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Identification the Pathogens Causing Rot Disease in Pomegranate (*Punica granatum* L.) in China and the Antifungal Activity of Aqueous Garlic Extract

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Abstract: Rot disease is a serious disease in pomegranate (*Punica granatum* L.) plantations in China. This disease usually weakens tree vigor, and seriously reduces the ornamental value, fruit yield, and quality. A better understanding of the pathogen that causes a disease is important for its control. Thus, the aims of this study were to isolate and identify the pathogen causing rot disease and to explore substances for its biological control. In this study, the morphology of the hyphae and spores of the pathogens was observed, and the pathogens were identified by morphological characteristics and the internal transcribed spacer (ITS) regions of their rDNA. Furthermore, the activity of an aqueous garlic extract as antifungal treatment for the identified pathogens was assessed. The results showed that the pathogens causing soft rot and dry rot in 'Xinjiang Big Seed' pomegranate were most probably *Aspergillus niger* and *Botryosphaeria dothidea*, respectively. In addition, the pathogenicity of *A. niger* was stronger than that of *B. dothidea.* The aqueous garlic extract had a strong antifungal effect on both pathogens by inhibiting mycelium growth in vitro, and the minimum inhibitory concentrations against *A. niger* and *B. dothidea* were 7.5 mg/mL and 10 mg/mL, respectively.

Keywords: pomegranate; rot disease; pathogen identification; antifungal activity; garlic extract

1. Introduction

Pomegranate (*Punica granatum* L.) trees have a long cultivation history. This species has strong adaptability and a wide cultivation area. They are widely planted in Shandong, Shaanxi, Henan, and Xinjiang provinces in northern China, and there is also a certain amount of pomegranate cultivation in southern China [1]. The pomegranate tree is one of the preferred trees for landscape greening and has high ornamental value. The pomegranate fruit, which is usually consumed either as fresh fruit or fruit juice, is nutritious with a high content of carbohydrates, flavonoids, vitamins, folic acid, potassium, iron, and other mineral substances [2,3]. In addition, the pomegranate fruit has relatively high amounts of pharmacologically and medicinally bioactive compounds, which contribute to the hypolipidemic, antioxidant, antiviral, anticancer, antibacterial, and vascular protection effects [3–6]. A recent study has also shown that the pomegranate metabolite Urolithin A(UroA) can alleviate inflammatory bowel disease [7]. Therefore, pomegranate fruit is increasing popular with consumers, and the market demand has recently risen. Nevertheless, pomegranate trees and fruit are susceptible to either bacterial or fungal diseases during growth. Of these diseases, rot disease is possibly the most serious one in China. The rot disease weakens tree vigor and leads to lower ornamental and greening value. In addition, the fruit yield and quality can be seriously reduced in the pomegranate cultivations. On the whole, the ecological and economic benefits of pomegranate trees are severely restricted by rot disease

Studies have identified different pathogens causing rot diseases in pomegranate in different countries. The identified pathogens that cause pomegranate rots include *Zythia versoniana* Sacc. [1], *Botrytis cinerea* [8], *Botryosphaeria dothidea* [9], *Pilidiella granati* [10], *Neofusicoccum parvum*, and *Alternaria alternata* [11]. These studies indicate that the pathogens that cause rot disease may vary with the natural environment of the disease. Since rot disease is becoming rather serious in pomegranate production areas in China, it is particularly urgent to isolate and identify these pathogens, with an emphasis on effective disease prevention and control. DNA sequences are key resources in fungal molecular identification, which usually relies on sequencing the nuclear ribosomal internal transcribed spacer (ITS) region [12]. By analyzing the sequence information of the ITS region, individual fungus would be able to be identified and classified quickly and effectively. In addition to molecular identification, morphological and physiological characteristics can also have differences that reflect species boundaries. These characteristics can be used not only as tools for identifying a species, but also for understanding its biology [13].

In recent years, rot diseases in pomegranate have been prevented by spraying with fungicides or other chemicals. This not only results in chemical residues that might be hazardous to human health, but also causes environmental pollution. Therefore, biological control has gradually become an alternative due to safety and environmental concerns. Garlic is a traditional Chinese vegetable that belongs to the family Liliaceae. Studies have shown that allicin in garlic has a broad range of biological activities in microbial, plant, and mammalian cells [14]. It has been confirmed that garlic extract has an antibacterial effect on human dental plaque microbiota [15,16]. Meanwhile, the strong inhibitory effect of garlic extract was also confirmed on dimorphic fungi such as *Sporothrix schenckii* [17]. Therefore, it is possible that garlic extract can exert its inhibitory effect on pathogens that cause rot disease in pomegranate. Moreover, garlic is an inexpensive material and widely available; therefore, it has potential for development and production as a natural fungicide.

In this study, two pathogens that can cause soft rot and dry rot were isolated from pomegranate fruit. The pathogens were identified by the morphological characteristics of colonies and through sequence analysis of the ITS regions. It was also confirmed that the aqueous garlic extract had a significant antifungal effect on these identified pathogens. This study provides a theoretical basis for the biological control of pomegranate rot disease.

