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Antifungal Activity of *Streptomyces hygroscopicus* JY-22 against *Alternaria alternata* and Its Potential Application as a Biopesticide to Control Tobacco Brown Spot

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Abstract: Tobacco brown spot caused by *Alternaria alternata* is a devastating fungal disease endangering plant production. To effectively control brown spot disease, an antagonistic Actinomyces strain, *Streptomyces hygroscopicus* JY-22, was isolated from rhizospheric soil, which remarkably restrains the growth of phytopathogenic fungus *Alternaria alternata.* This antagonistic strain and its culture filtrate showed significant antifungal activity against *Alternaria alternata*. Firstly, the confrontation culture method of strains JY-22 and *Alternaria alternata* showed that *Streptomyces hygroscopicus* JY-22 had noticeable antifungal activity against *Alternaria alternata*, including inhibition of mycelial growth and mycelial morphological changes, compared with the control group. Furthermore, the culture filtrate of *Streptomyces hygroscopicus* JY-22 showed substantial inhibition of the mycelial growth and spore germination of *Alternaria alternata* in a dose-dependent manner. Additional studies revealed that these antifungal actions were mainly related to membrane-active mechanisms that increased membrane permeability and damaged the cell membrane, leading to changes in certain cytoplasmic properties, such as extracellular conductivity and ergosterol, MDA and soluble protein content. In detached-leaf and field experiments, foliar spraying with culture filtrate resulted in smaller lesions and a lower disease index than the control group. Taken together, these results suggest that *Streptomyces hygroscopicus* JY-22 and its culture filtrate have the potential to be a safe biopesticide for the bio-control of tobacco brown spot.

Keywords: tobacco brown spot; bio-control; Actinomyces

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1. Introduction

Alternaria alternata is a devastating necrotrophic fungus with a high incidence and causes brown spots on many plant leaves and fruit, including citrus, cherry tomato and tobacco [1–3]. This notorious fungal pathogen is a common aggressive fungus that can sense specific stimuli, colonize leaves or fruit and over-secrete unique host-selective toxins in plants, eventually causing diseases on different host plants [4]. At the initial stage, *A. alternata* infection results in small yellowish-brown round spots on plants and then the spots gradually increase in size into bigger ones with the occurrence of decay, which results in a considerable reduction in plant yield and quality [5,6]. Specifically, tobacco brown spot disease, a destructive foliar disease caused by *A. alternata*, is a crucial fungal disease endangering tobacco yield that infects most of China's tobacco-growing fields. Currently, the most effective treatment for this disease is the application of traditional fungicides such as dimetachlone and mancozeb [3]. Unfortunately, these conventional fungicides gradually lead to increasingly serious environmental pollution and food security issues, which can be reversed by effective, biodegradable and safe natural fungicides [7].

Investigations have indicated that biopesticides can protect plants from pathogens and increase crop yields without adverse environmental impacts, so they are an appropriate alternative approach to traditional pesticides and can reduce the use of agricultural fungicides [8]. Natural antagonistic microorganisms for bio-control, including *Bacillus*, *Pseudomonas* and *Streptomyces*, are abundant in soil and can support plants not only by controlling plant pathogens but also by increasing the soil nutrient content [9,10]. In this context, *Streptomyces*, acting as Gram-positive bacteria, are significant bio-control agents and mainly thrive in decaying organic matter and soil. As bacteria of the genus Actinomyces, *Streptomyces* have slender hyphae without multi-core or isolated branches. Up to now, many *Streptomyces* have been used to produce various secondary metabolites, which are generally regarded as agro-antibiotics and antibiotics [11]. With the high relevance of *Streptomyces* in controlling many fungal pathogens, the use of its strains as bio-control agents for plant diseases is promising and has been popularized and commercialized in numerous areas and countries [12]. Many Actinomycete species, particularly the genus *Streptomyces*, can be used to control some pathogenic fungi. However, little is known about the antifungal effect of *Streptomyces hygroscopicus* (*S. hygroscopicus*) against the plant pathogen *A. alternata*.

In this study, an isolation of *S. hygroscopicus* was obtained from rhizospheric soil and named JY-22. The strain of *S. hygroscopicus* JY-22 was further used to verify its antagonistic antifungal properties and mechanism against *A. alternata*. Malondialdehyde (MDA) content, ergosterol content, soluble protein content and the integrity of the cell membrane of *A. alternate*, which are very important for the survival of eukaryotic microbes, were used to investigate the inhibition mechanisms of *S. hygroscopicus* against *A. alternate*. Its ability to control tobacco brown spot caused by *A. alternata* was also further evaluated in detached-leaf and field tests.

2. Materials and Methods

2.1. Antagonistic Actinomycetes and Pathogen Sources

The *Streptomyces hygroscopicus* strain, which was named JY-22 and used in the experiment, was isolated from the soil of Jinyun Mountain (29°41′ N, 106°18′ E, 895 m elevation, Chongqing, China). *A. alternata* (*Alternaria alternata* (Freis) Keissler) was isolated from naturally infected tobacco, provided by Professor Dou Yanxia, College of Plant Protection, Southwest University, and was identified as a highly pathogenic strain.

2.2. Plant Culture

Tobacco seeds (the cultivar "K326") were germinated in a growth chamber at a relative humidity of 60% and a temperature of 25 °C. Seedlings at the same stage were transferred into new pots filled with potting medium (Pindstrup Mosebrug A/S, Denmark) and incubated in a growth chamber at 22 °C with a cycle of 16 h light and 8 h dark [13]. The seedlings were used in experiments until 5–6 truly fully expanded leaves were observed. A total of 20 tobacco plants were used for each treatment. The pots were drenched with the same volume of deionized water to maintain the moisture content at 60% w/w. Each experiment was repeated three times.

2.3. In Vitro Antagonistic Assay

The ability of antagonistic microorganisms (*S. hygroscopicus* JY-22) to inhibit the mycelial growth of *A. alternata* in vitro was detected by the confrontation culture method with minor modifications. Here, a slide (7.5 cm × 2.5 cm) was placed in every petri dish, and PDA medium was added to the plates until it sufficiently flooded the slides. *S. hygroscopicus* JY-22 (JY-22) was inoculated onto PDA medium on one side of the slide. After three days of incubation, *A. alternata* was inoculated on the other side of the slide. The plates containing *A. alternata* only served as a control. Each experiment was repeated three times, and plates were incubated in an incubator at 28 ± 2 °C for 7 days. Finally,

the morphological changes of antagonistic microorganisms and *A. alternata* JY-22 were analyzed using a light microscope.

