Antibacterial mechanism of Aspergillus niger xj spore powder crude extract B10 against Agrobacterium tumefaciens T-37

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ABSTRACT
A crude extract (B10) of Aspergillus niger spores showed effective antibacterial activity against Agrobacterium tumefaciens T-37. The inhibition rate was 98.22%, and the half maximal inhibitory concentration was 0.035 ± 0.018 mg/mL. To study the primary antibacterial mechanism, this study used relative electric conductivity, release of proteins and nucleic acids, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and detection of reactive oxygen species (ROS). After adding B10, the relative electric conductivity increased in the supernatant indicating electrolyte transport from intracellular to extracellular space. Compared with the control group and the benzylpenicillin potassium (BP) group, the B10 group had a significant increase in extracellular nucleic acid and protein within 0–18 h, showing that the cell and cytoplasmic membranes were damaged by B10. The SDS–PAGE results confirmed that the extracellular protein and nucleic acid levels were in agreement with lower intracellular total protein. Finally, the intracellular levels of ROS indicated that B10 caused an increase in ROS. In summary, B10 showed clear antibacterial activity against A. tumefaciens through damaging cytoplasmic membranes. The research provided a scientific basis for the prevention and treatment of bacteria.

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KEYWORDS
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Introduction
An enormous number of plant pathogens worldwide cause many important plant diseases and are responsible for major crop losses. Every year, plant diseases cause an estimated 40 billion dollars of loss worldwide, either directly or indirectly [1]. At least 20%–40% of losses in crop yield are caused by pathogenic infections [2]. Chemicals are one of the main components in integrated pest management and are crucial in preventing loss and damage from plant diseases, as demonstrated by the increase in the number of fungicide specifications since 1960s [3]. However, with increasing public attention on agrochemical residues in recent years, side effects of chemical pesticides such as food contamination, environmental dispersal, and higher costs of food production, food safety and soil contamination are a major concern [4]. In addition, the increased resistance of pests and pathogens can lead to more severe future problems. Therefore, there is a great need for environmentally friendly substitutes for chemical pesticides. Among the alternatives, biological control of plant pests and pathogens appears the best option for low-cost, eco-friendly and sustainable management approaches for protection of plants and crops [5].

Agrobacterium tumefaciens has been recognized as a worldwide problem in agriculture for over 150 years, and its negative economic impact in causing crown gall disease is restricted to a limited number of horticultural species, such as perennial fruit, nut, ornamental and vine crops [6, 7]. Many strategies are used to manage crown gall disease, including chemicals, pre-plant application of soil sterilants, soil solarization, herbicides and soil amendments [8, 9]. Zhu et al. [10] reported for the first time that a fungal strain isolated from the soil of fruit tree roots in Xinjiang (China) was identified as Aspergillus niger and named Aspergillus
niger xj. Experiments showed that A. niger xj has antibacterial effect on A. tumefaciens T-37. A. niger was approved by the Food and Drug Administration of the United States as a safe industrial strain [11, 12]. Thus, A. niger is a potential substitute for pesticides used to prevent crown gall as well as being a traditional fungus in the food industry for producing citric acid and xylanase [13, 14]. However, there has been little research on its antibacterial function in the sphere of plant pathogens. Consequently, there are few data available concerning the detailed mechanism behind the antibacterial action of A. niger spores against A. tumefaciens. Our study aimed to determine the antibacterial component in A. niger spores and perform fundamental research on its antibacterial mechanism.

Materials and methods

Culture of strains

The A. niger xj was isolated and identified by the Institute of Fungi Resource, Guizhou University, and stored at the China Center for Type Culture Collection (CCTCC), CCTCC No. M206021, Patent number CN1847388. A. tumefaciens T-37 was purchased from the Soil and Fertilizer Institute of the Chinese Academy of Agricultural Science and stored in the Institute of Fungi Resource, Guizhou University. Strain T-37 was cultured at 37°C with shaking in nutrient broth (NB) media for 16 h and maintained on NB slant media at room temperature.