2. Materials and Methods

2.1. Plant Material

'Xinjiang Big Seed' pomegranate fruit showing symptoms of rot were collected from local pomegranate storage caves in December 2017 in Liquan County, Shaanxi Province, China. Fresh and healthy pomegranates were collected for pathogenicity testing from a well-managed orchard in October 2018 in Liquan County, Shaanxi Province, China. Mature 'Cangshan' garlic (*Allium sativum* L.) with a white dry peel was purchased from a local market located at Boxue Road, Yangling, Shaanxi Province, China.

2.2. Pathogen Isolation

The epidermal tissues that were taken from the margin of the rotted part of the pomegranate were sterilized with 70% ethanol and then were cut into small pieces (approximately 5 mm in diameter). The small pieces were transferred into potato dextrose agar (PDA) and incubated at 28 °C for 48 h. The mycelia were collected from the edge of the colony and transferred to new PDA plates for purification. The previous step was repeated twice, and the single colonies of each pathogen were isolated for further identification.

2.3. Identification of the Pathogens by Koch's Postulates

Spores or hyphae were taken from the third generation colony to prepare a spore suspension or a hyphae sample. The concentration of the spore suspension was adjusted to 1×10^6 cells/mL with sterilized double-distilled H_2O. Healthy pomegranate fruits were sterilized for 30 s with 70% ethanol and rinsed with sterile distilled water [9]. After the water droplets on the fruit surface were dried by tissue paper, the pomegranates were wounded (approximately 1 × 1 mm, 4 mm in depth) on the surface (two points per pomegranate) with a sterilized pipette tip. The spore suspension or hyphae sample was injected into each wounded point. After 30 min of inoculation, the injected pomegranates were stored in a dark chamber with constant temperature and humidity (28 °C, 90% relative humidity, RH). The morphological characteristics of the lesions were observed for 7 to 10 days. When the same symptoms appeared, small pieces of the rotten tissue were collected from the diseased junction of the pomegranate and cultured on potato dextrose agar (PDA) plates. Thereafter the colony growth characteristics were observed and recorded.

2.4. Morphological Identification of Pathogens

The colonies isolated from the third generation of isolated pathogens were subcultured on the new PDA plates and incubated at 28 °C for 12 days. The morphological characteristics of the colonies were observed every day. The morphology of the spores and hyphae was observed under an optical microscope. The morphological characteristics were compared with those observed in previous studies to identify the pathogens [18–20].

2.5. DNA Extraction

The genomic DNA of the third generation of isolated pathogens was extracted by the CTAB method [21]. Pathogens grew on PDA plates that were covered with cellophane, so that the samples could be collected easily for DNA extraction. Pathogen samples were collected in the early growth stage (3 days).

2.6. Polymerase Chain Reaction (PCR) and Sequencing

The universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGC-3′), ITS4 (5′-TCCTCCGCT TATTGATATGC-3′), and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) were used as primers for the PCR [22,23]. ITS4/ITS5 and ITS1/ITS4 were used to clone pathogens A and B, respectively. PCR was performed with 10 μL PCR mixture including 5 μL of PrimeSTAR Max DNA polymerase (Takara, Tokyo, Japan), 0.5 μL of ITS1 and 0.5 μL ITS4 for pathogen A, and 0.5 μL ITS4 and 0.5 μL of ITS5 primers, 1 μL of genomic DNA template, and 3 μL of double-distilled H_2O. The PCR was performed as follows: predenaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 45 s with 35 cycles, and extension at 72 °C for another 10 min. After the PCR, 5 μL of the PCR product was mixed with 1 μL of 6 × loading buffer (bromophenol blue) for staining. The stained PCR product was added to a 1.0% agarose gel and electrophoresed at 189 V, 255 mA for 15 min. Then, the target strips were observed under ultraviolet irradiation. After that, the target strips were collected and ligated to the pMD-19T vector and transferred to DH5α competent cells. After ampicillin resistance screening, monoclonal colonies were picked for shaking culture and sequenced by TSINGKE Biological Technology Co. Ltd. (Beijing, China) The sequencing results were analyzed online using the BLAST analysis program on the National Center for Biotechnology Information (NCBI) nucleic acid database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequenced ITS fragments of the isolated pathogens were aligned with similar sequences and other sequences of different species with the same genus from NCBI by the Clustal W multiple alignment. Phylogenetic tree analysis was conducted using the Neighbor-Joining (NJ) statistical method [24] and the maximum composite likelihood substitution model [25] on MEGA 7.0. The phylogenetic trees were inferred with 1000 bootstrap (BS) replicates.

2.7. Pathogenicity Test

A spore suspension was prepared, and the concentration was adjusted to 1×10^6 cells/mL with sterile double-distilled H_2O. Fresh pomegranates were wounded (approximately 1 × 1 mm, 4 mm in depth, two opposite holes in each fruit) with a sterilized pipette tip, and 20 µL of the spore suspension was injected into the wounded point. For pathogens without sporulation, mycelia disks of equal size (diameter of 5 mm) that were punched by a perforator from the PDA plates were attached to the wounded points. The control pomegranates were wounded in the same way and inoculated with an injection of 20 µL sterile double-distilled water. After 30 min of inoculation, all injected pomegranates were stored in a dark chamber at 28 °C and 90% RH. On the 3rd, 7th, and 10th day after infection, the lesion size was assessed by the cross method: lesion diameter = (lesion diameter A + diameter B perpendicular to diameter A)/2 [26]. For each unit treatment (infected and the control), five fruits were used as one replicate, the experiment was replicated three times, and two units of treatment were applied.