2.4. Preparation of Culture Filtrate

JY-22 was inoculated into sterilized PDA medium and cultivated in an incubator at 28 ± 2 °C for 4 days. Circular mycelial blocks (Φ = 8 mm) were obtained by punching at the edges of colonies with a hole punch. Then, the *S. hygroscopicus* blocks were inoculated into Erlenmeyer flasks containing sterilized broth medium (1 L sterilized medium contained glucose (20 g), cornmeal (15 g), soybean flour (30 g), NaCl (2.5 g) and $CaCO_3$ (2 g)). On the other hand, the pure sterilized broth medium (uninoculated) served as the control group.

All cultures were placed in a shaker incubator at 28 °C and 160 r/min for 72 h. The supernatant was collected by centrifugation at 12,000 r/min, and the medium residue was removed. This fermented liquid was filtered by a 0.22 μm drainage pin-type filter to remove impurities and obtain the culture filtrate. The JY-22 culture filtrate was kept at 4 °C before use.

2.5. JY-22 Culture Filtrate Antagonistic Assays

To investigate the antibacterial activity of the JY-22 culture filtrate, the culture filtrate was diluted with pure sterilized broth medium to different proportions ranging from 10 to 100 times (10, 20, 40, 80 and 100 times), and the relative inhibitory effect of the fermentation broth on the growth of an *A. alternata* colony was measured by a typical method [14]. Specifically, the culture filtrate suspension was added to the solid PDA medium (50 °C) at the final test concentrations mentioned above. The pure sterilized broth medium added to solid PDA medium was the control group. Next, the *A. alternata* block (Φ = 0.45 mm) was inoculated in the center of a PDA plate and incubated at 28 °C for 7 days. The colony diameters on agar plates were measured. The fungal relative inhibition rate was calculated by the following formula:

$$\text{Relative inhibition rate } (\%) = \frac{A - B}{A} \times 100\%$$

where A represents the control colony diameter and B represents the experimental colony diameter.

2.6. Inhibition of JY-22 Culture Filtrate on Spore Germination

To further explore the antagonistic ability of JY-22 culture filtrate on the spore germination of *A. alternata*, the inhibitory rate of germination was investigated. Briefly, 100 μL *A. alternata* spore suspension (2.0×10^5 CFU/mL) was added to a 96-well plate. Then, 100 μL fermented filtrate was diluted with pure sterilized broth medium and added to the 96-well plate for final dilution (10, 20, 40, 80 and 100 times). Meanwhile, 100 μL pure sterilized broth medium was added into the 96-well plate to obtain the control group. After being mixed completely, the test substances contained *A. alternata* spores and JY-22 culture filtrates; however, in the control group, only the *A. alternata* spores were present. Every experiment was repeated three times. After incubation at 28 °C for 36 h, the mixture was examined microscopically. The relative inhibition rate was calculated by the following formula:

$$\text{Relative inhibition rate of germination } (\%) = \frac{A - B}{A} \times 100\%$$

where A represents the number of germinated spores in the control group, and B represents the number of germinated spores in the experimental group.

2.7. Measurement of Extracellular Conductivity

Referring to a previous study [15], the membrane permeability was measured using the extracellular conductivity method with some modifications. Specifically, an *A. alternata*

block (Φ = 0.45 mm) was inoculated in medium and shaken at the speed of 160 r/min at 28 °C for 36 h. JY-22 culture filtrate was added to conical flasks containing *A. alternata* mycelia at diluted concentrations of 10, 20, 40, 80 and 100 times, respectively. The *A. alternata* mycelia treated with pure sterilized broth medium in conical flasks served as the control group. After the mycelia were treated with JY-22 culture filtrate in shakers for differing lengths of time (0, 30, 60, 90, and 120 min), the extracellular conductivity was measured by a conductivity meter (Shanghai Precision Scientific Instrument Co., Ltd., Shanghai, China). Every experiment was repeated three times.

2.8. Assays of Malondialdehyde (MDA)

The MDA assay was carried out according to a previous method [16] with minor modifications. Briefly, after treatment with JY-22 culture filtrate or pure sterilized broth medium (control group) as described in Section 2.6 above, the mycelia were cultivated for 96 h and separated by centrifugation. Next, the mycelia were gently washed with distilled water and the excess water was soaked up by filter paper. Then, the dried mycelia (2.0 g) were mixed with 2 mL trichloroacetic acid (20%, *w*/*v*) and barbiturate (0.5%, *w*/*v*) and ground adequately. The mixture was subsequently transferred into a centrifuge tube for centrifugation for 10 min (10,000 r/min). Next, the supernatant was boiled for 10 min. After cooling, the MDA content of *A. alternata* was calculated by measuring the absorbance of the supernatant at 450 nm, 532 nm and 600 nm. Every experiment was repeated three times. The formula was as follows:

$$\text{MDA (μmol/kg)} = 6.45 \times (OD_{532} - OD_{600}) - 0.56 \times OD_{450}$$

2.9. Determination of Ergosterol Content

The ergosterol content of *A. alternata* was investigated as previously described [17]. First, *S. hygroscopicus* culture filtrate was added to flasks containing *A. alternata* mycelia suspensions at the final test concentrations (10, 20, 40, 80 and 100 times for the JY-22 culture filtrate). In the control group, pure sterilized broth medium was used instead of JY-22 culture filtrate. After the treated *A. alternata* was cultured for 96 h, the mycelia were washed twice with distilled water and harvested. Then, the mycelium samples were gently dried with filter paper to avoid affecting the measurement of ergosterol in the plasma membrane. Next, the mycelium samples were added to methanol/trichloromethane at a ratio of 2:1, ground with liquid nitrogen and cooled at room temperature for 1 h. The samples were transferred to a mixture of sterile distilled water (6 mL), trichloromethane (6 mL) and phosphate buffer (6 mL) and incubated at 80 °C for another 4 h. Methanol (8 mL) and ethanol (2 mL) were added to these samples and mixed for saponification at 60 °C for 1 h. Then, petroleum ether (15 mL) was added, and the resulting solution was shaken for 20 min and extracted twice. Subsequently, the upper liquid was washed with sterilized water and the supernatant was removed and diluted with ethanol (95%) to 10 mL. Finally, the absorbance was measured at 282 nm by a UV/VIS spectrophotometer (TU-19, Beijing Purkinje General Instrument Co, Ltd., Beijing, China). Samples without the culture filtrate treatment were used as the control group. Every experiment was repeated three times. The formula to determine the inhibition rate of ergosterol was as follows:

$$\text{Inhibition rate (\%)} = (A - B)/A \times 100\%$$

where A represents the optical density of the control group, and B represents the optical density of the experimental group.

2.10. Soluble Protein Content Measurement

The proteins released into the supernatant were defined as soluble proteins, which usually reflect the integrity of cells. Here, the concentrations of soluble protein were assessed using the Bradford method (1976), using bovine serum albumin as standard [18]. Briefly, the mycelia were treated with different concentrations of JY-22 culture filtrate or pure sterilized

broth medium (control group), and the protein contents were determined by measuring the optical density (OD_{595}). The crude protein extracts were centrifuged at 12,000 rpm for 15 min at 4 °C. Next, the binding of the proteins and Coomassie Brilliant Blue was measured at 595 nm by a spectrophotometer (Beijing Purkinje General Instrument Co, Ltd., China). The experiment was repeated three times.

