The Lethal Effect of *Bacillus subtilis* Z15 Secondary Metabolites on *Verticillium dahliae*

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Abstract

*Bacillus subtilis* Z15 (BS-Z15), isolated from cotton rhizosphere soil, inhibits *Verticillium dahliae* and suppresses cotton Verticillium wilt in pot experiments. We investigated the influence of environmental factors, pH, temperature, ultraviolet light, protease, and incubation time on the stability of BS-Z15 secondary metabolites (SMs), and the mechanism underlying the cytotoxicity of BS-Z15 SMs on *V. dahliae*. BS-Z15 and its fermentation broth inhibited *V. dahliae*, and this effect was mediated by its SMs. These were shown to be stable to the influence of the above environmental factors. BS-Z15 SMs decreased the viability of *V. dahliae* conidia in a time-dependent manner. Scanning electron microscopy showed that BS-Z15 and its SMs resulted in flattened and depressed conidia. BS-Z15 SMs induced morphological abnormalities in the hyphae, which showed rough aberrant structures, reduced conidiophore production, and accelerated aging. Flow cytometry using Hoechst/propidium iodide double staining revealed that BS-Z15 SMs induced necrosis in *V. dahliae* in a time-dependent manner. Fluorescence microscopy showed that BS-Z15 SMs did not induce apoptotic bodies in the conidia of *V. dahliae* but caused significant changes in karyotypes, accompanied by nuclear lysis and nucleic-acid diffusion, which may play important roles in necrosis. In addition, 0.3 mg/mL BS-Z15 SMs had no effect on either the mitochondrial membrane potential or the synthesis of proapoptotic proteins, indicating that the SMs did not induce apoptosis in *V. dahliae*. Their lethal effect on *V. dahliae* was by inducing necrosis in its conidia and hyphae. BS-Z15 SMs thus have potential as biological pesticides to control Verticillium wilt in cotton.

Keywords

*Bacillus subtilis* BS-Z15, antifungal secondary metabolites, *Verticillium dahliae*, cytotoxicity, necrosis

Received: September 26th, 2020; Accepted: December 15th, 2020.

Verticillium wilt in cotton was first discovered in Tennessee and California in 1927 and 1930. Since then, the disease has been reported almost simultaneously from major cotton-growing areas of the world. In China, more than 40% of the cotton-growing area is threatened by Verticillium wilt, particularly cotton in the Xinjiang province of China. Verticillium wilt in cotton, caused by the soil-inhabiting fungus *Verticillium dahliae* Kleb., is difficult to control, due to the formation of microsclerotia, which exist in the soil for a long time. Cultural practices, use of resistant varieties, and application of chemical fungicides are the most common strategies used to control Verticillium wilt of cotton. Chemical fungicides, such as the traditional pesticide methyl bromide, have been gradually eliminated because of their threat to the ecological environment and human health. However, the new chemical pesticide control spectrum is insufficient, and there are still potential adverse effects on the environment. Biological control is a technology and method

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to control crop diseases and insect pests through the role of some microorganisms and microbial metabolites.6,7

Biological control is the most promising environmental strategy for controlling Verticillium wilt in cotton.8,9 This method is highly appropriate for controlling fungal growth and decreasing the levels of agrochemical residues during crop cultivation.10-12 The products extracted from microorganisms can be used as an ideal substitute for chemically synthesized antifungal agents to improve the quality of agricultural products.4

Biocontrol fungi such as *Trichoderma* spp.13 and biocontrol bacteria such as *Bacillus subtilis*,14,15 *B. amyloliquefaciens*,16 and *Pseudomonas* spp.8,17 are widely used for controlling Verticillium wilt. In particular, *B. subtilis* is considered to be a probiotic and causes no harm to the environment and human body.18 Therefore, *B. subtilis*, which can effectively antagonize *V. dahliae*, is an ideal strain to control Verticillium wilt in cotton.

