 127.0 (d, C-5), 118.3 (s, CN), 115.9 (s, C-7), 76.5 (d, C-2), 73.1 (d, C-8), 69.9 (d, C-16), 48.5 (d, C-1′), 47.8 (t, C-12), 45.8 (d, C-2′), 42.9 (t, C-14), 39.2 (t, C-17), 37.4 (t, C-10), 35.9 (t, C-3), 35.5 (d, C-15), 35.1 (d, C-9), 31.2 (t, C-5′), 29.6 (t, C-3′), 27.0 (d, C-13), 26.2 (d, C-11), 25.2 (t, C-4′), 20.2 (q, C-20), 18.2 (q, C-22), 16.9 (q, C-21), 14.9 (q, C-19). ESI-MS m/z 488 $[M - H]^-$; HRESI-MS m/z 512.2982 $[M + Na]^+$ (calcd $C_{28}H_{43}NNaO_6$ for 512.2983).

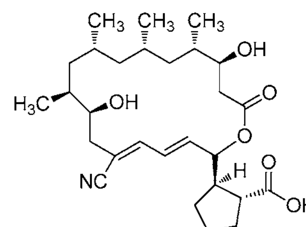

Figure 1. Structure of borrelidin.

Activity Evaluation of Borrelidin against *P. sojae* in Vitro. Borrelidin was dissolved in methanol and diluted with water to a different concentration, which was then added to CA media; a 0.2% methanol plate was used as negative control and metalaxyl at the different concentrations was used as positive controls. The fungus plug (5 mm diameter) of mycelial inocula was cut from the margin of the mycelium grown on CA medium and placed in the center of the agar plate. Experiments were performed in triplicate. After the control group was covered with fungal mass, the diameters covered with fungal mass were recorded and converted to inhibition activity rate, and the antifungal indices were calculated as follows

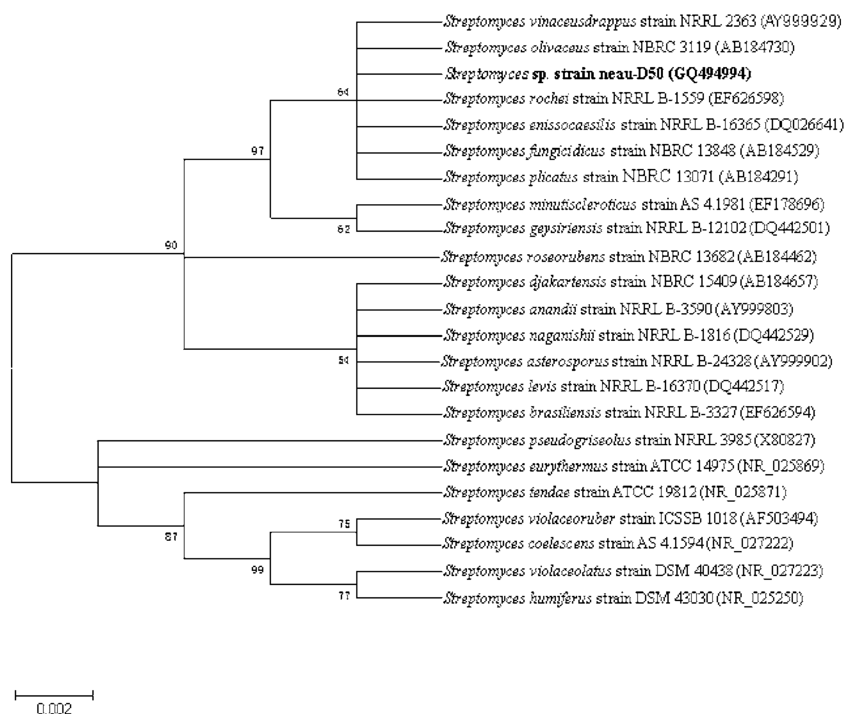

Figure 2. Neighbor-joining tree showing the phylogenetic position of *Streptomyces* sp. neau-D50 and related taxa based on 16S rDNA gene sequences. The numbers on the branches are confidence limits (expressed as percentages) estimated from a bootstrap analysis with 1000 replicates. The bar represents 0.002 substitutions per nucleotide position.

$$\text{antifungal index (\%)} = (1 - D/D_c) \times 100$$

where D is the diameter of growth zone in the experimental dish (cm) and D_c is diameter of growth zone in the control dish (cm). Each test was repeated three times and the average was calculated. Regression equation was conducted according to the colony diameter at the different concentrations of compounds. The effective concentrations required for 50% inhibition (EC_{50}) and 95% inhibition (EC_{95}) for each *P. sojae* race were calculated on the basis of the linear regression equations.