2.11. Detached-Leaf Assays

This experiment was performed to determine the protective effect and curative effect of the culture filtrate. An amount of 2 mL JY-22 culture filtrate (the final test diluents were 10, 20, 40, 80 and 100 times) or 50% carbendazim was sprayed onto tobacco leaves. The same volume of pure sterilized broth medium was used for control treatment. At 24 h before or after spraying, each leaf was inoculated with an *A. alternata* block. After cultivating in a plant growth chamber at a temperature of 25 °C and a relative humidity of 85–90%, the stalks were drenched with moist gauze to stay hydrated. After 5 days, the control effect of JY-22 culture filtrate on *A. alternata* in vivo was observed. Every assay was performed three times.

2.12. Field Experiments

The field experiment was carried out in Fuling, Chongqing, China (29°43′3.61″ N and 107°36′11.45″ E). The tobacco cultivar K326, which is susceptible to *A. alternate*, was planted to test the control effect of JY-22 culture filtrate against *A. alternata*. The fertilizer was applied following a standard, and all plants were cultivated under the same amount of well water irrigation in the field management. During the entire growth period, no fungicides were used except for the experimental treatment. The field experiments were conducted to determine protective and curative effects as follows: Protective effect: Tobacco plants were sprayed with JY-22 culture filtrate, 50% carbendazim or well water (control group). After 24 h of spraying, an *A. alternata* spore suspension (2.0×10^5 cfu/mL) was inoculated onto tobacco plants by the spraying method. Disease indexes for three different treatments were calculated to evaluate the protective effect. Curative effect: After inoculated with *A. alternata* for 24 h, tobacco was sprayed with the JY-22 culture filtrate or well water (control group) to evaluate the curative effect. The disease severity was evaluated for each treatment. The experiments were performed as randomized blocks with four replicates. The field experiment was repeated in 2020, 2021 and 2022.

2.13. Data Analysis

All experiments included at least three independent replicates. Statistical differences were estimated by SPSS 19.0 using independent-sample t-tests for comparison between two treatments or one-way analysis of variance (ANOVA) for multiple treatments. All data were recorded as the mean ± standard deviation with * and ** representing statistical significance at the levels of $p < 0.05$ and $p < 0.01$, respectively.

3. Results

3.1. Bio-Control Effects of JY-22 In Vitro

The confrontation culture experiment between the bio-control bacterium (JY-22) and fungus *A. alternata* showed noticeable inhibition on the pathogen of *A. alternata* (Figure 1). Specifically, *A. alternata* profusely colonized the mycelia after being inoculated in the medium (Figure 1a), but the mycelial growth was significantly suppressed when in proximity to JY-22 (Figure 1c), and the diameter of the inhibition zone was approximately 22.5 mm. Furthermore, morphological changes in *A. alternata* were observed. The morphology of the control samples was smooth and uniform and their hyphae were linear with constant diameter (Figure 1b). On the contrary, the pathogen hyphae near the inhibition zone underwent several morphological changes, including protoplasmic collapse (Figure 1g), shriveled hyphae with moniliform malformations (Figure 1e–g), increases in quantity and shortened hyphal branches (Figure 1f,d), and even the broken plasmalemma was detached from the

cell wall, resulting in the outflow of intracellular contents and empty mycelia (Figure 1g), all of which indicate that the strong antibacterial activity of JY-22 against *A. alternata* may be attributed to its secondary metabolites.

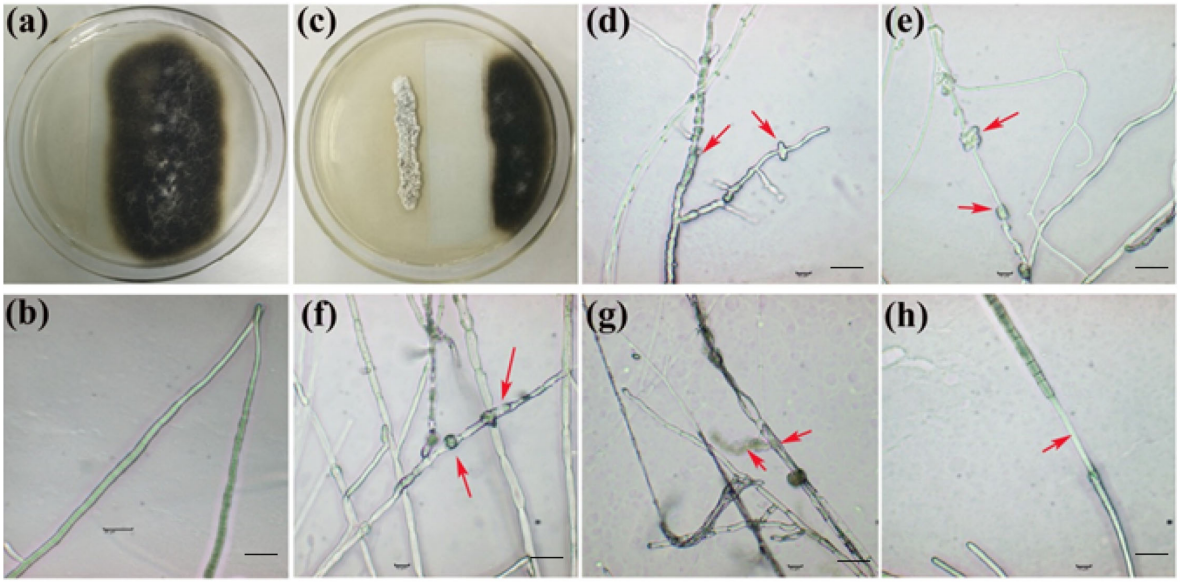

Figure 1. Confrontation culture of *Alternaria alternate* with blank (**a**,**b**) or *Streptomyces hygroscopicus* JY-22 (**c**–**h**). (**a**,**c**) display the antagonistic activity of JY-22 against *Alternaria alternata* after co-culture on PDA plates. (**b**,**d**–**h**) show the mycelial features of *Alternaria alternata* after confrontation culture with blank and JY-22. Red arrows in (**d**–**h**) show the malformations of hyphae, including swelling (**d**–**f**), twist (**e**), collapse (**g**) and thinning (**h**). The scale bar is 12 μm.