*Bacillus subtilis* shows promise as an environmentally safe biocontrol agent and promotes the growth of rhizobacteria.19 It produces various antifungal compounds including ribosomally synthesized (lantibiotics) and nonribosomally synthesized (lipopeptides) peptides, as well as nonpeptide compounds such as polyketides. Lipopeptides have considerable antifungal activity.20-22 Tang examined the mechanism underlying the cytotoxicity of fengycin on *Rhizopus stolonifer* and reported that low and high concentrations of fengycin trigger apoptosis and necrosis, respectively.23 Jiang demonstrated that surfactin damages cells, causing leakage of nucleic acids and proteins and inducing apoptosis in *Fusarium moniliforme*.24 Antifungal protein agglutinin attacks the cell wall and plasma membrane, decreasing the mitochondrial membrane potential and inducing programmed cell death in *Rhizoctonia solani*.25 Understanding the antifungal mechanism of biological control agents will provide theoretical support for the efficacy of biological pesticides. However, the biocontrol of pathogenic fungi can occur via a variety of mechanisms by the use of different *B. subtilis* strains, so it is necessary to study the antifungal mechanism of this bacterium to screen for effective biocontrol agents against *V. dahliae*.

In the early stage of this study, a bacterial strain was isolated from the soil of a continuously cropped cotton field in Heshuo County, Xinjiang; the strain, named BS-Z15, was identified as *B. subtilis*, based on its genome sequence (Figure S1(c) provided in the Supplemental Material). We found that the fermentation product of BS-Z15 had a good control effect on Verticillium wilt in the laboratory26 and had a strong antagonistic effect on *V. dahliae* by the dural culture method on potato dextrose agar (PDA) plates with pathogens.27 Although BS-Z15 has a high efficacy to control Verticillium wilt in the laboratory, it is necessary to study its cytotoxic mechanism to pathogenic bacteria in order to popularize it into production practice. In this study, we investigated the cytotoxic mechanism of BS-Z15 secondary metabolites (SMs) against *V. dahliae*. Our findings will facilitate the biological control of Verticillium wilt of cotton using BS-Z15 and its SMs.

Results

The Inhibitory Substances of BS-Z15 on *V. dahliae* Are Its Secreted Secondary Metabolites

BS-Z15 was isolated from cotton rhizosphere soil and stored in our laboratory (Figure S1(a) provided in the Supplemental Material). As shown by scanning electron microscopy (SEM), BS-Z15 is a typical short rod bacterium without flagella or capsule (Figure S1(b) provided in the Supplemental Material).

The antifungal activities of BS-Z15 fermentation liquid and cell-free fermentation liquid were determined by the double-plate agar diffusion assay. Both BS-Z15 fermentation liquid and cell-free fermentation liquid strongly antagonized *V. dahliae* (Figure 1(A)). Thus, the effect of BS-Z15 on *V. dahliae* is

Figure 1. Antifungal activities of BS-Z15 and its SMs. (A) Antifungal activity of BS-Z15 against *Verticillium dahliae*. 1: Anti-*V. dahliae* activity of BS-Z15; 2: anti-*V. dahliae* activity of sterile fermentation broth. (B) Effect of the nitrogen source on BS-Z15 growth and antifungal SMs in basic medium. I: no nitrogen source; II: 1% peptone; III: 1% yeast extract; IV: 1% KNO3; V: 0.5% KNO3; VI: 0.25% KNO3. (*P < 0.05, **P < 0.01 compared with no nitrogen source-treated group). Values are mean ± SD, where n = 3; significant values are given by the analysis of variance (Dunnett’s *t*-test). The medium composition is provided in the Supplemental Material Table S1. KNO3, potassium nitrate; SM, secondary metabolites.
mediated by its metabolites. We next investigated BS-Z15 growth and production of antifungal metabolites in the presence of different nitrogen sources. BS-Z15 growth was strong when either 1% peptone or 1% yeast extract was used as the nitrogen source (Figure 1(C)), and the cell-free fermentation liquid inhibited *V. dahliae* (Figure 1(B)). BS-Z15 showed strong growth but did not inhibit *V. dahliae* when either 0.25% or 0.5% potassium nitrate (KNO₃) was used as the nitrogen source (Figure 1(B) and (C)). This suggests that the antifungal metabolites of BS-Z15 are not a prerequisite for the growth of the strain, which indicates that the antifungal substances are secondary metabolites (SMs).