Acquisition of crude extract

The spore culture of A. niger was provided by the Institute of Fungi Resource, with a total weight of spores of 500 g. Spores of A. niger were extracted three times using acetone, with proportions of solute and solvent of 1:5 (w/v). The extraction temperature was maintained at 60°C, and the extraction time was 3 h each time. The supernatant was filtered through a double layer of filter paper and the solvent removed using a rotatory evaporator. There was 14.75 g of remaining crude extract.

Separation process

The crude extract was isolated by normal silica gel column chromatography. The crude extract was dissolved in acetone and then stirred with a 40–80 mesh size of silica gel on a water bath at 65°C to remove the acetone. Then, the silica gel mixture was poured into a silica gel column (length: 1 m; diameter: 16 cm) and washed with petroleum ether about twice the volume of the column. Then, the column was washed using a gradient of ratios of petroleum ether to acetone of 1:0, 50:1, 20:1, 10:1, 5:1, 2:1, 1:1 and 0:1. Each gradient washed 3 times the column volume and the flow rate was in normal pressure. Different samples were acquired using vacuum evaporation and divided into different groups according to analysis using thin-layer chromatography. Finally, 12 groups of samples were acquired, named B1–B12.

Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis of B10 was performed on an Agilent 6890 GC apparatus (HP6890/5975C GC/MS, Agilent, USA) with a 5975C mass spectrometer detector. The chromatographic column was ZB-5MSI 5% phenyl-95% dimethylpolysiloxane (30 m × 0.25 mm; film thickness: 0.25 μm). The operating conditions were as follows: column temperature was maintained at 50°C for 2 min, raised to 310°C at 5°C/min and maintained for 4 min; run time was 58 min; vaporizing chamber temperature was 250°C; carrier gas was high purity He (99.999%); column pressure was 7.06 psi; carrier gas flow rate was 1.0 mL/min; split ratio was 20:1; solvent delay time was 5 min; ion source was El(Electron impact ion source) of temperature 230°C; quadrupole temperature was 150°C; electric energy was 70 eV; emission current was 34.6 μA; multiplier voltage was 1482 V; interface temperature was 280°C; and mass range was 29–500 amu. The compounds of sample B10 were identified by comparing their peaks with a computer retrieval system and verified using standard mass spectrograms Nist2005 and Wiley275. Then, the relative mass fractions of the various components were determined by peak area normalization method.

Inhibition activity and the half maximal inhibitory concentration

We used ultraviolet absorption to measure the inhibitory activity of samples to A. tumefaciens T-37. The preparation of T-37 inoculation suspension was inoculated into 20 mL of NB medium and cultured with shaking at 30°C and 150 r/min for 16 h. The necessary amount of bacterial suspension was taken, centrifuged at 4500 r/min (Allegra X-30R, Beckman Coulter, USA) for 5 min to remove the supernatant, and sterile water was added to dilute the bacterial suspension to 10⁸ CFU/mL. All samples were dissolved in dimethyl sulfoxide (DMSO), and then, these sample solutions were
added into the T-37 inoculum suspension to achieve a final concentration 0.8 mg/mL, which was incubated at 37 °C with shaking at 150 r/min for about 22 h. The absorbency of the bacterial suspension was determined by ultraviolet spectrophotometer (BIOMATE 3S, Thermo Fisher, USA) at 400 nm. The control was set up using the normal inoculum suspension with benzylpenicillin potassium (BP) with the same final concentration as the experiment group. Bacterial suspension without sample that was added the same volume of DMSO served as a control group, and the value of inhibition activity was calculated using the following formula:

\[
\text{inhibition rate(\%)} = \left( \frac{OD_{ck} - OD_{sample}}{OD_{ck}} \right) \times 100\%
\]

According to the tested inhibition rate, the half maximal inhibitory concentration (IC50) was calculated as follows. The groups of inoculum were given different final concentrations of B10 in a gradient of 0.8, 0.4, 0.2, 0.1 and 0.05 mg/mL for T-37. This experiment had three parallel groups with the same conditions. The value of IC50 was calculated by SPSS 19.0. The graphs were processed by GraphPad Prism 5.