In addition, *A. alternata* was inoculated on PDA agar plates with different concentrations of JY-22 culture filtrate (diluted 10, 20, 40, 80 and 100 times), and the mycelial inhibition rate of each group was investigated by measuring the colony diameter of each group and its control group. Notably, compared to the control, the growth of the pathogen colonies was markedly dependent on the concentration of JY-22 culture filtrate (Figure 2a). At 10-times dilution, JY-22 culture filtrate exhibited the highest degree of bio-control on *A. alternata*, with a 71.39% inhibition rate. In contrast, the dilution effects of 20-, 40-, 80- and 100-times concentrations were relatively weaker, 41.79%, 30.31%, 18.01 and 9.10%, respectively.

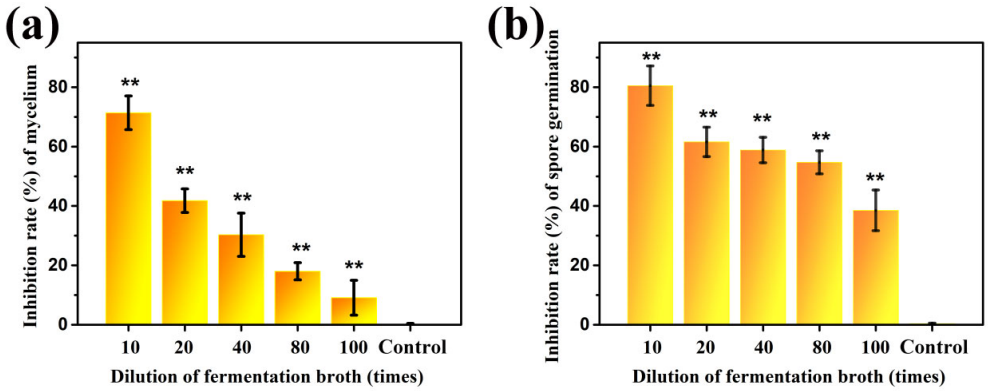

Figure 2. Inhibition rate of JY-22 culture filtrate on mycelial growth and spore germination of *Alternaria alternata*. (**a**) Histogram showing the inhibition rate (%) of mycelium of *Alternaria alternate* under different JY-22 culture filtrate treatment. (**b**) Histogram showing the inhibition rate (%) of spore germination of *Alternaria alternate* under different JY-22 culture filtrate treatment. Error bars indicate SE of three biological replicates. Statistical analysis of data was performed based on one-way ANOVA for data from multiple treatments. The *p*-values are indicated by stars, **: $p < 0.001$.

The results of the *A. alternata* spore germination experiment were similar to those of the colony growth experiment (Figure 2). The JY-22 culture filtrate at a dilution of 10 times exhibited the greatest inhibition on the spore germination of the pathogen, with an inhibition rate of 80.52%, whereas the inhibition rates of 20-, 40-, 80-times dilution were 61.59%, 58.84% and 54.71%, respectively. In contrast, the inhibition rate of the corresponding 100-fold diluent was only 38.52% (Figure 2b). Therefore, these results confirmed that almost all fungal growth and spore germination were noticeably inhibited by directly adding JY-22 culture filtrate to the culture medium in a dose-dependent manner.

3.2. Extracellular Conductivity

To confirm the inhibition mechanism of the phytopathogen *A. alternata*, we compared the changes in conductivity, which can be used to judge cell membrane permeability. A large increase in extracellular conductivity was measured when the fungus crossed JY-22 culture filtrate (Figure 3a). During the first 4 h, when exposed to 10 to 100 times of JY-22 culture filtrate diluents, the extracellular conductivity was 206–179 μs/cm, and these values increased remarkably compared with the extracellular conductivity of the control group (166 μs/cm). Moreover, as the treatment time increased, the conductivity of the suspension increased rapidly after incubation with JY-22 culture filtrate. After 20 h of treatment, the conductivity of the 10-times diluent reached 226.33 μs/cm and was 1.33 times higher than that of the control group, indicating that JY-22 culture filtrate caused the electrolyte exosmosis of *A. alternata* and may lead to cellular lysis and death of the phytopathogen.

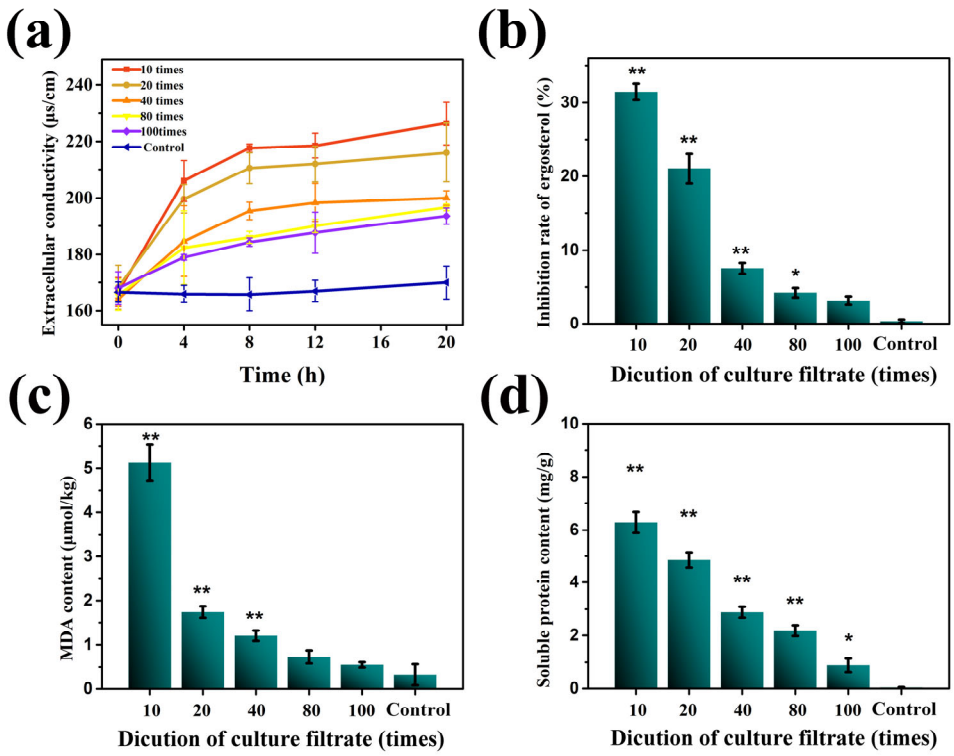

Figure 3. Effects of JY-22 culture filtrate on (**a**) extracellular conductivity, (**b**) inhibition rate of ergosterol content, (**c**) MDA content and (**d**) soluble protein content of Alternaria alternata. SE of three biological replicates. Statistical analysis of data was performed based on one-way ANOVA for samples from multiple treatments. The *p*-values are indicated by stars, *: $p < 0.05$, **: $p < 0.001$.