### Influence of Environmental Factors on the Stability of BS-Z15 SMs

When the pH was adjusted to 7, the precipitate dissolved, and the antifungal activity did not significantly decrease. At pH >4.5, the BS-Z15 SMs did not precipitate and retained high anti-*V. dahliae* activity. At pH >11, the activity was significantly decreased (Figure 2(A)). Thus, the BS-Z15 SMs remained active under acidic or weak alkaline conditions; but under strong alkaline conditions, the BS-Z15 SMs underwent a conformational change, resulting in a significant decrease in anti-*V. dahliae* activity. After protease K and pepsin treatment at 37 °C for 3 hours, the antifungal activity of the BS-Z15 SMs did not decrease (Figure 2(B)), indicating that the active component is not proteinaceous. Treatment of the BS-Z15 SMs for 1 hour at −80 °C to 100 °C did not affect its activity (Figure 2(C)). The activity was also not significantly decreased by ultraviolet (UV) irradiation at 254 and 189 nm, at a power of 30 W for 2 hours (Figure 2(D)).

Therefore, BS-Z15 SMs have strong stability for different pH values, against protease, temperature, UV irradiation, and incubation duration on BS-Z15 cell density and so have great development and utilization value in the subsequent role.

### Effect of BS-Z15 SMs on *V. dahliae*

The *V. dahliae* fungal block grew well on Czapek-Dox agar in the absence of BS-Z15 SMs, and its diameter increased consistently (Figure S2(a) provided in the Supplemental Material). However, mycelial growth was significantly inhibited on Czapek-Dox agar containing BS-Z15 SMs (Figure S2(b) provided in the Supplemental Material), with no change observed in the diameter of the fungal block (Figure 3(A)).

The effect of BS-Z15 SMs on the spore activity of *V. dahliae* was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The absorbance at 560 nm of untreated *V. dahliae* increased over time, indicating an increase...
in the number of viable cells (Figure 3(B)). Spore activity decreased significantly in a time-dependent manner after treatment with 0.3 mg/mL BS-Z15 SMs. Therefore, BS-Z15 SMs significantly reduced the spore activity of *V. dahliae*.

**Effect of BS-Z15 SMs on the Morphology of *V. dahliae* Conidia**

*Verticillium dahliae* conidia were treated with 0.3 mg/mL BS-Z15 SMs for 12, 24, 48, and 72 hours. The control conidia showed satiation and had a smooth surface. Those conidia with an ellipsoidal shape were preparing for germ tube elongation (Figure 4(A-1)), while the smaller rod-shaped conidia were not (Figure 4(A-2)). Other conidia had a germ tube and sunken surface, showing signs of aging (Figure 4(A-3)). A large proportion of the treated conidia had a crinkled, squashed, and sunken surface, and the magnitude of this appearance increased over time (Figure 4(B)–(E)). Therefore, BS-Z15 SMs are capable of killing *V. dahliae* conidia.

*Verticillium dahliae* conidia were incubated with BS-Z15 for 24 and 48 hours, and their morphology was visualized by SEM. As shown in Supplemental Material Figure S3(a), most of the untreated conidia appeared normal, whereas a few were crinkled, indicating loss of viability. After 24 hours, the conidia had shrunk and had a depressed surface, the magnitude of which was greater after 48 hours (Figure S3(b) and (c)) provided in the Supplemental Material). Furthermore, most BS-Z15 cells adhered to the surface of *V. dahliae* conidia. Therefore, BS-Z15 is capable of strongly adhering to *V. dahliae* conidia. This phenomenon has not been reported for antagonistic fungi of *B. subtilis*.

The lethal effect of strain BS-Z15 on *V. dahliae* was mediated by SMs and was not dependent on the presence of viable BS-Z15 cells.

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**Figure 3.** Antifungal activity of BS-Z15 secondary metabolites. (A) Diameter of a *Verticillium dahliae* block on Czapek-Dox agar (*P* < 0.05, **P** < 0.01 compared with control group). (B) Conidia activity of *V. dahliae*. (●) Control and (○) Czapek-Dox containing 0.3 mg/mL BS-Z15 (*P* < 0.05, **P** < 0.01 compared with control group). Values are mean ± SD, where *n* = 3; significant values are given by analysis of variance (Dunnett’s t-test).