Efficacy of Borrelidin against *P. sojae* in Situ. The efficacy of borrelidin against *P. sojae* in situ was assessed as previously described.[30] Soybean seeds were sterilized with 10% NaOCl for 20 min, rinsed three times with sterilized water, and germinated aseptically in an incubator at 30 °C until the root length of each seed was longer than 10 mm. Batches of five germinated seeds were placed evenly on water-soaked filter papers in Petri dishes. Borrelidin was diluted in water with 1% Tween 80 at concentrations of 1, 5, and 10 mg/L, and 25% metalaxyl WP dissolved in water at concentrations of 4, 20, and 40 mg/L (i.e., 1, 5, and 10 mg a.i./L, respectively) was used as positive control. Then, 2 mL of the diluted borrelidin, 1% Tween 80 sterile water (negative control 1), and 25% metalaxyl WP were spread on the germinated seed roots, respectively. A fresh mycelial disk (7 mm in diameter) of *P. sojae* was placed directly on the seminal roots of soybean and removed after 24 h. The dishes were kept for 48 h at 28 °C, and the disease severity was rated using a 5-class scale: 0, healthy or no apparent discoloration; 1, less than 25% discoloration of the root; 2, 25−50% discoloration of the root; 3, 50−75% discoloration of the root; and 4, more than 75% discoloration of the root or dead plants. In order to observe the root length, 1% Tween 80 sterile water without *P. sojae* was used as negative control 2. The mean disease index and control efficiency were calculated using the following equations:

$$\text{disease index (\%)} = \Big[\sum (\text{plant numbers with the same grade of disease severity} \times \text{grade level}) / (\text{total plants} \times \text{number of rating scale}) \Big] \times 100$$

$$\text{disease control efficiency (\%)} = [1 - (\text{disease index of the treatment groups} / \text{disease index of the negative control group})] \times 100$$

Statistical Analysis. Results are expressed as means ± standard deviations (SDs). Data were analyzed using one-way analysis of variance (ANOVA), followed by each pair of Student's t tests for multiple comparisons. Differences were considered significant if $p < 0.05$. All analyses were performed using SPSS for Windows, version 13 (SPSS, Chicago, IL).

RESULTS

Isolation and Identification of Strain Neau-D50. In order to search for antifungal compounds against *P. sojae*, which cause severe soybean losses throughout the world, strain neau-D50 was isolated from healthy soybean root. The morphology of the strain neau-D50 on ISP3 medium is typical of *Streptomyces* with a gray color of the mycelium. Comparison of the almost complete 16S rDNA nucleotide gene sequence of strain neau-D50 (1518 nt) with corresponding streptomycete sequences clearly shows that the organism belongs to the genus *Streptomyces*. The isolate was most closely related to *Streptomyces rochei* strain NRRL B-1559, and the two strains shared a 16S rDNA gene sequence similarity of 99.87%, a value which corresponds to 2 nt differences at 1487 sites. The phylogenetic analysis of the 23 (22 references and 1 clone) aligned sequences was conducted with a 1K bootstrap. These

strains selected through a BLAST search showed maximum sequence homology percentage and query coverage as well as the lowest *E* value. The analysis of a 16S rDNA sequence homology report through BLAST and neighbor-joining (NJ) tree construction from aligned data identified the strain neau-D50 as one strain of *Streptomyces* (Figure 2). The 16S rDNA sequence of this strain has been submitted to the GenBank database under accession number GQ494994. Due to the potential application as a biocontrol agent, strain neau-D50 has been deposited in the China General Microbiological Culture Collection Center (accession number CGMCC 4.6963).

Activity Evaluation of Culture Extracts of Neau-D50 against *P. sojae*. In vitro antifungal activity assay demonstrated that the isolate showed particularly strong activity against *P. sojae.* As shown in Figure 3, the methanol extract from the mycelium of strain neau-D50 which was diluted 20-fold could completely inhibit the growth of *P. sojae*.