3.3. Ergosterol Content in Plasma Membrane

The plasma membrane plays a significant role in environmental balance, signal and energy transformation, and material exchange, which eventually guarantees cell health and vitality. Ergosterol, a major sterol component, is responsible for maintaining fungal cell function and membrane integrity. The effect of JY-22 culture filtrate on ergosterol content

in the plasma membrane of *A. alternata* is shown in Figure 3b. According to these results, the culture filtrate can lead to a considerable decrease in ergosterol biosynthesis of the phytopathogen. In contrast to the control, the ergosterol content decreased by 31.46%, 21.04%, 7.51%, 4.24% and 3.18% for 10, 20-, 40-, 80- and 100-times dilutions of JY-22 culture filtrate, respectively. Hence, our observations revealed that the plasma membrane might be a vital antifungal target of JY-22 culture filtrate.

3.4. MDA Content in Plasma Membrane

Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation and the level of MDA in eukaryotic cell is often used as a parameter to evaluate the damage to eukaryotes' cells due to stress. To obtain much stronger evidence to confirm the efficiency and toxicity of JY-22 culture filtrate, the MDA content of *A. alternata* was further detected. As shown in Figure 3c, the MDA content of *A. alternata* increased with the incubation dose of JY-22 culture filtrate. In contrast to the control, the MDA content of phytopathogens was significantly increased in the presence of JY-22 culture filtrate at specific diluents of 10-, 20- and 40-times concentrations, and the corresponding MDA contents were 5.12 μmol/kg, 1.74 μmol/kg and 1.2 μmol/kg, respectively.

3.5. Soluble Protein Content

The content of some soluble proteins can increase under a broad range of stress conditions and is often used as a marker to evaluate the damage to eukaryotes' cells. Thus, we measured soluble protein content to investigate whether JY-22 culture filtrate damaged mycelium cell membranes because once cell membranes were destroyed by external factors, proteins and other large molecules would leak out. Compared with the control, after interaction with JY-22 culture filtrate, the protein leakage of *A. alternata* was obviously concentration-dependent (Figure 3d). Notably, the soluble protein in the 10-fold culture filtrate was as high as 6.28 mg/g. As shown in Figure 3d, compared with the control, the protein leakage rate remarkably reached 164.26 times, indicating that JY-22 culture filtrate may cause protein leakage through damaging the integrity of fungal structures and eventually lead to cell death.

3.6. Bio-Control Efficacy of JY-22 Culture Filtrate

Assessment of the bio-control effect of JY-22 culture filtrate (diluents from 10 to 100 times) against *A. alternata* was investigated through detached-leaf and field experiments. As shown in Figure 4 and Table 1, JY-22 culture filtrate had a certain control effect on *A. alternata* in terms of disease treatment or prevention. In the three years of the prevention and disease treatment experiments, the lesions of leaves treated with filtrate diluents (from 10 to 40 times) were significantly smaller than those of the control group. However, there were no significant differences in the lesions between the leaves treated with the 100 times diluents and the control group. Specifically, in the prevention experiment (pre-inoculation treatment), the leaves treated with JY-22 culture filtrate diluent (10 to 40 times) and carbendazim developed smaller lesions, with lengths of 4.13, 4.60, 4.70 mm and 5.51 mm, while the length of the lesions in the control group was 27.85 mm. Furthermore, a similar trend was observed in the disease treatment experiment (post-inoculation treatment). In the disease treatment experiment, the lesion lengths of leaves treated with three concentrations of JY-22 culture filtrate diluent (10 to 40 times) and carbendazim were 10.60, 13.33, 15.32 mm and 9.55 mm, respectively, while that of the control group was 29.12 mm.

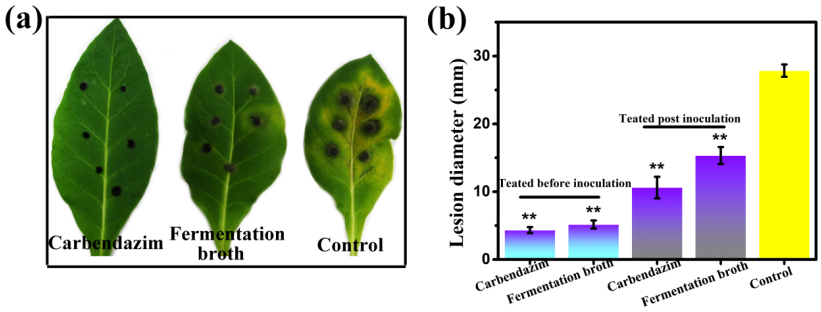

Figure 4. Lesion diameter development 7 days post-inoculation with JY-22 culture filtrate following preventative and curative treatments in vivo. (**a**) Phenotypic images of the preventative effect of JY-22 culture filtrate against A. alternata in vivo; (**b**) lesion length following preventative and therapeutic treatments. SE of three biological replicates. Statistical analysis of data was performed based on one-way ANOVA for samples from multiple treatments. The *p*-values are indicated by stars, **: $p < 0.001$.

Table 1. Lesion diameter results of JY-22 culture filtrate against tobacco brown spot in vivo.

Treatments	Lesion Diameter (mm)	
	Tobacco Treated before Inoculation	Tobacco Treated Post-Inoculation
50% Carbendazim	4.33 ± 0.44 **	9.55 ± 1.59 **
Fermentation broth 1:10	4.13 ± 0.48 **	10.60 ± 0.45 **
Fermentation broth 1:20	4.60 ± 0.28 **	13.33 ± 0.90 **
Fermentation broth 1:40	5.15 ± 0.61 **	15.32 ± 1.25 **
Fermentation broth 1:80	16.33 ± 1.3 **	25.05 ± 1.59
Fermentation broth 1:100	22.28 ± 2.47	28.50 ± 2.08
Control	27.85 ± 0.91	29.12 ± 2.01

**: *p*-value < 0.01. All the means of DSIs and control efficacy followed by asterisks (**) are significantly different from the data of control according to independent-sample t-tests.

The field trial results of the bio-control efficacy experiment for different years are shown in Table 2. Compared with the control, JY-22 culture filtrate diluents (10 to 40 times) remarkably reduced the destructiveness of tobacco brown spot disease. In the prevention experiment, the disease indexes of JY-22 culture filtrates diluted 10 times, 20 times and 40 times were 8.96, 13.94 and 15.3, respectively, significantly lower than that of the control group (66.86). In addition, for the disease treatment experiment, the disease indexes of 10-, 20- and 40-times diluents were 10.26, 14.86, 16.26 and 17.87, respectively, whereas the disease index of the control group was 65.38. For JY-22 culture filtrate diluted 40 times, the relative control effects in the prevention experiment and disease treatment experiment were 77.10% and 72.67%, respectively, significantly greater than that of the control and similar to that of the positive control carbendazim (87.91% and 84.31%, respectively). Therefore, JY-22 culture filtrate had an excellent effect as a biopesticide to control tobacco brown spot.

Table 2. Control efficacy of JY-22 culture filtrate against tobacco brown spot under field conditions.