2.8. Antifungal Assays

The fresh garlic was peeled and cut into pieces (approximately 2 mm thickness), and dried in a freeze dryer (LABCONCO, USA) for 72 h. The dried garlic pieces were ground into a powder, and 250 g of garlic powder was accurately weighed and put into an Erlenmeyer flask to which 1000 mL of sterile distilled water was added. The mixture was shaken at 28 C and 200 r/min for 2 h and filtered with four layers of sterilized gauze (pore size 1 mm), to obtain a raw extract. The raw extracts were used immediately.

The antifungal assays were determined by the changes in the diameter of fungal colonies on the Petri dishes. The raw extract was divided into 50 mL tubes and centrifuged at 2000 g for 10 min at 20 °C. Then the supernatant was collected and diluted with the sterilized PDA media before the media was poured into Petri dishes to obtain the final concentration gradient corresponding to 1, 2.5, 7.5, 10, 50, and 100 mg garlic powder per mL media as the different treatments. The aqueous garlic extract was evenly mixed with the sterilized media. The PDA media without the aqueous garlic extract was used as a control. A Petri dish of 55 mm inner diameter was used for pathogen cultivation and each dish contained approximately 10 mL culture media with different concentrations of the aqueous garlic aqueous extract. Thereafter, a 0.5 mm culture media cube with mycelia was removed from the edge of a 3 day old, actively growing colony, and placed in the center of each dish, and then the dishes were sealed with Parafilm and placed in an incubator at 28 °C. For each pathogen, five Petri dishes were prepared for each concentration gradient, and the treatment was repeated three times. When the mycelia of the control group grew over the entire culture dish, the colony diameter of each treated dish was determined by the cross method. Thereafter, the minimum inhibitory concentration (MIC) was recorded, and the inhibitory rate was calculated by the following formula:

$$\text{Inhibitory rate} = \frac{\text{The growth of control group} - \text{The growth of treatment group}}{\text{The growth of control group}} \times 100\%.$$

2.9. Statistical Analysis

All the statistical analyses were performed using Minitab 17 (Minitab Ltd., State College, PA, USA), and one-way analysis of variance (ANOVA) was applied to the lesion size data from the infected pomegranates for each pathogen. The differences were determined by using Tukey's test and were considered statistically significant if $p < 0.05$. Similarly, significant differences in the inhibition rate between pathogens were analyzed with the above methods.

3. Results

3.1. Observation of the Rot Symptoms

Two types of rot disease symptoms which were observed on the pomegranates: soft rot and dry rot. The soft rot lesions were soft, with yellowish brown skin and a dark color at the edge (the blue dotted line indicates the rotten area in Figure 1a). At the late stage of infection, black spotted mildew appeared on the lesion (Figure 1a). In the case of dry rot, the lesions were dry and brown, with an irregular shape (Figure 1b).

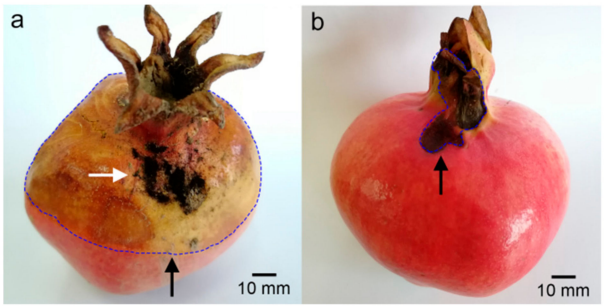

Figure 1. Symptoms of soft rot (**a**) and dry rot (**b**) in pomegranate under natural conditions.

After purification, two main pathogens were isolated from the rotten pomegranate fruit. These pathogens were identified as pathogens A and B, which could cause soft rot and dry rot, respectively.

3.2. Pathogenicity Identification of the Pathogens

The purified pathogens A and B were reinfected into fresh pomegranates, and the same symptoms as in the rot sample pomegranates appeared at the inoculation sites (Figure 2). In the pathogen A-injected pomegranate, as the rot increased, the development of soft lesions occurred. Soft lesions with yellowish brown skin and black spotted mildew appeared, and the black mildew spread to the internal part of the lesion (Figures 2b and 3b). The inside of the dry rot pomegranate showed light pink or off-white seed discolorations (Figure 3c). The corresponding pathogens were isolated again from the diseased parts, and it was confirmed that pomegranate rot was induced by these two pathogens.

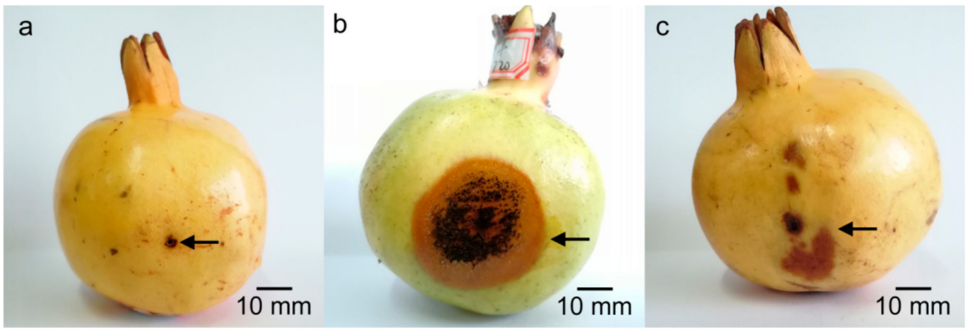

Figure 2. Control pomegranates (**a**), and pomegranates infected by pathogen A (**b**) and infected by pathogen B (**c**) after 6 days in the chamber (28 °C, 90% RH).