**Antibacterial mechanism**

**Cell membrane permeability**

The relative electric conductivity was used to indicate the change in cell membrane permeability and was determined according to a previously described method [15] with some modification. The inoculum suspension was composed of the logarithmic phase of strains incubated at 37 °C with shaking at 150 r/min for 16 h. The strains were separated by centrifugation at 4,500 r/min (Allegra X-30R, Beckman Coulter, USA) for 5 min and washed with phosphate-buffered saline (PBS) until their electric conductivities were 1. Then, B10 was added to the suspension with a final concentration of 0.8 mg/mL. The control was set up using the normal inoculum suspension with BP with the same final concentration as the experiment group. After mixing, the samples were incubated at 37 °C with shaking at 150 r/min for 5 h, and then, the conductivity of bacterial lipids was measured using a conductivity meter (DDBB303A, INESA Scientific Instrument Co., China).

**Integrity of cell membranes**

The effect of B10 on the T-37 cell membrane was determined by measuring the release of cell constituents in the supernatant using a spectrophotometer (BIOMATE 3S, Thermo Fisher, USA). B10 was added into the T-37 inoculum suspension to a final concentration 0.8 mg/mL. Then, samples were incubated at 37 °C with shaking at 150 r/min for 18 h, and 3-mL samples were removed every 3 h from each. All samples were centrifuged at 4,500 r/min (Allegra X-30R, Beckman Coulter, USA) for 5 min after incubation was complete, and the absorption of the supernatants was immediately determined at 280 and 260 nm. The control was conducted as described in the cell membrane permeability assay.

**SDS–PAGE**

The change in intracellular proteins was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The B10 was added to the T-37 inoculum suspension to sample concentrations as described in the cell membrane permeability assay. Then, the solution was kept at 37 °C with shaking for 16 h, and 1-mL samples were removed every 4 h. Samples were centrifuged at 12,000 r/min (Allegra X-30R, Beckman Coulter, USA) for 2 min, and the supernatants discarded and deposits collected. The deposits were placed in Tris–HCl buffer and kept at 95 °C for 10 min. The control group and the BP group were prepared as described in the cell membrane permeability assay. The results of the experiment were observed by SDS–PAGE followed by Coomassie Brilliant Blue staining. The images were processed through the BIO-RAD gel imaging system.

**Determining the change in reactive oxygen species (ROS) by fluorescence microscope**

The ROS value indicated the quantity of oxygen free radicals, including superoxide anion, hydrogen peroxide, hydroxide and peroxide in a cell. We determined the degree of cell damage by examining T-37 after interaction with B10 for the presence of ROS using a fluorescence microscope (OLYMPUS IX73, Olympus Corporation, Japan). Following the user’s guide of Reactive Oxygen Species Assay Kit (Nanjing Jiancheng Bioengineering Institute, China), we cultured the T-37 in NB liquid media at 37 °C with shaking for 22 h, and we took 1.5 mL of inoculum solution to centrifuge at 12,000 r/min for 5 min, discarded the supernatant and then washed the deposits twice with PBS. The deposits were dissolved in 10 μmol/L 2,7-dichlorofluorescin diacetate (DCFH-DA) and incubated at 37 °C for 30 min, with shaking for 3–5 min. Then, the deposits were washed three times with PBS to remove the residual DCFH-DA outside cells. Finally, B10 was added to the prepared solution at a concentration of 0.8 mg/
mL, and the signal of samples was detected five times, with a time gap between each 10 min. The internal reference (ROSUP) used was the prepared agent provided in the kit. The control was conducted as described in the cell membrane permeability assay.