**Figure 4.** Effect of BS-Z15 secondary metabolites (SMs) on the morphology of *V. dahliae* Conidia. (A) Untreated *V. dahliae* Conidia (control), 1, *V. dahliae* conidia preparing for germ tube elongation; 2, *V. dahliae* conidia not preparing for germ tube elongation; 3, *V. dahliae* conidia with a germ tube. (B–E), *Verticillium dahliae* conidia treated with 0.3 mg/mL BS-Z15 SMs for 12, 24, 48, and 72 hours. Red boxes in the row I panels encompass the areas enlarged in the row II panels below (magnifications: I, 2700×; II, 8000×).
Effect of BS-Z15 SMs on the Morphology of *V. dahliae* Hyphae

Untreated hyphae grew normally and were constant in diameter. Moreover, hyphal tips produced well-developed conidiophores with a smooth surface (Figure 5(A)). After treatment with 0.3 mg/mL BS-Z15 SMs for 12 hours, hyphae were rough, expanded, and aged, with unevenly distributed contents and with either decreased or absent conidiophore production (Figure 5(B)). After treatment with BS-Z15 SMs for 72 hours, *V. dahliae* hyphal networks were either collapsed or distorted, hyphae enlargements were ruptured, and conidia were damaged; the magnitude of these effects increased over time (Figure 5(C)). Compared with the control, many small vesicles (possibly vacuoles) were observed on numerous hyphae.

BS-Z15 SMs Induce Necrosis of *V. dahliae*

*Verticillium dahliae* conidia were treated with 0.3 mg/mL BS-Z15 SMs for 1, 3, 6, 12, 24, 36, 48, 60, and 72 hours and stained with Hoechst/propiudim iodide (PI). Treatment for 72 hours caused a significant decrease in the proportion of viable conidia (from 98.1% to 35.6%) and a significant increase in the proportion of necrotic conidia (from 1.2% to 60.4%) (Figure 6; Figure S4 provided in the Supplemental Material). Therefore, BS-Z15 SMs exert a lethal effect on *V. dahliae* by inducing necrosis and targeting the nuclear membrane (Figure 7).

BS-Z15 SMs Do Not Induce Apoptosis of *V. dahliae*

To confirm that BS-Z15 SMs do not induce apoptosis of *V. dahliae* conidia, we evaluated mitochondrial membrane potential by staining with the tracer dye Rh123. When *V. dahliae* conidia were treated with BS-Z15 SMs for 12, 24, 36, and 48 hours, there was no significant change in fluorescence intensity compared with the nontreated control conidia (Figure 8), indicating that BS-Z15 SMs do not influence either mitochondrial membrane potential or induce apoptosis of *V. dahliae* conidia. This finding is different from previous reports indicating that *B. subtilis* antifungal substances cause loss of fungal mitochondrial membrane potential.28

Caspases, and particularly caspase 3, are crucial mediators of apoptosis.29 To determine whether caspase-related apoptosis was induced by BS-Z15 SMs, caspase activation was examined by Western blotting. Caspase 3 was produced in the control, possibly due to spontaneous apoptosis of *V. dahliae* conidia. The caspase 3 level was increased slightly but not significantly by BS-Z15 SMs compared with the control (Figure S6 provided in the Supplemental Material). Therefore, BS-Z15
Figure 6. Hoechst/propidium iodide double staining of *Verticillium dahliae* and flow cytometry. (A) Untreated *V. dahliae* conidia (control); (B–E) *V. dahliae* conidia treated with 0.3 mg/mL BS-Z15 secondary metabolites for 6, 24, 48, and 72 hours; (F) the summary of conidia population data (*P* < 0.05, **P** < 0.01 compared with control group). Values are mean ± SD, where *n* = 3; significant values are given by analysis of variance (Dunnett’s *t*-test).

Figure 7. Hoechst/propidium iodide double stained fluorescence microscopy images. (A) Untreated *Verticillium dahliae* conidia (control); (B–E) *V. dahliae* conidia treated with 0.3 mg/mL BS-Z15 secondary metabolites for 6, 24, 48, or 72 hours.
SMs do not induce either proapoptotic proteins or apoptosis of *V. dahliae*.