Treatments	Protective Effect		Therapeutic Effect	
	Disease Severity Index [1] (%)	Control Efficacy [2] (%)	Disease Severity Index [1] (%)	Control Efficacy [2] (%)
Carbendazim	8.08 ± 0.50 **	87.91 ± 0.75 **	10.26 ± 0.70 **	84.31 ± 1.06
Fermentation broth 1:10	8.96 ± 0.37 **	86.59 ± 0.55 **	14.86 ± 1.48 **	77.27 ± 2.26
Fermentation broth 1:20	13.94 ± 1.34 **	79.15 ± 2.01 **	16.26 ± 1.47 **	75.13 ± 2.25
Fermentation broth 1:40	15.31 ± 1.28 **	77.10 ± 1.92 **	17.87 ± 1.43 **	72.67 ± 2.18
Control	66.86 ± 4.59	——	65.38 ± 3.93	——

[1] Disease severity index (DSI) is used to estimate the disease severity, where DSI (%) = [sum (class frequency × score of rating class)]/[(total number of plants) × (maximal disease index)] × 100. [2] Control efficacy (%) = {(DSI of control − DSI of treatment)/DSI of control at the same application time} × 100. All the means of DSIs and control efficacy followed by asterisks (**) are significantly different from the data of control according to independent-sample *t*-tests. **: *p*-value < 0.01.

4. Discussion

Recently, plant fungal disease has caused massive threats and losses to agricultural productivity worldwide. Currently, due to the abuse of fungicides and continuous cropping, plant diseases have become a great threat to human survival. Moreover, other great threats, such as antibiotic resistance of pathogens and environmental risk factors caused by the inappropriate application of fungicides, have emerged. Thus, eco-friendly and effective agents that control fungi are urgently needed, and biological control has attracted extensive research interest in the sustainable management of fungal plant diseases [12,14,19]. Usually, the main method underlying bio-control to suppress plant disease is to utilize antagonistic living organisms to defeat plant pathogens [20].

As bio-control agents, Actinomycetes have been found to have promising effects on a number of crop diseases [9]. Therein, *Streptomyces hygroscopicus*, as a vital Actinomycete, inhibits the growth of target microorganisms because of its robust viability and can utilize various compounds as the sole C-source, efficiently competing for space and nutrients [9,21]. In agricultural and horticultural protection, *Streptomyces* spp. produce more than 60% of antibiotics that serve as inhibitors of phytopathogens [21]. Hence, it is still crucial to develop bio-control agents and study their antifungal activities.

The bio-control strain in our study (Actinomyces strain JY-22) was identified as *S. hygroscopicus*. Pathogen infection and reproduction proceed with mycelium growth, owing to the asexual reproduction of mycelium [22]. Inhibiting fungal growth is a typical method to reduce fungal proliferation. Dual culture experiments showed that JY-22 and *A. alternata* competed for limited space and nutrients, thereby interfering with pathogen growth. In contrast, at the ultrastructural level, we observed obvious morphological alterations of *A. alternata* hyphae by light microscopy and revealed that the JY-22 strain had strong antifungal activity against *A. alternata* by curbing or damaging the hyphae. The strong activities of the chitinase, cellulase and β-1,3-glucanase of *S. hygroscopicus* could destroy or disintegrate the cell walls of fungal pathogens [23,24]. Furthermore, the injuries might have caused the cellular breakdown of *A. alternata*, which undoubtedly affected the survival of the fungus.

Actinomycetes as bio-control agents are usually directly applied to plants through spore suspensions or culture filtrates [25]. More importantly, researchers have speculated that the key factors contributing to the antifungal activities of this bio-control microbial strain are metabolites [9,12]. Hence, the antifungal activities of *S. hygroscopicus* fermentation filtrate on both mycelial growth and spore germination have been primarily assessed. All tested JY-22 culture filtrate diluents (at concentrations of 10–100 times) exhibited marked antifungal effects against *A. alternata*. Therefore, these observations confirmed that both JY-22 and its fermentation filtrate can inhibit the spore germination and mycelial membrane formation of *A. alternata*. Some possible mechanisms underlying the antifungal properties of JY-22 culture filtrate against bio-control strains have been proposed. Previous reports have disclosed that *S. hygroscopicus* is an efficient bio-control agent because it produces a large number of secondary metabolites. In this case, antibiotic production is one of the key functions, including various antimicrobial compounds [14]. *S. hygroscopicus* could produce approximately 650 types of secondary bioactive metabolites, some of which were applicable as antimicrobial agents to combat pathogenic bacteria in plants [25]. Among these bioactive metabolites, rapamycin and clethramycin have antifungal activity [26,27]. Therefore, according to these works within the literature, metabolites from the JY-22 culture filtrate are speculated to some possess some antibiotic properties. Moreover, microbes produce abundant secondary metabolites during the fermentation process, and these metabolites may change with a change in environment and further suppress pathogen growth [27]. Herein, it was speculated that one or more metabolites produced in the fermentation process of JY-22 can inhibit *A. alternata* growth. However, the exact mechanism needs to be investigated.

Furthermore, several studies have shown that the mechanism of antifungal agents against plant pathogens is related to membrane disruption by some highly lipophilic

and low-molecular-weight components that can pass through the cell membranes easily and lead to intracellular leakage, eventually resulting in fungal cell death [12,28,29]. As expected, the extracellular conductivity and ergosterol and MDA contents in our study conform to the above mechanism. The conductivity of the group incubated with JY-22 culture filtrate was higher than that of the control group during the experiment, and the values increased with time and fermentation concentration. Moreover, ergosterol is a major sterol component of the cell membrane, vital to cell integrity and normal function [17]. JY-22 culture filtrate exhibited a strong inhibition on ergosterol content for 10–80 times diluents, revealing that it had strong permeation and cell membrane disruption. The results of this study were similar to those of recent studies on plant-derived antifungal agents [15]. Collectively, the physical and morphological structures of *A. alternata* hyphae were changed after treatment with JY-22 culture filtrate, largely attributed to the effect of the filtrate on the permeability and integrity of the pathogen's cell membrane. In addition, JY-22 culture filtrate resulted in mass protein loss from cells, further indicating irreversible damage to cytoplasmic membranes.

In the detached-leaf and field tests, the disease severity index (DSI) in the control group reflected that the tobacco plants were under disease pressure. Tobacco brown spot is a leaf disease, and a reduction in this disease means an increase in tobacco production. Hence, these results further suggested that JY-22 culture filtrate was effective against tobacco brown spot disease, suggesting that it is a promising bio-control agent against *A. alternata*. Interestingly, our data demonstrated that the preventative treatment of the culture filtrate had a better control effect than the curative treatment. Previous studies suggested that with the application of the fermented organic fertilizer *S. hygroscopicus* B04, the B04 strain could better adapt to the strawberry rhizosphere and protect plants from pathogens [30]. In this regard, JY-22 culture filtrate might have potential applications as an accelerator to increase the colonization of some other *S. hygroscopicus* bio-control strains, eventually increasing fungal densities and inhibiting pathogens. In addition to its broad-spectrum antifungal ability, which includes activity against *Fusarium oxysporum*, *S. hygroscopicus* can also be extensively used in crop production and has economic development benefits due to its advantages in plant and fruit growth and production promotion [9,30,31]. Overall, the culture filtrate of *S. hygroscopicus* may be a potential bio-control agent against tobacco brown spot disease. Nevertheless, some challenges remain, including determination of its effective components, specific antifungal mechanisms and safety during usage in plant protection.