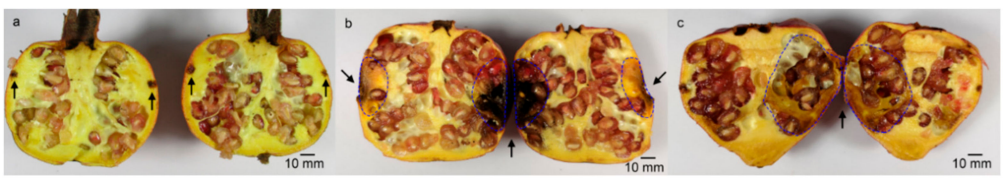

Figure 3. Interior symptoms of the control pomegranate (**a**), the soft rot pomegranate (**b**), and the dry rot pomegranate (**c**) after 10 days of pathogen infection in the chamber (28 °C, 90% RH).

3.3. Morphological Identification of the Pathogens

The two pathogens grew vigorously on PDA plates at 28 °C, but their morphological characteristics were different. The mycelia of the pathogen A were white and creeping, and black vesicles were produced after 2 to 3 days of culture (Figure 4a,b). The microscopic observation results showed that the conidial head was subsphaeroidal to radial and dark brown to deep dark brown in color. The conidia were spherical, transparent, and colorless (Figure 5a,b). For pathogen B, the mycelia were slender and loose, and the aerial mycelia were flourishing (Figure 4c,d). The microscopic results showed that the hyphae of pathogen B branched more than those of pathogen A. However, no spores were observed (Figure 5c,d). In addition, other treatments including ultraviolet and scratch treatments were applied, but they did not show spore development.

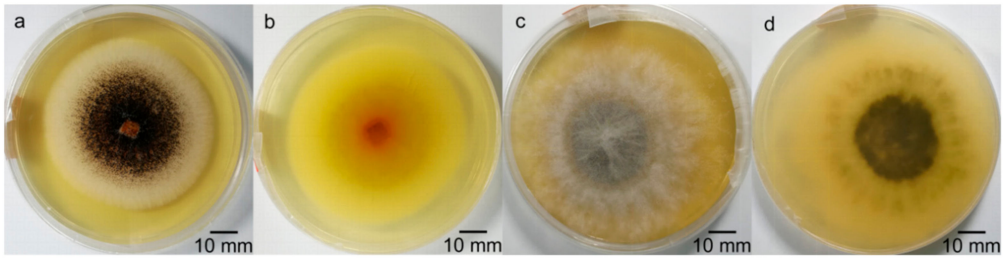

Figure 4. Morphology of pathogen A (5 days) and B (3 days) on PDA plates. Front (**a**) and back (**b**) of pathogen A on PDA plates; front (**c**) and back (**d**) of pathogen B on PDA plates.

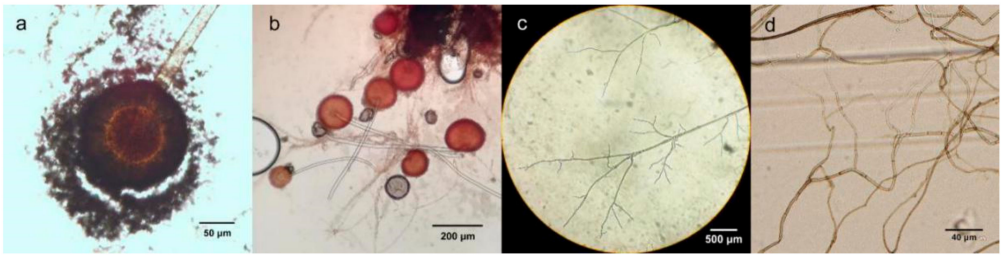

Figure 5. Morphology of pathogens A and B under the microscope. (**a**,**b**) Vesicles of pathogen A (5 days); (**c**,**d**) Hyphae of pathogen B (3 days).

According to the analysis of the morphological characteristics, including the colonies, hyphae, and spores, it was suggested that pathogen A was *Aspergillus niger* and pathogen B was *Botryosphaeria dothidea.*

3.4. Molecular Biology Identification

The fungal rDNA ITS regions (ITS1-5.8s-ITS2) of the two pathogens were cloned and sequenced. The rDNA ITS fragments of pathogens A and B were 623 bp and 470 bp, respectively (Figures S1 and S2). According to the BLAST result from NCBI, the ITS fragment of pathogen A had maximum query coverage (100%) and high identity (100%) with the genus *A. niger* (MH109325.1) (Figure S1). Meanwhile, the ITS fragment of pathogen B had 100% identity (with 100% query cover) with that of *Botryosphaeria* sp. Ponipodef 09 (HQ731645.1) (Figure S2). In addition, the pathogen B ITS fragment also had very high identity (99.79% with 100% query cover) with that of other *B. dothidea* strains or isolates (MF040305.1 and HQ660462.1).