**Discussion**

*Bacillus subtilis* is a potent and safe biocontrol agent that is distributed across a variety of habitats. Bacillus subtilis can antagonize their target either directly or by producing SMs that exert a similar effect. In this study, BS-Z15 and its SMs inhibited the growth of *V. dahliae*. In addition, the antagonism of BS-Z15 against *V. dahliae* was mediated by SMs. The lack of information on the mechanisms underlying the cytotoxic effects of most antifungals hampers the biocontrol of crop diseases.

Iiturin and fengycin are the main antifungal lipopeptides produced by *B. subtilis*, whereas surfactins show antibacterial activity but no marked fungitoxicity. Iturin acts mainly on the cell membrane. Fengycin exerts antifungal activity against filamentous fungi by altering cell membrane structure and permeability in a dose-dependent manner. Actinomycin D exerts an antifungal effect via a membrane-splitting mechanism. Essential oils exert an antifungal effect by influencing the cell wall and membrane of *Candida albicans*. The mechanism underlying the antifungal activity of BS-Z15 differs from those of other *B. subtilis* strains. BS-Z15 SMs increased cell membrane permeability and induced nuclear karyolysis, nuclear membrane rupture, and necrosis in *V. dahliae*. This indicates that the antifungal compounds of BS-Z15 are novel; therefore, structural and functional studies are needed.

*Verticillium dahliae* is an obligate phytopathogenic fungus that infects roots. A few hyphopodia differentiate from large numbers of hyphae after conidia germination on the root surface for further infection. The severity of cotton Verticillium wilt is associated with pathogenicity, the number of hyphae, and sporulation capacity. The morphological status of the fungus plays an essential role in the pathogenesis of fungal infections and in the response to antifungal treatment. According to SEM images, BS-Z15 SMs altered the morphology of *V. dahliae* conidia and hyphae. BS-Z15 and its SMs produced crumpled conidia with a rough surface, and the magnitude of this effect increased over time. BS-Z15 SMs induced collapse and aging of hyphae, reducing pathogenicity. Therefore, the BS-Z15 SMs show promise as a biological pesticide.

Apoptosis is intentional cellular suicide based on a genetic mechanism. Morphologically, the cell shrinks and internucleosomal double-stranded chromatin (DNA) breaks, but the integrity of the plasma membrane is preserved. In contrast, necrosis does not involve any regular DNA and protein degradation pattern and is accompanied by swelling of the entire cytoplasm (oncosis) and the mitochondrial matrix. Necrosis and apoptosis reduce cell activity and death. In this study, the nuclear morphology, mitochondrial membrane potential, and level of a proapoptotic protein indicated that 0.3 mg/mL BS-Z15 SMs induces necrosis, but not apoptosis, of *V. dahliae*.
BS-Z15 SMs may cause disintegration of the nucleus of *V. dahliae* by rupturing the nuclear membrane. Therefore, the nuclear membrane is an important target of BS-Z15 SMs.

Cell death occurs via apoptosis at low drug concentrations and via necrosis at high drug concentrations. Phillips evaluated the response of *C. albicans* to environmental stresses and amphotericin B and discovered a dose-dependent relationship between apoptosis and necrosis. Reiter examined the cytotoxicity of viral killer toxins in yeast, concluding that a low toxin concentration induces apoptosis, and a high concentration induces necrosis, of yeast cells. Whether a lower concentration of BS-Z15 SMs induces apoptosis of *V. dahliae* requires further study.

**Conclusion**

In this study, we investigated the mechanism underlying the antifungal activity of BS-Z15 SMs against *V. dahliae*. BS-Z15 SMs induced necrosis rather than apoptosis and targeted the nuclear membrane. Therefore, BS-Z15 SMs show promise in the biological control of cotton Verticillium wilt. A study of the molecular mechanism of the antifungal effect of BS-Z15 SMs is warranted.

**Materials and Methods**

**BS-Z15 and Production of SMs**

BS-Z15 was inoculated into beef extract-peptone medium (3 g/L beef extract, 10 g/L peptone, 5 g/L sodium chloride [NaCl], pH 7.2) and cultured at 37 °C with shaking at 180 rpm for 18 hours. The antifungal activity of BS-Z15 against *V. dahliae* was assayed by the double-plate agar diffusion method at 28 °C for 48 hours, after which the diameter of the zone of inhibition was measured.

