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Isolation and Identification of Pear Ring Rot Fungus and Resistance Evaluation of Different Pear Varieties

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Abstract: *Botryosphaeria dothidea* is a significant plant pathogen responsible for causing ulcers, wilt, and fruit decay across a wide range of host plants. One notable fungal disease attributed to *B. dothidea* is pear tree ring rot, which currently ranks among the most severe diseases affecting pear trees in China. This pathogen primarily targets branches and fruits, occasionally impacting leaves as well, leading to tree weakening, fruit rot, and leaf drop. The annual repercussions of this disease severely affect both the yield and quality of pear fruits, thereby impeding the healthy development of the pear industry. Recent studies have indicated that other species within the *B. dothidea* complex can also induce pear ring rot; however, specific physiological strains of *B. dothidea* remain unreported. Consequently, this study collected tissues from pear trees infected with ring rot from orchards located in Liaoning, Hebei, Shandong, and other regions throughout China. Through morphological characterization combined with pathogenicity assessments and DNA sequence comparisons involving partial internal transcribed spacer (ITS), translation elongation factor (TEF), and β-tubulin (TUB) genes, 21 strains belonging to the *Botryosphaeria* spp. were identified. These 21 strains served as research subjects for inoculating dormant annual branches from 30 germplasm resources of pear trees in vitro. The results demonstrated that all tested strains could induce lesions on the branches which were characterized by dark brown spots. Furthermore, inoculation experiments involving these 21 strains were conducted to evaluate the resistance levels of various pear varieties against ring rot disease. The resistance was assessed by inoculating different isolates onto distinct pear varieties; this approach established the criteria for evaluating resistance while minimizing identification errors stemming from the variable responses exhibited by certain varieties towards individual strains. Ultimately, this study aims to provide a theoretical foundation for effective prevention and treatment strategies against pear ring rot.

Keywords: pear; ring rot disease; *Botryosphaeria dothidea*; pathogen isolation and identification; resistance evaluation

Citation: Wang, C.; Zhang, Y.; Ou, C.; Wang, F.; Ma, L.; Wang, C.; Jiang, S. Isolation and Identification of Pear Ring Rot Fungus and Resistance Evaluation of Different Pear Varieties. *Horticulturae* **2024**, *10*, 1152. https://doi.org/10.3390/horticulturae10111152

Academic Editors: Dalia Aiello and Seiya Saito

Received: 29 September 2024
Revised: 22 October 2024
Accepted: 29 October 2024
Published: 30 October 2024

1. Introduction

The cultivated area and pear yield mean it is ranked third among fruit trees in China. According to the National Bureau of Statistics, the cultivated area in 2021 was 940,700 hm^2, while the output in 2022 reached 19,265,300 t. Disease resistance is a crucial characteristic of pear fruit and represents one of the primary objectives in pear breeding. *Pyrus* have very rich germplasm resources—at least 22 major pear species have been reported—but only *P. bretschneideri*, *P. pyrifolia*, *P. communis*, *P. sinkiangensis*, and *P. ussuriensis* are cultivated for actual fruit production [1]. Despite these numerous varieties with attractive fruit characteristics and favorable taste profiles, their poor disease resistance leads to increased costs associated with chemical disease control measures and contributes to environmental pollution. There is an urgent need for non-chemical methods to manage these diseases

effectively. Through resistance identification in different pear varieties, screening out the resistant germplasm for disease-resistance breeding is the most effective measure for disease control [2].

Pear ring rot is one of the major diseases affecting pear trees and is caused by the *Botryosphaeria* spp. This disease is prevalent in all pear-producing regions of China. It damages the branches, fruits, and even the leaves of pear trees [3], leading to premature aging and resulting in a significant loss of leaves and fruit. It has been estimated that pear ring rot can reduce production by approximately 25% annually [4]. In severe cases, it can affect up to 80% of the fruit in an entire orchard [5]. When a tree becomes infected it exhibits two types of symptoms. The first occurs when the infection affects the branches, fruits, and leaves of the pear tree, resulting in a ring pattern symptom known as ring rot [6]. The second manifests as an ulceration of infected branches and is referred to as dry rot. Zhai [7] identified four pathogens responsible for these symptoms: *B. dothidea*, *B. rhodina*, *B. obtusa*, and *B. parva*, with *B. dothidea* being the dominant species causing ring rot disease. Xiao [8] demonstrated that the pathogen responsible for pear ring rot closely resembles that of apple ring rot. In addition to *B. dothidea*, *B. kuwatsukai* was also present, and its virulence was significantly stronger than that of *B. dothidea*. Pathogens were isolated from samples exhibiting pear wheel pattern symptoms and dry rot symptoms. Only *B. kuwatsukai* was isolated from the samples with pear wheel pattern symptoms, while both *B. dothidea* and *B. kuwatsukai* were isolated from the samples showing dry rot symptoms.