The phylogenetic tree showed that pathogen A clustered into a large group with *A. niger* strain CBS 117.36 (MH855726.1), *A. niger* isolate SMP2 (MG675233.1), *A. niger* strain WM 10.86 (HQ014690.1), and *A. niger* strain EIM-6 (FJ040211.1) (Figure S3). Pathogen B had high identity with other *B. dothidea* strains or isolates, which had rather high similarity with each other. After phylogenetic analysis, pathogen B clustered into a large group with *Botryosphaeria* sp. Ponipodef 09 (HQ731645.1) and sp. Ponipodef 03 (HQ731639.1), four other *B. dothidea* isolates (HQ660462, KJ381064.1, KP183165.1, and

KY393146.1), and five *B. dothidea* strains (KC960931.1, KF293923.1, MH013509.1, NNRO01000251.1, and MF040305.1) (Figure 6).

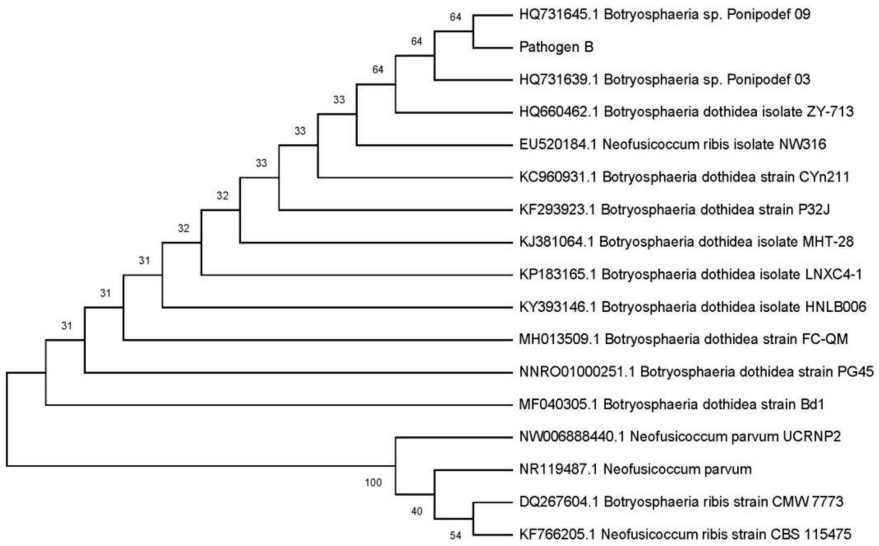

Figure 6. Phylogenetic tree of *Botryosphaeria* species. The evolutionary history was inferred using the neighbor-joining method [24]. The evolutionary distances were computed using the maximum composite likelihood method [25], and the numbers are shown near the branches.

According to the above analysis, pathogen A is likely to be *Aspergillus niger*, and pathogen B is likely to be *Botryosphaeria dothidea*.

3.5. Pathogenicity Test

In the first 3 days after infection, the pomegranates infected by both fungi did not show visual symptoms, but on day 10 after infection, the lesion diameter of rot from *A. niger* infection was as high as 22 mm, while that from *B. dothidea* infection was only 7.8 mm (Figure 7).

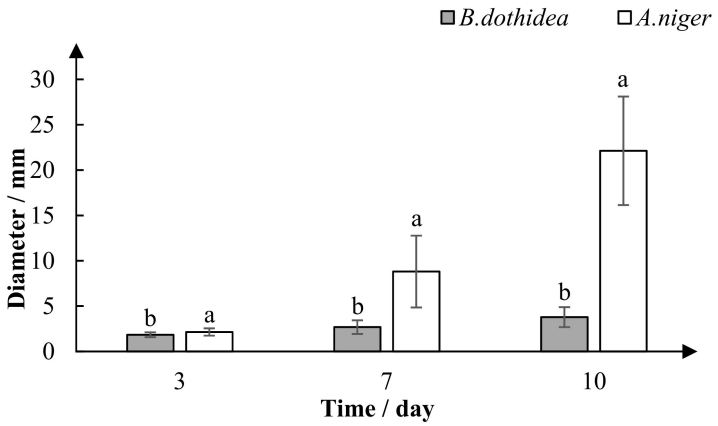

Figure 7. The change in lesion diameter from pomegranates infected by *Aspergillus niger* and *Botryosphaeria dothidea.* (The values are the means, and error bars indicate the standard deviations. Different letters (a/b) indicate significant differences between two pathogens, n = 18).

3.6. Antifungal Assays

The aqueous garlic extract showed a strong inhibitory effect on the growth of *A. niger* and *B. dothidea* in vitro. With the increase in the concentration of the aqueous garlic extract, the inhibitory effect on both pathogens increased gradually. The growth of the black vesicles produced by *A. niger*

and the aerial mycelium produced by *B. dothidea* were significantly reduced (Figures 8 and 9). On day four of growth, the colony diameter of *A. niger* of was 42 mm, 28 mm, and 25 mm under the 1 mg/mL, 2.5 mg/mL, and 5 mg/mL aqueous garlic extract treatments, respectively, compared with a 40.5 mm colony diameter in the control (Figure 8). For *B. dothidea*, the control colony spread to the whole plate (55 mm) on day three, and the colony diameter under the 1 mg/mL, 2.5 mg/mL, and 5 mg/mL aqueous garlic extract treatments was 42.5 mm, 28.5 mm, and 20.5 mm, respectively (Figure 9). When the concentration of the aqueous garlic extract reached 7.5 mg/mL, the colony edge of *B. dothidea* began to grow irregularly.