The growth rate of BS-Z15 and inhibition of *V. dahliae* by its fermentation broth were assessed in the presence of different nitrogen sources (1% peptone, 1% yeast extract, 1% KNO₃, 0.5% KNO₃, and 0.25% KNO₃) in basic medium (1.5 g/L glucose, 0.5 g/L NaCl, 0.1 g/L Na₂HPO₄·NaH₂PO₄) at 37 °C for 18 hours. The absorbance at 600 nm and the diameter of the resulting fungal colonies was measured using a hemocytometer.

**Preparation of BS-Z15 SMs**

BS-Z15 SMs were extracted following a published method and used in subsequent experiments at 0.3 mg/mL. This concentration is equivalent to the concentration of secondary metabolites in the original fermentation broth.

**Effect of BS-Z15 SMs on V. dahliae Mycelial Growth**

*Verticillium dahliae* was cultured on PDA for 7 days, and a 6-mm *V. dahliae* block was inoculated in Czapek-Dox agar containing 0.3 mg/mL BS-Z15 SMs and cultured in the dark at 28 °C. Similar experiments using sterile water as a substitute for SMs were conducted as controls in triplicate. The diameter of the resulting fungal colonies was measured using an electronic ruler after 3, 5, 7, 9, 11, 13, and 15 days.

**Influence of Environmental Factors on the Stability of BS-Z15 SMs**

The effect of pH: 14 mL, 0.3 mg/mL BS-Z15 SMs were divided into 14 groups, with each group having 1 mL. The pH was adjusted from 1 to 14 using either 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide and returned to the original value (pH 7.0) after incubation overnight at 4 °C. Antifungal activity was assayed using the perforated-plate method, in triplicate.

The effect of temperature and storage time: 8 mL, 0.3 mg/mL BS-Z15 SMs was maintained at −80 °C, −20 °C, 0 °C, 20 °C, 40 °C, 60 °C, 80 °C, and 100 °C for 60 minutes, and the antifungal activity was assayed as above. We also stored the SMs at room temperature for 20, 40, and 60 days and assayed the antifungal activity in triplicate.

The effect of UV light: 9 mL, 0.3 mg/mL BS-Z15 SMs was added to Petri dishes and exposed to UV light (400 nm) for 0, 20, 40, 60, 80, 100, and 120 minutes. The antifungal activity was assayed in triplicate.

The effect of protease: 3 mL, 0.3 mg/mL BS-Z15 SMs was transferred to glass tubes to which were added, 1.5 µL double distilled water, 1 g/mL protease K solution, and 1 g/mL pepsin solution, and the tubes were incubated at 37 °C for 30 minutes. Finally, antifungal activity was assayed by the perforated-plate method.

The effect of incubation duration on BS-Z15 cell density and BS-Z15 SMs: the cells were cultured in beef extract-peptone medium for 12, 24, 36, 48, and 60 hours, and the absorbance was measured at 600 nm. According to the above extraction method, we obtained the BS-Z15 SMs after different fermentation times and assayed the antifungal activity by the perforated plate method.

**V. dahliae Spore Activity**

Zhao reported a rapid and sensitive MTT colorimetric detection system for fungal spore activity based on *Metarhizium anisopliae*. The effect of BS-Z15 SMs on *Verticillium dahliae* conidia was evaluated by MTT assay. *Verticillium dahliae* conidia were prepared using the method of Zhao. Conidia (1.0 × 10⁷ cfu/ml, determined using a hemocytometer) were added to Czapek-Dox agar containing 0.3 mg/mL BS-Z15 SMs and incubated at 28 °C with shaking at 180 rpm for 3 days in the dark. As the control, sterile deionized water was used in place of the BS-Z15 SMs. Spore suspensions cultured for 0, 6, 12, 18, 24, 48, 72, 96, and 120 hours were added to 1 mg/mL MTT solution for 2 hours at 50 °C, and 500 µL 1 M HCl was added. The conidia were collected and suspended in 1000 µL isopropanol for 10 minutes, followed by centrifugation for 10 minutes at 13 000 rpm. The supernatant was seeded in the wells of
96-well plates, and the absorbance was measured at 560 nm with a microplate reader.