In "Descriptors and Data Standard for Pear (*Pyrus* spp.)" [9], the methodology employed for evaluating the resistance of pear fruit to ring rot involved the spray inoculation of the fruit with conidia from the pathogenic fungus. However, it is noteworthy that no established criteria exist for assessing the resistance of leaves and stems to ring rot. According to various scholarly sources [10–12], the incidence rate and disease index associated with the inoculation of mycelial blocks using punctured branches were higher than those resulting from the conidia inoculation method. This alternative method is advantageous as it does not require the continuous cultivation of the fungus under light conditions for approximately ten days to produce conidia, thereby rendering it a more rapid and straightforward approach for evaluating disease resistance. Furthermore, some researchers assessed the resistance of different pear germplasm resources through physiological indicators, such as measuring the accumulation of primary phenolic compounds in pear fruits and observing the alterations in these compounds following inoculation with *Botryosphaeria* spp. [13,14]. Currently, there is no consensus on the evaluation criteria for pear ring rot resistance. Therefore, establishing a rapid and comprehensive evaluation system for branch ring rot resistance is of considerable importance for the breeding of resistant pear varieties.

This research aimed to (i) gather and isolate the pathogenic fungi implicated in ring rot disease across the provinces of Shandong, Hebei, and Liaoning; (ii) identify the strains through morphological examination and multilocus analysis; (iii) evaluate the virulence of the isolated strains and formulate grading criteria for the virulence of *B. dothidea*; and (iv) investigate the resistance of different pear cultivars to ring rot disease, while also establishing the criteria for assessing resistance to this affliction.

2. Materials and Methods

2.1. Isolation and Purification of Pathogenic Fungi

Commencing in August 2022, tissue samples were collected from various regions, including Shandong, Hebei, and Liaoning. These samples, which comprised leaves, fruits, and branches, were individually stored in ziplock bags at a temperature of 4 °C within a refrigerator. Following the separation of tissues, infected samples were thoroughly washed and dried using sterile water, and subsequently disinfected with 75% ethanol. For the leaf or fruit samples, a sterilized scalpel was utilized to excise a tissue block approximately 5 mm in diameter from the interface of the diseased and healthy tissue. In the case of the branch samples, the tumor was carefully excised with a scalpel and then bisected along its diameter using sterilized scissors to obtain the requisite tissue block. The tissue blocks

were immersed in a 0.1% mercuric chloride solution for 30 s, followed by a 30 s rinse with sterile water. They were then soaked in 75% ethanol for an additional 30 s and subsequently rinsed twice with sterile water for 30 s each [15]. Following these procedures, the tissues were cultured in Potato Dextrose Agar (PDA) medium at a temperature of 28 °C. After a growth period of three days, a pure single colony was selected and transferred to a new PDA medium for further cultivation.

2.2. Morphological and Molecular Identification

2.2.1. Observation of Mycelial Growth Status and Induction of Conidia

The characteristics of the colony and the growth state of the mycelia were critical criteria for identification. The aerated mycelia of this genus, when cultured on PDA medium, displayed a progressive color change from gray to black as the fungus matured. Simultaneously, the reverse side of the colony transitioned from white to yellow, followed by a change to dark green, and ultimately to black. To facilitate the observation of colony morphology, three distinct media were utilized for culturing the strains.

The colony morphology of each strain was meticulously documented. Hyphal blocks, measuring 5 mm in diameter, were excised from the periphery of the purified colonies and cultured on PDA medium for seven days. Following this incubation, the growth characteristics of the colonies on both sides of the Petri dish were evaluated. Mycelia cultured on PDA for seven days were subsequently analyzed under an Olympus IX51 inverted microscope to assess their morphological features. The colony was cultured on PDA medium for three days, and the mycelium pieces with a diameter of 5 mm were selected and placed on PDA, OA (Oatmeal Agar), and WA (Water Agar) plates (each plate with a diameter of 90 mm and a medium volume of 25 mL; all culture medium allocation methods are shown in Table S1) and incubated at a constant temperature and in darkness at 28 °C [16]. The process was repeated three times with each strain. The growth morphology of the mycelium was observed every 48 h.