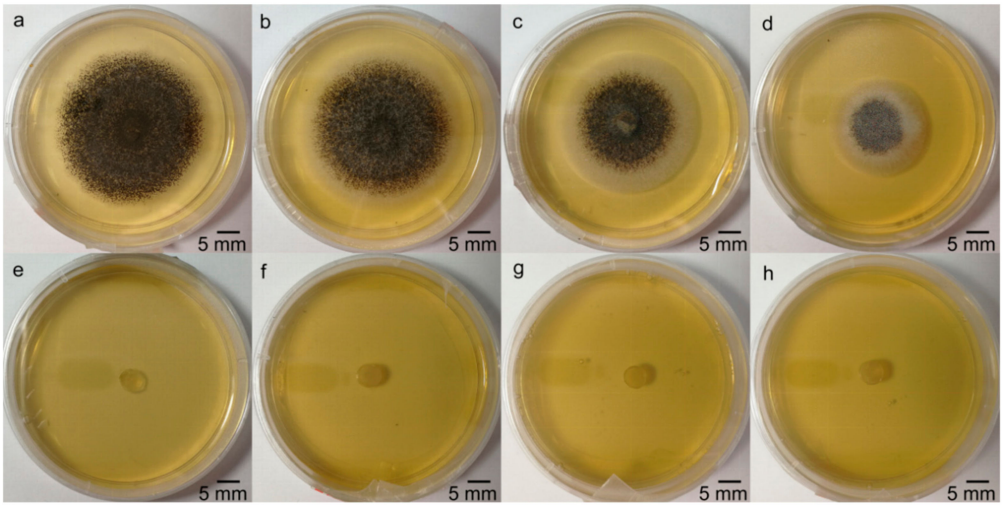

Figure 8. In vitro growth status of *A. niger* (4 days) under different concentrations of aqueous garlic extract (the Petri dish inner diameter is 55 mm). (**a**) control; (**b**) 1 mg/mL; (**c**) 2.5 mg/mL; (**d**) 5 mg/mL; (**e**) 7.5 mg/mL; (**f**) 10 mg/mL; (**g**) 50 mg/mL; (**h**) 100 mg/mL.

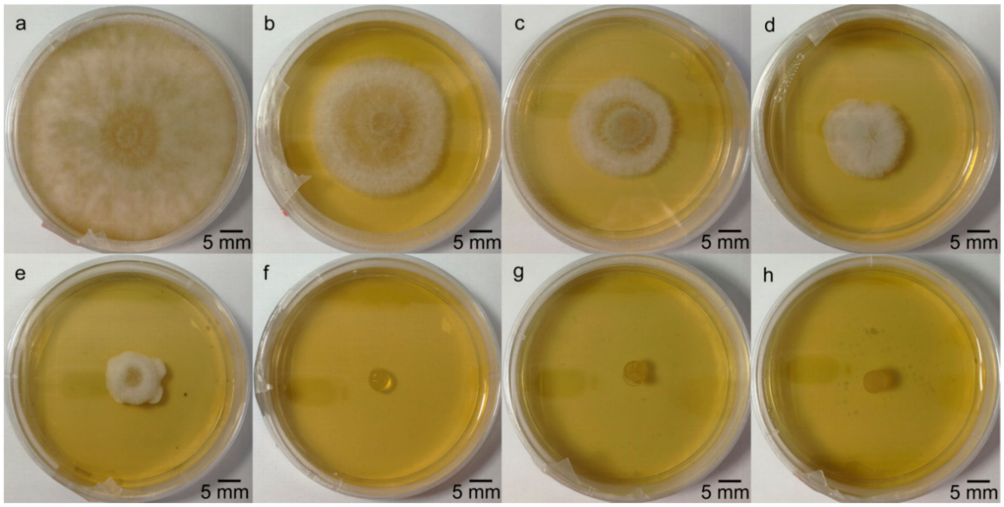

Figure 9. In vitro growth status of *B. dothidea* (3 days) under different concentrations of aqueous garlic extract (the Petri dish inner diameter is 55 mm) (**a**) control; (**b**) 1 mg/mL; (**c**) 2.5 mg/mL; (**d**) 5 mg/mL; (**e**) 7.5 mg/mL; (**f**) 10 mg/mL; (**g**) 50 mg/mL; (**h**) 100 mg/mL.

The results showed that 2.5 mg/mL aqueous garlic extract had a significant inhibitory effect on both *A. niger* (41%) and *B. dothidea* (57%). In addition, the minimum inhibitory concentration (MIC) of aqueous garlic extract on *A. niger* and *B. dothidea* was 7.5 mg/mL and 10 mg/mL, respectively. (Figure 8e–h, Figure 9f–h, and Figure 10).