Results and discussion

Chemical composition of B10

The peak area was selected as the analytical index of the relative content, together with the retention index of the peaks. Seventeen compounds were confirmed (Figure 1, Table 1), representing 91.291% of the total area. Ethyl linoleate was the main compound with the highest peak area percentage of 23.444%; ethyl oleate (11.812%) was the second major compound, followed by No. 15 (9.016%). Other compounds were found at trace levels, including 2, 3, 13, 16 and 17.

Antibacterial activity of samples

The B10 group had considerable antibacterial activity to T-37, and its inhibition rate reached 98.22%, which exceeded that of BP (Figure 2). The B11 had a lower inhibitory effect than B10 with 69.04%. Samples B1 and B7–B9 had good inhibitory effects of near 50%. The other samples had relatively low inhibitory activity. The IC50 of B10 against T-37 was 0.035 ± 0.018 mg/mL, while the IC50 of BP against T-37 was 0.06 ± 0.03 mg/mL (Table 2). B10 had better antibacterial activity against T-37 than BP group. Of the 12 samples of the crude extract, B10 showed particularly high inhibition of T-37.

Cell membrane permeability

The relative electric conductivity represented the change in cell membrane permeability after treatment with B10 (Figure 3). The conductivity of B10 and BP groups increased over time within 5 h after treatment of the bacterial suspension. There was a clear increasing trend for B10 compared with the control. Moreover, the value of relative electric conductivity for B10 exceeded that for the BP group, indicating a change in membrane fundamental structure, increased
permeability of the cell membrane. Selective permeability is one of the cell membrane’s most important properties, and it plays a key role for maintaining intracellular ion homeostasis. The bacterial cytoplasmic membrane provides a permeability block to small ions such as K^+, Na^+ and H^+, which are crucial to facilitate cell membrane functions and maintain enzyme activity and normal metabolism [16]. Maintenance of ion homeostasis is of great importance for the energy status of the cell as it is significant to energy relevant processes such as solute transport, control of metabolism, management of turgor pressure and motility [17]. Hence, cytoplasm permeability plays an important role in cell growth, and even a relatively minor variation in membrane structure can have severe consequences for the cell [18]. In our study, the relative electric conductivity of strain T-37 after treatment with B10 showed gradual increases in cell membrane permeability with treatment time, indicating that there was some site of action for B10 on the cell membrane that caused the release of cytoplasmic inclusions. Finally, the relative electric conductivity in the extracellular environment rose.

**Integrity of the cell membrane**

The absorbance value of nucleic acids (OD: 260 nm) of T-37 significantly increased from 0 to 0.022 after 18 h in the presence of B10 at the IC_{50} concentration (Figure 4). The effect gradually increased with time and reached its maximum at 18 h, exceeding the BP group and the control group. The absorbance value of protein (OD: 280 nm) for T-37 also reached the maximum at 18 h, surpassing the BP group and the control group, indicating that the amount of protein released increased (Figure 5). The B10 sample had a greater effect on cells and plasma membranes than the BP group, damaging the membrane structure and leading to the release of cell constituents into the cell suspension; this disorders the process of transcription and translation and results in cell metabolic chaos that eventually caused cell death. We concluded that B10 was an even more effective bactericide than BP, especially in regard to cell membrane destruction.

![Table 2. The IC_{50} of B10 against T-37.](image)

| Concentration (mg/mL) | B10     | BP       |
|-----------------------|---------|----------|
| 0.8                   | 95.05 ± 3.80 | 82.60 ± 3.30 |
| 0.4                   | 94.19 ± 3.77 | 77.45 ± 3.10 |
| 0.2                   | 72.69 ± 2.91 | 83.09 ± 3.23 |
| 0.1                   | 71.00 ± 2.84 | 72.30 ± 2.89 |
| 0.05                  | 61.51 ± 2.46 | 53.92 ± 2.70 |
| IC_{50} (mg/mL)       | 0.035 ± 0.018 | 0.06 ± 0.03 |

*B*, benzylpenicillin potassium.