Figure 3. The antifungal activity of the methanol extract of *Streptomyces* sp. neau-D50 against *P. sojae* race 1. The test phytopathogen was incubated at 28 °C for 7 days. 1, methanol (negative control); 2, 50-fold diluted methanol extract; 3, 20-fold diluted methanol extract; 4, 10-fold diluted methanol extract; 5, methanol extract.

Structure Elucidation of the Antifungal Compound. An antifungal activity-guided separation of the components in the methanol extract of strain neau-D50 against *P. sojae*, using the in vitro antifungal assay, led to the isolation of compound **1** as its active principle. Compound **1** (Figure 1) was obtained as light-yellow crystals. Its mass spectrum ($[M - H]^-$ m/z = 488) and HRESI-MS ($[M + Na]^+$ m/z = 512.2982) were in accordance with a molecular formula of $C_{28}H_{43}NO_6$. The ^1H NMR spectrum of **1** showed three olefinic proton signals at δ 6.21 (1H, m), 6.39 (1H, dd, J = 14.5, 11.2 Hz), 6.83 (1H, dd, J = 11.2 Hz); three oxymethine protons at δ 4.98 (1H, dd, J = 10.7, 3.2 Hz), 4.12 (1H, d, J = 9.6 Hz), 3.87 (1H, br d, J = 9.7 Hz); and four aliphatic doublet methyls at δ 1.05 (3H, d, J = 6.4 Hz, 9-CH_3), 0.84 (3H, d, J = 6.4 Hz, 11-CH_3), 0.83 (3H, d, J = 6.7 Hz), 0.80 (3H, d, J = 6.2 Hz). Its ^{13}C NMR spectrum displayed 28 carbon resonances, including one carboxyl carbonyl at δ 180.1; one ester carbonyl at δ 172.2; eight methylenes at δ 47.8, 42.9, 39.2, 37.4, 35.9, 31.2, 29.6, 25.2; nine aliphatic methines (including three oxygenated ones) at δ 76.5, 73.1, 69.9, 48.5, 45.8, 35.5, 35.1, 27.0, 26.2; and four methyl carbons at δ 20.2, 18.2, 16.9, 14.9, in addition to five downfield carbon signals at δ 144.0 (d), 138.5 (d), 127.0 (d), 118.3 (s), 115.9 (s). The IR spectrum of **1** in KBr showed absorptions at 3443, 2958, 2213, 1723, 1638, 1460, 1379, 1253, 1175, 1034, and 972 cm^{-1}. Furthermore, the UV spectrum of **1** in ethanol gave UV maxima at 257 nm. These ^1H NMR, ^{13}C NMR, UV, and IR data were identical to those of borrelidin[31] and were used to assign the structure of **1** as borrelidin.

Antifungal Activity of Borrelidin against *P. sojae*. The in vitro antifungal activity of borrelidin against eight races of *P. sojae* was determined at various concentrations. The EC_{50} and EC_{95} values of borrelidin were evaluated and are listed in Table 1. The inhibitory effect was enhanced with increased concentrations, indicating that the antifungal activity of borrelidin was dose-dependent. The findings were in agreement with the results obtained from the in vitro antifungal assay of methanol extract, and it further confirmed that borrelidin was the main antifungal constituent produced by strain neau-D50.

In a simulated in situ disease control experiment, borrelidin displayed great efficacy in control of soybean root disease caused by *P. sojae* race 1 (Table 2). In comparison to that of the negative control 1 (soybean inoculated with *P. sojae*, in the absence of test chemicals), the length of cortical browning of the roots was shortened and lessened significantly after treatment with borrelidin. The control efficacy of borrelidin was 94.72% to *P. sojae* race 1 at 10 mg/L, which was significantly higher than that obtained metalaxyl at 10 mg/L (87.70%). The length of roots treated with borrelidin at 10 mg/L were 6.81 ± 0.21 cm, which showed little change in comparison to the negative control 2 (soybean inoculated without *P. sojae* and test chemicals) (6.88 ± 0.35 cm). Thus, borrelidin had not an effect on soybean seedling root growth at the concentration of 10 mg/L.