Moreover, each strain was inoculated onto PDA, OA, and WA. Once the colonies had fully colonized the culture dish, the mycelium was carefully scraped using a sterile bamboo skewer. The conidial morphology was then examined microscopically while subjected to continuous ultraviolet irradiation within a constant-temperature incubator maintained at 28 °C for 45 days [17].

2.2.2. Molecular Identification and Phylogenetic Analysis

Different strains were activated on PDA medium for three days, and white mycelia with edges were selected and transferred to a new PDA medium for propagation. After five days, a small amount of mycelia were selected and the total genomic DNA was extracted by the Chelex-100 method [18]. The internal transcribed spacer (ITS) [19], translation elongation factor (TEF) [20], and β-tubulin (TUB) [21] were amplified using the primers in Table S2.

The PCR system was 50 μL, including 25 μL of premix Taq (TaKaRa, Biotechnology, Dalian, China), 1 μL of each primer, 1 μL of DNA template solution, and 22 μL of dd H_2O. The procedure was as follows: initial denaturation at 94 °C for 5 min; followed by 30 cycles at 94 °C for 30 s, primer annealing at a suitable temperature for 30 s (58 °C for ITS, 60 °C for TEF and TUB), and primer extension at 72 °C for 45 s; and extension at 72 °C for 10 min. The PCR product was observed under UV illumination on a 1% agarose gel containing ethidium bromide (0.5 mg/mL) and amplicons were sequenced by Taihe Biotechnology (Beijing, China). The sequencing results were compared and homologously analyzed with the known sequences in the GenBank database using the BLAST program. The sequences were spliced according to the ITS-TEF-TUB sequence, and the sequences were spliced and aligned using ACOPTloos. Using *Neofusicoccum parvum* as the outgroup, MEGA7.0 [22] was applied to construct the phylogenetic tree of the three-gene join by the neighbor-joining method [23], and 1000 repeats were performed by the bootstrap method [24].

2.3. Pathogenicity Determination of Pathogenic Fungi

The pathogenicity of the strains was tested according to Koch's rule. Initially, the surfaces of the dormant annual branches from randomly selected pear tree varieties were disinfected using 75% alcohol, followed by two rinses with sterile water. After allowing the branches to dry, they were cut into segments measuring 120 mm, with both ends sealed using paraffin wax. Wounds were created in the middle of the branches, extending to the xylem, while avoiding the flower buds. The isolated strains, cultured on PDA medium for five days, were then formed into disks using a 5 mm diameter hole punch. The mycelial surface of these disks was subsequently inoculated at the designated points on the branches, with each strain being tested in ten replicates. A blank PDA medium served as a control. The inoculated branches were then incubated in an artificial-climate chamber maintained at 28 °C, where the incidence of infection was monitored and recorded. Pathogenic fungi were isolated and purified from the re-infected branches using the previously described method, ensuring that the isolates were consistent with the originally inoculated strains.

To assess the differences in virulence among the strains, the spread of lesions caused by different strains on the 'Hongxiangsu' variety was analyzed through cluster analysis. The 21 strains were categorized into three virulence levels: strong, medium, and weak. Following the aforementioned inoculation method, the virulence of each strain was observed across different pear tree varieties. According to the lesion extension length at 21 d, Origin v2022 software was used for a systematic clustering analysis, and combined with the distribution of branches of different disease grades, the classification standard of virulence was determined.

2.4. Identification of Resistance of Different Pear Germplasm Resources

A total of 30 pear germplasm resources (Table 1) were selected from the experimental orchard of the Pear Breeding Research Group of the Research Institute of Pomology of the CAAS (Xingcheng) in January 2024. The strains were inoculated on the annually dormant branches of the 30 pear germplasm resources. The treatment method was as referred to in Section 2.3, with the process repeated ten times for each variety and a blank PDA medium inoculated as the control. The length of lesions on the branches were measured at 14, 21, and 28 days after inoculation.