![Figure 2. Inhibition activity of crude extracts against T-37.](image)

Note: B1–B12 are different parts from the crude extract, and BP was benzylpenicillin potassium, N.S. p < 0.05, *p < 0.05, **p < 0.01.

![Figure 3. Relative electric conductivity of T-37 for B10, BP (benzylpenicillin potassium) and control (DMSO) treatments over time.](image)

Note: In the control treatment, bacterial suspension was added the same volume of DMSO as the B10 group.
cytoplasmic membrane is important to cell metabolism, especially in maintaining a stable intracellular environment, which provides a benign environment for cell growth. The leakage of cell constituents including proteins and nucleic acids, which were measured using absorbance at 280 and 260 nm, respectively, is an indicator of membrane integrity [19]. Our results showed that the cytoplasmic membrane was malfunctioning as reflected by the constant rise in extracellular proteins and nucleic acids following treatment with B10, likely due to the cytoplasmic membrane being unable to function normally.

**SDS–PAGE**

The inoculum solutions were treated with B10 for 4, 8, 12 and 16 h, respectively, and SDS–PAGE was used to determine the changes in total protein in T-37. The total protein quantity of the B10 group and the BP group decreased with time (Figure 6). The control check group, that is bacterial suspension with the same volume of DMSO as the B10 group, had generally steady values, with little decrease. We concluded that total protein content of T-37 was decreased by B10, perhaps because the damage to cytoplasmic and cell membranes caused the release of nucleic acids and intracellular proteins. The SDS–PAGE results confirmed the result of constituent leakage from the perspective of total protein. The B10 caused membrane malfunction and breakdown, which decreased the quantity of intracellular proteins and nucleic acids, further affecting the translation of protein and finally leading to cell death.

**ROS analysis**

Addition of B10 caused a large response in ROS after addition to the T-37 suspension, with a rapid and effective ROS signal during the first 40 min (Figure 7). However, the ROS signal in the BP group was clearly less than that in the B10 group. At high intracellular concentrations, ROS will cause oxidative damage to membranes. Based on the results (Figure 7) we
hypothesize that B10 disturbed the ROS balance and so led to damage of cell membranes by causing malfunction of the domains involved in transmembrane transport or to decomposition of the phospholipid bilayer. ROS are very important products in cell metabolism [20]. ROS are normal by-products of metabolism in a normal or stressed intracellular environments, and play an important part in cell growth, adaptive stress response and programmed cell death [21]. Excessive intracellular accumulation of ROS causes oxidative damage to membrane lipids, proteins, and nucleic acids, and triggers or accelerates cell apoptosis [22]. In our study, B10 generated an increase in ROS signals, further confirming our hypothesis concerning the mechanism of membrane disruption.

The increase in the use of chemical antiseptics has led to increased public discussion. The misuse of chemical antiseptics not only results in public health risks such as allergy and carcinogenesis, but also endangers the environment [23]. In seeking replacements to apply for crop protection, we found that *A. niger*, a fungus used in the food industry, has enormous antibacterial activity against *A. tumefaciens* T-37, which is a species of major concern as a plant pathogen [6]. Our study on the antibacterial mechanism of B10, and the results demonstrating its inhibitory activity indicated that some component of the B10 sample had an inhibitory function against T-37. Next, we will explore and isolate the main antibacterial active substances of B10.

**Conclusions**

With increasing public attention on food health matters, such as agrochemical residues, the adoption of chemical agents to prevent plant pathogenic diseases is currently undesirable for sustainable development. Our study showed high inhibitory activity on *A. tumefaciens* growth and the capacity to damage cytoplasmic membranes, indicating a benign disruptive effect, and finally causing cell death. Hence, B10 could be an effective antibacterial component for traditional chemical pesticides for preventing *A. tumefaciens* T-37. Further study is required to determine the effective monomer in B10 and further explore the antibacterial mechanism, which could promote application of B10 in crop disease prevention.
Disclosure statement
The authors declared that they have no conflict of interests to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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