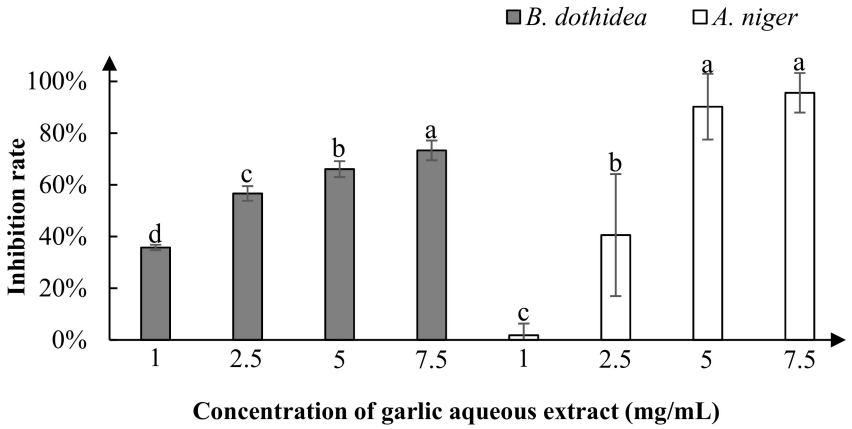

Figure 10. The inhibition rate of the aqueous garlic extract on *A. niger* and *B. dothidea.* (The values are the means and the error bars indicate the standard deviations. Different letters (a/b) indicate significant difference between different concentrations of aqueous garlic extract within each pathogen, $n = 3$).

3.7. Mycelium Growth Rate of the Two Pathogens under Different Concentrations of Aqueous Garlic Extract

As shown in Figure 11, compared with the control, the growth rate of *A. niger* in the media containing the aqueous garlic extract was slower in the initial stage of growth, however, there was no difference in the middle stage. *A. niger* did not grow in the first 24 h in the 5 mg/mL aqueous garlic extract, whereas *B. dothidea* had no growth in the first 24 h in the 5 mg/mL and 7.5 mg/mL aqueous garlic extracts. In addition, when the concentration of garlic aqueous extract reached 10 mg/mL, the mycelium did not grow for either pathogen. In the control, the growth rate of *B. dothidea* was higher than that of *A. niger* (Figures 11 and 12).

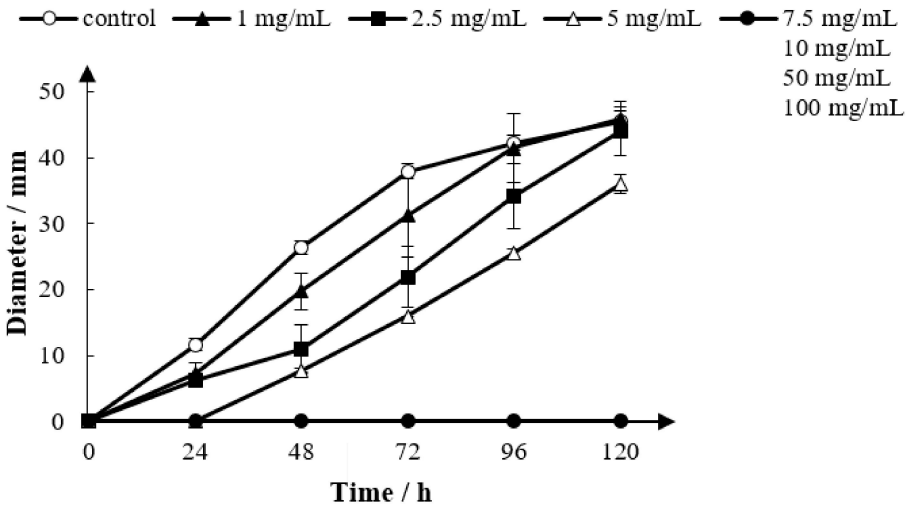

Figure 11. Growth curve of the *A. niger* colony on PDA plates under different concentrations of garlic extract ($n = 15$).

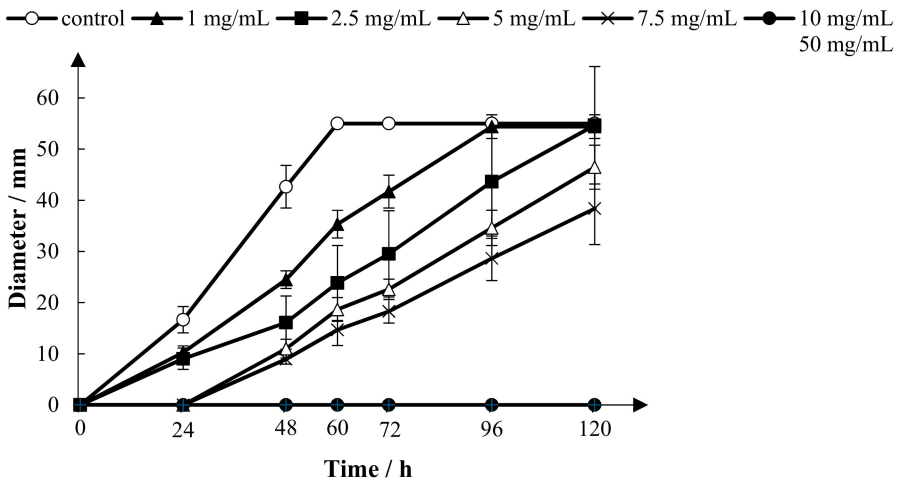

Figure 12. Growth curve of the *B. dothidea* colony on PDA plates under different concentrations of garlic extract (n = 15).