**The Effect of BS-Z15 SMs on the Morphology of V. dahliae Conidia**

The effect of BS-Z15 SMs on *V. dahliae* conidia was investigated by SEM. *Verticillium dahliae* conidia (1.0 × 10^7 cfu/mL) were cultured in Czapek-Dox medium containing 0.3 mg/mL BS-Z15 SMs for 12, 24, 36, and 72 hours. Spore suspensions were centrifuged at 13 000 rpm for 10 minutes and fixed in 2.5% glutaraldehyde solution for 2 hours. Fixed conidia were washed 3 times with phosphate buffer (pH 7.2) for 30 minutes each, immersed in 1% osmic acid for 2 hours, dried, glued, coated, and observed by SEM.

The effect of BS-Z15 on the morphology of *V. dahliae* conidia was also investigated. *Verticillium dahliae* conidia (1.0 × 10^7 cfu/mL) were co-cultured with BS-Z15 fermentation liquid at 28 °C for either 24 or 48 hours; as a control, sterile deionized water was used in place of the BS-Z15 fermentation liquid.

**The Effect of BS-Z15 SMs on the Morphology of V. dahliae Hyphae**

The effect of BS-Z15 SMs on the morphology of *V. dahliae* hyphae was examined by SEM. *Verticillium dahliae* was cultured in PDA for 7 days, after which a 6-mm fungal block was inoculated in Czapek-Dox agar, and a 1-cm coverslip was inserted into the agar and incubated for 3 days in the dark at 28 °C. The coverslip was transferred to Czapek-Dox agar, and 0.3 mg/mL BS-Z15 SMs were added. Sterile deionized water served as the control. The coverslip was removed after 12 and 72 hours, immersed in 1% osmic acid for 2 hours, dried, glued, coated, and observed by SEM.

**Hoechst/PI Staining to Detect the Lethal Effect of BS-Z15 SMs on the Conidia of V. dahliae**

*Verticillium dahliae* conidia (1.0 × 10^7 cfu/mL) were incubated in Czapek-Dox medium containing 0.3 mg/mL BS-Z15 SMs for 1, 3, 6, 12, 24, 36, 48, 60, and 72 hours at 28 °C and 180 rpm. Untreated conidia served as the control. Conidia (1.0 × 10^7 cfu/mL) were subjected to staining with 5 µL Hoechst reagent and 5 µL PI and incubated for 30 minutes in the dark. The necrotic ratios of *V. dahliae* conidia were assessed by flow cytometry, and nuclear morphology was visualized by fluorescence microscopy.

**Mitochondrial Membrane Potential Assay**

*Verticillium dahliae* conidia (1.0 × 10^7 cfu/mL) were added to Czapek-Dox agar containing 0.3 mg/mL BS-Z15 SMs and incubated for 3 days at 28 °C in the dark. Untreated conidia served as the control. Spore suspensions were cultured for 12, 24, 36, 48, and 72 hours, collected, resuspended in fresh medium containing 10 µg/mL rhodamine 123 (Rh123), and incubated for 30 minutes in the dark at 37 °C. The cells were washed with PBS and analyzed by flow cytometry.

**Western Blot Analysis**

*Verticillium dahliae* conidia were treated with 0.3 mg/mL BS-Z15 SMs for 12, 24, 36, and 48 hours. Untreated conidia served as the control. Total protein was extracted by the trichloroacetic acid method, and protein concentrations were determined by the bicinchoninic acid method. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk in Tris-buffered saline with Tween 20 (TBST), the membranes were incubated overnight at 4 °C with an anti-caspase 3 primary antibody. Next, the membranes were washed in TBST and incubated for 2 hours with a horseradish peroxidase-conjugated secondary antibody. After washing, immunoreactive protein bands were detected by enhanced chemiluminescence.

**Acknowledgment**

The authors thank Dr Xiaorong Li, Xinjiang Academy of Agricultural Sciences, for providing the strain of *Verticillium dahliae*.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by Grants from the Open Project of Key Laboratory in Xinjiang Uygur Autonomous Region (No. 2020D04010), the National Natural Science Foundation of China (No. U1703112), the Graduate Scientific Research and Innovation Project of Xinjiang Normal University (No. XSY202002010) and the Entrepreneurship Training Program for College Students (No. 201910762019).

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**Supplemental Material**

Supplemental material for this article is available online.

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