5. Conclusions

Overall, both in vitro and in vivo results showed that JY-22 isolated from rhizospheric soil is a great potential alternative as a fungicide against *A. alternata* and can serve as a biopesticide for biological control of tobacco brown spot in the future (Figure 5). Both the strain and its culture filtrate showed superior antifungal properties against the growth of *A. alternata*, and this inhibition increased with increases in culture filtrate concentration. This antifungal action resulted in the inhibition of mycelium growth and spore germination. Furthermore, the permeability of the *A. alternata* cytomembrane, which exhibited a degree of leakage, was changed, and the MDA, soluble ergosterol and soluble protein contents were altered, ultimately resulting in fungal death and remarkably reducing tobacco brown spot diseases under field conditions.

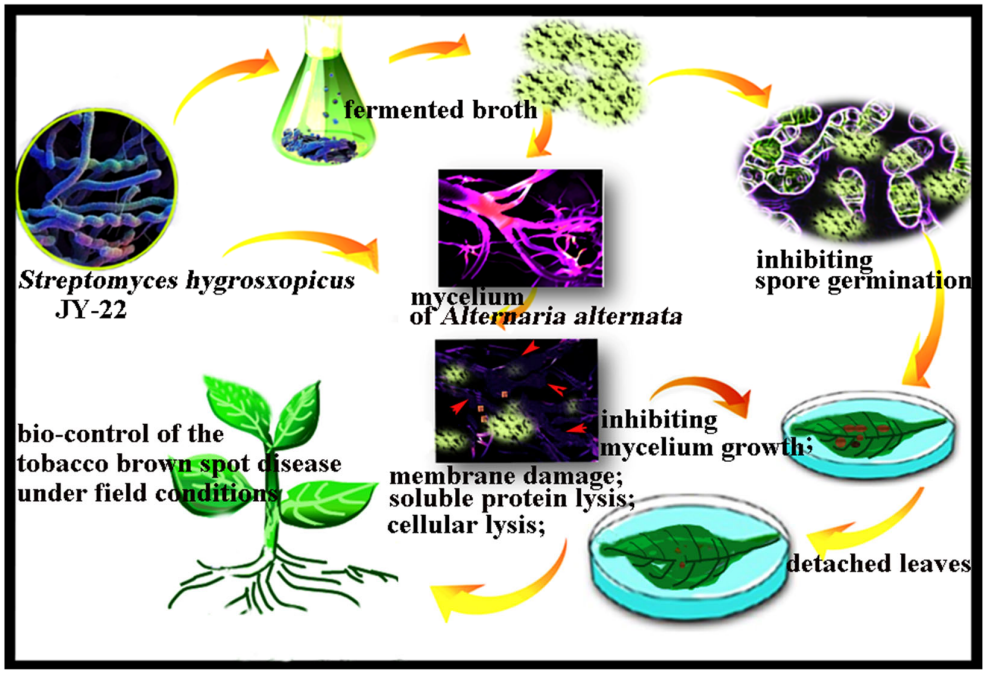

Figure 5. Schematic representation of application of *Streptomyces hygroscopicus* JY-22 to control tobacco brown spot caused by *A. alternata*.

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References

1. Yang, S.L.; Chung, K.-R. The NADPH oxidase-mediated production of hydrogen peroxide (H_2O_2) and resistance to oxidative stress in the necrotrophic pathogen *Alternaria alternata* of citrus. *Mol. Plant Pathol.* **2012**, *13*, 900–914. [CrossRef] [PubMed]
2. Yan, F.; Hu, H.; Lu, L.; Zheng, X. Rhamnolipids induce oxidative stress responses in cherry tomato fruit to *Alternaria alternata*. *Pest Manag. Sci.* **2016**, *72*, 1500–1507. [CrossRef] [PubMed]
3. Jing, C.; Zhao, J.; Han, X.; Huang, R.; Cai, D.; Zhang, C. Essential oil of *Syringa oblata* Lindl. as a potential biocontrol agent against tobacco brown spot caused by *Alternaria alternata*. *Crop Prot.* **2018**, *104*, 41–46. [CrossRef]
4. Kohmoto, K. Correlation of resistance and susceptibility of Citrus to *Alternaria alternata* with sensitivity to host-specific toxins. *Phytopathology* **1991**, *81*, 719–722. [CrossRef]
5. Cheng, D.-D.; Jia, Y.-J.; Gao, H.-Y.; Zhang, L.-T.; Zhang, Z.-S.; Xue, Z.-C.; Meng, Q.-W. Characterization of the programmed cell death induced by metabolic products of *Alternaria alternata* in tobacco BY-2 cells. *Physiol. Plant.* **2011**, *141*, 117–129. [CrossRef]
6. Chen, Y.-H.; Lu, M.-H.; Guo, D.-S.; Zhai, Y.-Y.; Miao, D.; Yue, J.-Y.; Yuan, C.-H.; Zhao, M.-M.; An, D.-R. Antifungal Effect of Magnolol and Honokiol from Magnolia officinalis on *Alternaria alternata* Causing Tobacco Brown Spot. *Molecules* **2019**, *24*, 2140. [CrossRef]
7. Wang, Y.; Zeng, X.; Zhou, Z.; Xing, K.; Tessema, A.; Zeng, H.; Tian, J. Inhibitory effect of nerol against *Aspergillus niger* on grapes through a membrane lesion mechanism. *Food Control* **2015**, *55*, 54–61. [CrossRef]
8. Muslim, A.; Horinouchi, H.; Hyakumachi, M. Biological control of Fusarium wilt of tomato with hypovirulent binucleate *Rhizoctonia* in greenhouse conditions. *Mycoscience* **2003**, *44*, 77–84. [CrossRef]