Table 1. Pear germplasm resources tested in this study.

P. bretschneideri	*P. pyrifolia*	*P. ussuriensis*	*P. communis*	*P. sinkiangensis*	**Stock**
Zaosu	Huobali	Jingbaili	Wujiuxiang	Kuerlexiangli	Duli
Jinfeng	Lvxiu	Nanguoli	Jinxiang		Zhongai 1
Hongxiangsu		Huagaili	Zhongaihongli		Zhongai 3
Yuluxiang			Bayuehong		
Huangguan			Zaohong Comice		
Pingguoli			Fenlao		
Zaojinsu			Zhongjia 1		
Jinhua			Bali		
Huasu			Conference		
Dangshansuli			Zaojinxiang		
Zaosuhong					

2.5. Statistical Analysis

The experimental data were compared by means of a one-way analysis of variance (ANOVA) and SPSS statistical software 26.0 ($p < 0.05$). The homogeneity test of variance was conducted before ANOVA.

A cluster analysis was carried out using Origin v2022's systematic clustering.

3. Results

3.1. Isolation of Pathogenic Fungi

A comprehensive collection of 65 samples exhibiting ring rot was conducted, encompassing leaves, fruits, and branches. The manifestations of the disease are illustrated in Figure 1. Infected leaves and fruits displayed dark brown lesions, while branches affected by the fungus exhibited cracking of the bark and the formation of warty growths.

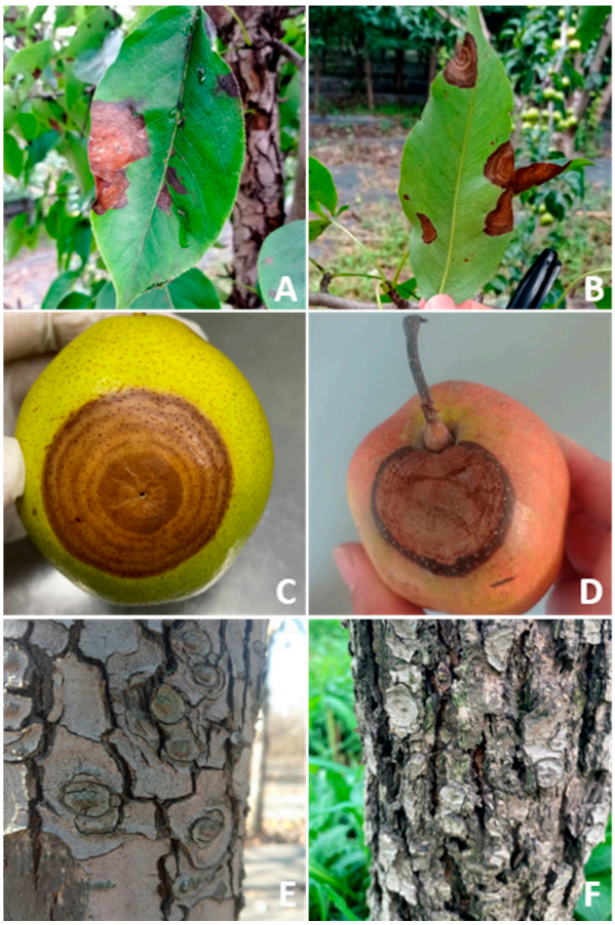

Figure 1. Ring rot disease symptoms on pears. (**A**,**B**) leaf; (**C**,**D**) fruit; and (**E**,**F**) trunk symptoms in the field.

3.2. Morphological Analysis of Pathogenic Fungi

The purified strains were inoculated onto PDA medium. After 7 days, the entire surface of the plates was colonized, with strains LN-ZJ4, LN-ZJ5, and HB-ZJZ3 exhibiting nearly complete white coverage, while the majority of the other colonies displayed gray and white coloration. The growth patterns across these plates were relatively uniform; however, strain HZ-SZ2162 demonstrated a highly irregular growth, with colonies aging rapidly and distinct color demarcations becoming apparent on the plate. Notably, while the peripheral mycelium remained white, the central mycelium exhibited significant blackening. Upon examining the reverse side of the plate, it was observed that colonies with a white coloration corresponded to a yellowish-gray hue on the back, whereas the off-white colonies were associated with a grayish-black appearance.