Table 1. EC_{50} and EC_{95} Values of Borrelidin and Metalaxyl against Races of *P. sojae*[a]

	borrelidin		metalaxyl	
P. sojae race[bc]	EC_{50} (mg/L)	EC_{95} (mg/L)	EC_{50} (mg/L)	EC_{95} (mg/L)
race 1	0.0056 ± 0.0002	0.026 ± 0.004	0.35 ± 0.03	6.82 ± 0.10
race 3	0.0051 ± 0.0003	0.021 ± 0.004	0.17 ± 0.02	4.63 ± 0.08
race 4	0.0038 ± 0.0002	0.017 ± 0.002	0.18 ± 0.04	8.03 ± 0.09
race 5	0.0052 ± 0.0004	0.020 ± 0.003	0.30 ± 0.02	11.58 ± 0.40
race 9	0.0053 ± 0.0003	0.020 ± 0.003	0.34 ± 0.04	11.81 ± 0.19
race 13	0.0057 ± 0.0003	0.021 ± 0.001	0.32 ± 0.03	7.55 ± 0.13
race 44	0.0047 ± 0.0002	0.018 ± 0.003	0.28 ± 0.03	12.25 ± 0.25
race 54	0.0057 ± 0.0005	0.021 ± 0.003	0.30 ± 0.02	13.28 ± 1.11

[a]Data are the average of three experiments and they were analyzed using one-way ANOVA ($p < 0.05$). [b]Races 1, 3, 4, 5, 9, 13, 44, 54 are from Zhang et al.[4] [c]All eight races were grown for 7 days in the presence of test chemicals on CA medium at 28 °C. Zones of inhibition were measured in cm.

Table 2. Antifungal Activity of Borrelidin against *P. sojae* Race 1 in Situ[a]

treatments	dose (mg/L)	root length (cm)	lesion length (cm)	disease control efficiency (%)
control		4.15 ± 0.71	3.34 ± 0.64	
borrelidin	1	6.24 ± 0.75	1.91 ± 0.76	56.25 ± 4.38
	5	6.53 ± 0.46	0.73 ± 0.58	80.76 ± 2.29
	10	6.81 ± 0.21	0.25 ± 0.52	94.72 ± 0.28
25% metalaxyl WP	4	6.21 ± 0.69	2.57 ± 1.18	45.52 ± 4.05
	20	6.48 ± 0.26	1.34 ± 0.66	71.97 ± 1.85
	40	6.73 ± 0.71	0.62 ± 0.71	87.70 ± 3.07

[a]Soybean seeds were germinated aseptically in an incubator at 30 °C for 4 days, and then 2 mL of test chemicals were spread on the germinated seed roots. A fresh mycelial disk (7 mm in diameter) of *P. sojae* was placed directly on the seminal roots of soybean, and removed after 24 h. Root length and lesion length (cm) were measured after 48 h of incubation. Dose (a.i.) of 25% metalaxyl WP = dose of 25% metalaxyl WP × 0.25. Data are the average of three experiments and they were analyzed using one-way ANOVA ($p < 0.05$).

DISCUSSION

Endophytic actinobacteria, which exist in the inner tissues of living plants, are known to produce a variety of bioactive metabolites with antibiotic, enzymatic, and plant growth-promoting or inhibiting activities.[32] There is evidence that actinomycetes are fairly prevalent in crops, such as maize,[33] wheat,[34] Chinese cabbage,[35] and tomato plants,[36] and some of these isolates have been shown to possess the ability to inhibit a wide variety of harmful fungi. Thus, there is great application value to develop antifungal agents from endophytic actinobacteria. Until now, many antifungal antibiotics have been isolated from endophytic actinobacteria. 6-Prenylindole, a simple molecule isolated from the culture broth of endophytic strain *Streptomyces* sp. TP-A0595, exhibited significant antifungal activity against plant pathogen *Fusarium oxysporum*.[37] Two novel compounds, cedarmycins A and B, were also isolated from the strain *Streptomyces* sp. TP-A0456, and showed in vitro antifungal activity against *Candida glabrata* with a MIC value of 0.4 μg/mL.[37] Actinomycin D was produced by endophytic *Streptomyces* sp. Tc022, and it displayed strongly inhibited *Colletotrichum musae* and *Candida albicans*.[38] Recently, a new antimycotic compound, saadamycin, was isolated from endophytic *Streptomyces* sp. Hedaya48, and it exhibited significant antimycotic activity against dermatophytes and other clinical fungi.[39] These studies listed above reinforced the assumption that endophytic actinobacteria could be a promising source of antifungal substances.