9. Shen, T.; Wang, C.; Yang, H.; Deng, Z.; Wang, S.; Shen, B.; Shen, Q. Identification, solid-state fermentation and biocontrol effects of *Streptomyces hygroscopicus* B04 on strawberry root rot. *Appl. Soil Ecol.* **2016**, *103*, 36–43. [CrossRef]
10. Cuesta, G.; García-de-la-Fuente, R.; Abad, M.; Fornes, F. Isolation and identification of actinomycetes from a compost-amended soil with potential as biocontrol agents. *J. Environ. Manag.* **2012**, *95*, S280–S284. [CrossRef]
11. Demain, A.L. Antibiotics: Natural products essential to human health. *Med. Res. Rev.* **2009**, *29*, 821–842. [CrossRef] [PubMed]
12. Shi, L.; Nwet, T.T.; Ge, B.; Zhao, W.; Liu, B.; Cui, H.; Zhang, K. Antifungal and plant growth-promoting activities of *Streptomyces roseoflavus* strain NKZ-259. *Biol. Control* **2018**, *125*, 57–64. [CrossRef]
13. Cai, L.; Liu, M.; Liu, Z.; Yang, H.; Sun, X.; Chen, J.; Xiang, S.; Ding, W. MgONPs Can Boost Plant Growth: Evidence from Increased Seedling Growth, Morpho-Physiological Activities, and Mg Uptake in Tobacco (*Nicotiana tabacum* L.). *Molecules* **2018**, *23*, 3375. [CrossRef]
14. Cong, Y.; Fan, H.; Ma, Q.; Lu, Y.; Xu, L.; Zhang, P.; Chen, K. Mixed culture fermentation between *Rhizopus nigricans* and *Trichoderma pseudokoningii* to control cucumber Fusarium wilt. *Crop Prot.* **2019**, *124*, 104857. [CrossRef]
15. Tian, J.; Wang, Y.; Zeng, H.; Li, Z.; Zhang, P.; Tessema, A.; Peng, X. Efficacy and possible mechanisms of perillaldehyde in control of *Aspergillus niger* causing grape decay. *Int. J. Food Microbiol.* **2015**, *202*, 27–34. [CrossRef] [PubMed]
16. Li, J.; Lei, H.; Song, H.; Lai, T.; Xu, X.; Shi, X. 1-methylcyclopropene (1-MCP) suppressed postharvest blue mold of apple fruit by inhibiting the growth of *Penicillium expansum*. *Postharvest dBiol. Technol.* **2017**, *125*, 59–64. [CrossRef]
17. Wang, L.; Hu, W.; Deng, J.; Liu, X.; Zhou, J.; Li, X. Antibacterial activity of *Litsea cubeba* essential oil and its mechanism against *Botrytis cinerea*. *RSC Adv.* **2019**, *9*, 28987–28995. [CrossRef] [PubMed]
18. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
19. Rong, S.; Xu, H.; Li, L.; Chen, R.; Gao, X.; Xu, Z. Antifungal activity of endophytic *Bacillus safensis* B21 and its potential application as a biopesticide to control rice blast. *Pestic. Biochem. Physiol.* **2020**, *162*, 69–77. [CrossRef]
20. Coakley, S.M.; Scherm, H.; Chakraborty, S. Climate change and plant disease management. *Annu. Rev. Phytopathol.* **1999**, *37*, 399–426. [CrossRef]
21. Hwang, B.K.; Lim, S.W.; Kim, B.S.; Lee, J.Y.; Moon, S.S. Isolation and in vivo and in vitro antifungal activity of phenylacetic acid and sodium phenylacetate from *Streptomyces humidus*. *Appl. Environ. Microbiol.* **2001**, *67*, 3739–3745. [CrossRef] [PubMed]
22. Yan, F.; Xu, S.; Guo, J.; Chen, Q.; Meng, Q.; Zheng, X. Biocontrol of post-harvest *Alternaria alternata* decay of cherry tomatoes with rhamnolipids and possible mechanisms of action. *J. Sci. Food Agric.* **2015**, *95*, 1469–1474. [CrossRef] [PubMed]
23. Ruiz-Herrera, J.; Elorza, M.V.; Valentãn, E.; Sentandreu, R. Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. *FEMS Yeast Res.* **2006**, *6*, 14–29. [CrossRef] [PubMed]
24. Zhao, J.; Xue, Q.-H.; Niu, G.-G.; Xue, L.; Shen, G.-H.; Du, J.-Z. Extracellular enzyme production and fungal mycelia degradation of antagonistic *Streptomyces* induced by fungal mycelia preparation of cucurbit plant pathogens. *Ann. Microbiol.* **2013**, *63*, 809–812. [CrossRef]
25. Lima, S.M.A.; Melo, J.G.D.S.; Militão, G.C.G.; Lima, G.M.S.; Lima, M.D.C.A.; Aguiar, J.; Araújo, R.; Braz-Filho, R.; Marchand, P.; Araújo, J.M.; et al. Characterization of the biochemical, physiological, and medicinal properties of *Streptomyces hygroscopicus* ACTMS-9H isolated from the Amazon (Brazil). *Appl. Microbiol. Biotechnol.* **2016**, *101*, 711–723. [CrossRef]
26. Geng, H.; Liu, H.; Liu, J.; Wang, C.; Wen, J. Insights into the metabolic mechanism of rapamycin overproduction in the shikimate-resistant *Streptomyces hygroscopicus* strain UV-II using comparative metabolomics. *World J. Microbiol. Biotechnol.* **2017**, *33*, 101. [CrossRef]
27. Furumai, T.; Yamakawa, T.; Yoshida, R.; Igarashi, Y. Clethramycin, a new inhibitor of pollen tube growth with antifungal activity from *Streptomyces hygroscopicus* TP-A0623. *J. Antibiot.* **2003**, *56*, 700–704. [CrossRef]
28. Marchese, A.; Barbieri, R.; Coppo, E.; Orhan, I.E.; Daglia, M.; Izadi, M.; Abdollahi, M.; Nabavi, S.M.; Ajami, M. Antimicrobial activity of eugenol and essential oils containing eugenol: A mechanistic viewpoint. *Crit. Rev. Microbiol.* **2017**, *43*, 668–689. [CrossRef]
29. Hu, Y.; Zhang, J.; Kong, W.; Zhao, G.; Yang, M. Mechanisms of antifungal and anti-aflatoxigenic properties of essential oil derived from turmeric (*Curcuma longa* L.) on *Aspergillus flavu*. *Food Chem.* **2017**, *220*, 1–8. [CrossRef]
30. Gopalakrishnan, S.; Pande, S.; Sharma, M.; Humayun, P.; Kiran, B.K.; Sandeep, D.; Vidya, M.S.; Deepthi, K.; Rupela, O. Evaluation of actinomycete isolates obtained from herbal vermicompost for the biological control of *Fusarium* wilt of chickpea. *Crop Prot.* **2011**, *30*, 1070–1078. [CrossRef]
31. Goudjal, Y.; Toumatia, O.; Yekkour, A.; Sabaou, N.; Mathieu, F.; Zitouni, A. Biocontrol of *Rhizoctonia solani* damping-off and promotion of tomato plant growth by endophytic actinomycetes isolated from native plants of Algerian Sahara. *Microbiol. Res.* **2014**, *169*, 59–65. [CrossRef] [PubMed]