Microscopic examination of the mycelium after 7 days of culture at a magnification of 400× revealed that the mycelium was colorless and transparent, exhibiting a flat and straight morphology, with branching structures, including thin branches, and a consistent thickness throughout. It could be seen that the mycelium was associated with the state of the colony (Figure 2).

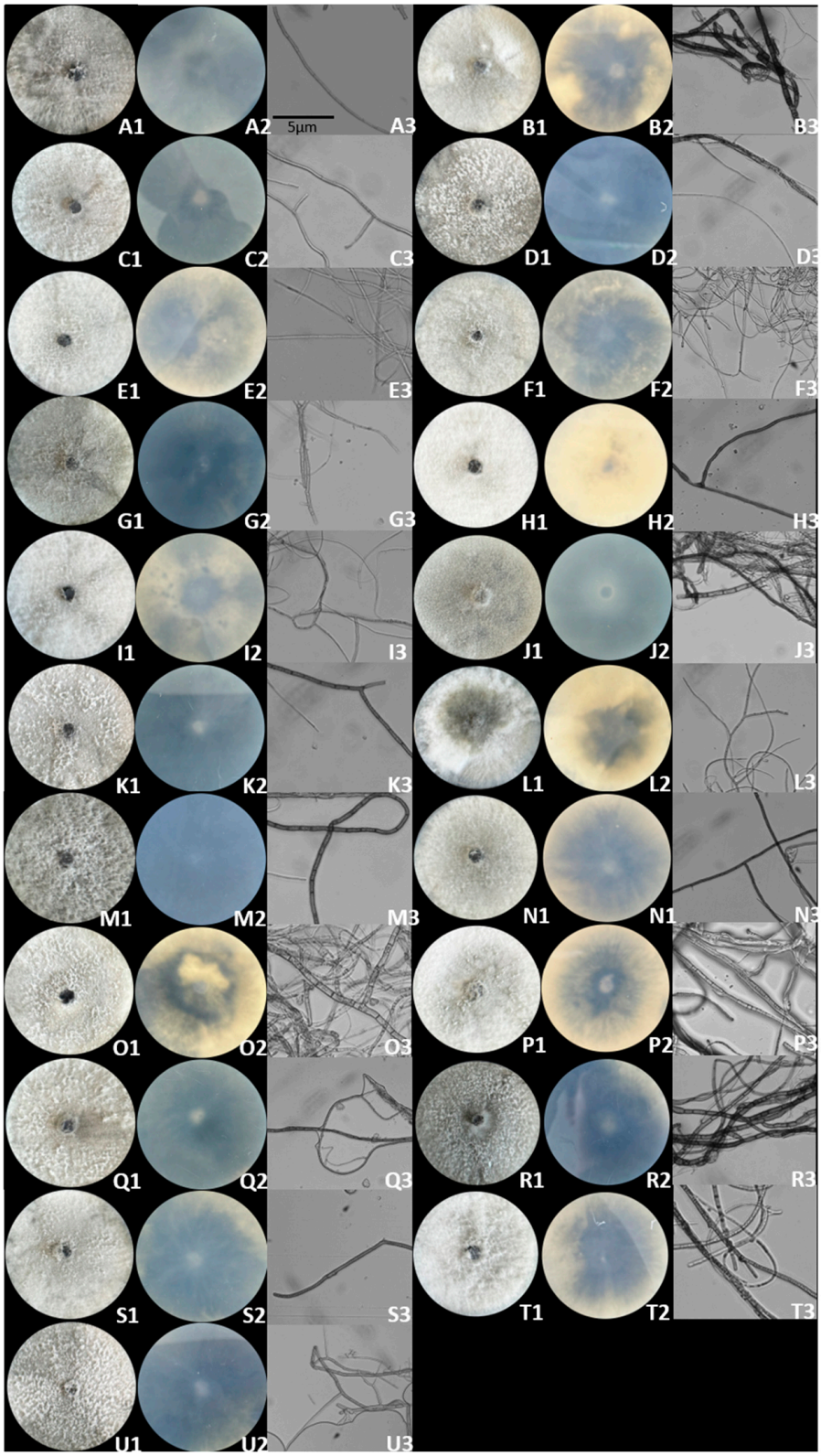

Figure 2. Colony and mycelial morphology on PDA medium: (**A**) LN-ZJ1, (**B**) HB-CZ6, (**C**) LN-ZJ2, (**D**) HB-SJZ2, (**E**) LN-ZJ4, (**F**) HB-ZJZ3, (**G**) LN-ZJ5, (**H**) GGS-K1, (**I**) LN-ZJ6, (**J**) HZ-MJ231, (**K**) LN-ZJ8, (**L**) HZ-SZ2162, (**M**) LN-SDGZ, (**N**) HB-CZ3, (**O**) LN-HS, (**P**) HB-HDWX, (**Q**) HB-CZ1, (**R**) HB-ZX, (**S**) HB-CZ2, (**T**) SD-YM, and (**U**) HB-CZ5 isolates; 1—fronts of colonies cultured on PDA, 2—backs of colonies cultured on PDA, and 3—mycelial morphology observed via a microscope at 400×.

The strains were inoculated on the media of PDA, OA, and WA for morphological observation. The results showed that all colonies on the PDA medium were white at the beginning of the period and showed radial growth, irregular edges, and grew outward layer by layer. The entire colony was snowflake-shaped, and some strains developed yellow pigments at 3 d, especially LN-ZJ5, LN-ZJ6, LN-ZJ8, HB-CZ2, HB-CZ5, and HB-ZX, which turned olive green in the middle of the plate. This showed that these strains aged quickly. The growth rate of different strains was also different. LN-ZJ4 and LN-HS had covered the whole plate by three days, while GGS-K1 and HZ-MJ231 only expanded by about 3 cm. All strains had overgrown the entire plate at 5 d, and the mycelium was dense and gradually changed to white and green. On the PDA medium, over time, the mycelium gradually changed from white to olive green, and finally completely changed to pure black. There are two kinds of air mycelia. The first kind are LN-ZJ4, LN-HS, LN-ZJ1, HB-ZJZ3, HB-ZX, and SD-YM, and the mycelia grew vertically and reached the top of the Petri dish. The second kind grew close to the underside of the plate. In OA medium, the colony expanded faster, the mycelia were dense, and the edges were neat, but the mycelia did not grow in the middle of the plate, and the interior showed concentric circles of black loss. The airborne mycelium was very abundant at the beginning, but with time, the number of mycelia gradually decreased, and more mycelia were present only in the periphery of the plate at 9 d.