Phytophthora root and stem rot of soybean caused by *P. sojae* has a long history in China. The results obtained in this study demonstrated that an endophytic strain, *Streptomyces* sp. neau-D50 antagonistic to *P. sojae* was isolated.

To learn more about the chemical nature of the antifungal activity of the culture filtrate, the active compound borrelidin was finally obtained. Borrelidin was first isolated from a soil sample of *S. rochei* and subsequently identified from other *Streptomyces* species such as *Streptomyces* sp. C2989, *Streptomyces albovinaceous*, *Streptomyces griseus* BS1325, *Streptomyces candidus* Y21007-2, *Streptomyces parvulus* Tü4055, and *Streptomyces californicus*.[16,19,22,31,40−42] To our knowledge, this is the first report that borrelidin can be produced by an endophytic *Streptomyces*.

Borrelidin has been demonstrated to have various biological activities. However, it is not a successful human pharmaceutical due to the high cytotoxicity.[21] In 2001, Shiang et al. first described the in vitro antifungal activity of borrelidin against phytopathogenic fungi, such as *Pythium aphanidermatum*, *Pythium splendens*, *Pythium sylvaticum*, *Pythium ultimum*, and *Phytophthora capsici*, with EC_{50} values ranging from 0.01 to 0.10 mg/L, and proposed that borrelidin might be developed as an antifungal agent used in agriculture. In this paper, we tested the in vitro antifungal activity of borrelidin against eight races of *P. sojae*, with EC_{50} values ranging from 0.0038 to 0.0057, which were 1−2 orders lower than those against other reported fungi.[22] In addition, EC_{50} and EC_{95} values of borrelidin were manyfold lower than those of the commercial fungicide metalaxyl, respectively. In order to validate the applicability of the method employed in an in vitro antifungal activity test method, we evaluated the antifungal activity of borrelidin against *P. capsici*. The result (EC_{50} = 0.025 mg/L) was consistent with the value (0.02 mg/L) reported by the patent,[22] which supported the validity of the test method used in this study. The in situ antifungal activity of borrelidin against *P. sojae* race 1 treated with 1, 5, and 10 mg/L was determined (Table 2). The control efficacy of borrelidin was 94.72% to *P. sojae* race 1 at 10 mg/L, which was much higher than that of the positive control (87.70%). On the basis of the length of roots tested, the in situ phytotoxic rating of borrelidin was similar to that of a water control, and thus, borrelidin was not phytotoxic at a concentration of 10 mg/L.

Phenylamide fungicides including the acylalanines (metalaxyl, furalaxyl, and benalaxyl), the butyrolactones (ofurace and cyprofuram), and oxazolidinone (oxadixyl) showed a high and selective activity against *P. sojae*.[43] Among of them, metalaxyl was most effective when applied to the soil, as it allows the plant to take it up through the roots and elongate the control period, and it can prevent the spores of *P. sojae* from entering the soybean plant tissues.[44] However, due to its single site of action and years of use, resistant strains have been selected.[15] Thus, there is a continuing need for more effective fungicides, especially those with novel modes of action, and natural products have a key role in the search for such compounds. Recently, a natural compound, farinomalein, was isolated from the entomopathogenic fungus *Paecilomyces farinosus*, which showed potent inhibition of *P. sojae*, with an MIC value of 5 μg/disk.[45] In the present study, our results showed that borrelidin exhibited higher antifungal activity than that of metalaxyl against *P. sojae* in vitro and in situ, which suggest that borrelidin might be useful as a candidate pesticide for the treatment of *Phytophthora* stem rot in soybean and will stimulate further studies using similar borrelidin-producing strains as biocontrol agents against *P. sojae*. Further research is needed to study the mode of action of borrelidin against *P. sojae*. Furthermore, the efficacy of in situ disease control provided by borrelidin should be confirmed in field trials.

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Notes

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