4. Discussion

In recent years, rot disease in pomegranate has attracted extensive attention from researchers, and more studies have focused on pomegranate fruit in particular. Some studies suggested that the pathogen causing pomegranate dry rot was *Z. versoniana* Sacc. [1] or *Coniella granati* [27,28]. It has also been confirmed that *B. dothidea* caused pomegranate rot [9]. Different methods have been applied for fungus recognition and identification, including morphological characteristics (such as the shapes and sizes of various structures), physiological characteristics (colony diameter, production of colored metabolites, and so on), and molecular tools [13]. Soft rot and dry rot diseases are a severe threat in the pomegranate orchards of Shaanxi Province in China. In this study, one pathogen that caused soft rot in pomegranate fruit was isolated, and according to the analysis of its morphological and molecular characteristics, this pathogen is most probably *A. niger*. The pathogen isolated from dry rot was identified as *B. dothidea*. The symptoms of dry rot due to *B. dothidea* are different from those caused by *Z. versoniana* Sacc. Pomegranates infected by *Z. versoniana* Sacc. showed high water loss and shrinkage, leading to stiff fruit, with dense black conidia inside and outside the stiff fruit [1]. In this study, the pathogenic symptoms of the dry rot caused by *B. dothidea*, were smooth, and the dry rot occurred in the early stage. As the infection time increased, the infected part became soft at the later stage of infection. Therefore, morphological characteristics should be observed carefully to distinguish different pathogens.

During the cultivation, it was found that the vesicles produced by *A. niger* had a strong diffusion ability and maintained activity for up to 2 months. Due to the production of a large number of vesicles by *A. niger*, which spreads rapidly in the field and in cold storage, soft rot becomes a major disease threat during pomegranate growth and storage. According to our observations, in the case of insufficient nutrient conditions in the late growth stage, *A. niger* produces its hypopus, the rhizomorph, to adapt to adverse environments. It has also been found that when using vesicles for subculture, it was easy to produce multiple growth points, which also interfered with the observation of colony morphology. *B. dothidea* showed no spores in the whole culture process, and no sporulation was induced by external treatment, including scratching and ultraviolet irradiation. This suggests that the propagation pattern for *B. dothidea* might be asexual reproduction.

Previous studies have found that aqueous garlic extracts have inhibitory effects on bacteria and some fungi [15,16]. Even for *A. niger* and *B. dothidea*, it has been proven that different garlic extracts showed antifungal activities [29–32], but the antifungal effect is related to the extraction solvent, the state of the garlic, the extraction method, and so on. The aqueous and petroleum ether garlic extracts possessed stronger activity and a broader fungicidal spectrum against eight fungal strains compared to a methanol extract [29]. In addition, the ethanol extracts showed more activity than aqueous extracts

on selected fungi of *Fusarium* spp. and *Rhizopus* spp. [33]. However, from the perspective of cost saving and environment-friendliness, antifungal research of aqueous extracts has significant practical value. On the other hand, extracts of dehydrated garlic showed a stronger inhibitory ability than the fresh forms because they contained higher amounts of sulfur compounds by dry weight [34]. In this study, dehydrated garlic powder was used to make the raw aqueous extract, and the MIC was 7.5 mg/mL on *A. niger*, which was much lower than the MIC (325 mg/mL) of the aqueous extract from fresh garlic [30]. In terms of practical application, the dehydrated garlic powder is also convenient for storage and industrial processing. In this study, the garlic extracts showed an increase in fungal inhibition effects with increasing concentrations, which is consistent with previous research results [29,33]. In addition, the inhibitory effect of the aqueous garlic extract was mainly observed in the early stage of mycelium growth. This may be due to the decreased antifungal activity of the aqueous garlic extract over a longer culture period. Garlic contains powerful organosulfuric compounds, such as allicin, alliinase, alliin, and their derivatives, and these compounds were assumed to be involved in the antifungal activity [29,35]. Therefore, further studies are needed to analyze the composition of raw garlic extract in order to reveal its antifungal mechanism.

5. Conclusions

In the pomegranate production area of Shaanxi Province in China, rot disease is the main problem for pomegranate trees and fruit. The isolated pathogens were most probably *A. niger* and *B. dothidea*, based on morphological characteristics and ITS identification. The aqueous garlic extract from dehydrated garlic powder showed a strong inhibitory effect on both pathogens. This indicated the potential of dehydrated garlic powder as an alternative to synthetic fungicides. Moreover, it is necessary to perform more research to identify the specific antifungal substances and reveal the inhibitory mechanism of the garlic extract.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/1/34/s1, Figure S1: Multiple sequence alignment of ITS fragments of pathogen A and four other similar *Aspergillus* species, Figure S2: Multiple sequence alignment of ITS fragments of pathogen B and four other similar *Botryosphaeria* species, Figure S3: Phylogenetic tree of *Aspergillus* species.

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