The strain grew slowly on WA medium, only a small amount of mycelia grew closely to the medium, the stratification was not obvious, and the aging rate of the mycelia was slow. Only HB-CZ6 colonies turned black at 5 d, the other strains only had melanin precipitation at 7 or 9 d, and the colonies did not overgrow the entire plate at 9 d (Figure S1).

The strains cultivated on PDA, OA, and WA failed to produce conidia, despite the mechanical disruption of the mycelium using a bamboo skewer and the continuous exposure to ultraviolet light for 45 days. The induction of conidia in the context of pathogens proved challenging under the experimental conditions employed. Furthermore, alternative methods aiming to enhance fungal conidia production were found to be ineffective for these pathogens.

3.3. Molecular Identification and Phylogenetic Analysis

The DNA from 21 strains was extracted by the Chelex-100 method, and the ITS, TEF, and TUB of the strains were amplified by primers. Bands of 568, 277, and 437 bp were obtained, respectively, and then compared by Blast on NCBI. The homology with *B. kuwatsukai* and *B. dothidea* was more than 99%, which indicated that the scientific classification status of the new isolates was clear. The sequence of strains with high homology and 58 strains of *Botryosphaericeae* and other species were selected as the reference objects (Table S3: Gene bank used in constructing the phylogenetic tree). Through phylogenetic analysis, the sequences of each strain were analyzed in tandem according to ITS–TEF–TUB, and a combined phylogenetic tree of multiple genes was constructed (Figure 3). The 21 isolates were clustered in the same clade as *B. kuwatsukai*. At the same time, they all had a close kinship with *B. dothidea*.

3.4. Strain Virulence Test

Twenty-one isolates were inoculated onto healthy annual dormant branches of pear trees, which could cause the host branches to develop disease at 14 d, producing obvious reddish-brown to black-brown concentric circle disease spots. Subsequently, these lesions expanded from the inoculation site towards both ends of the branches in an oval configuration, leading to the formation of epidermal folds and even cracks in the areas where the lesions proliferated. The boundary between health and sickness was clear (Figure 4). At 21 d there was a marked toroidal lesion.

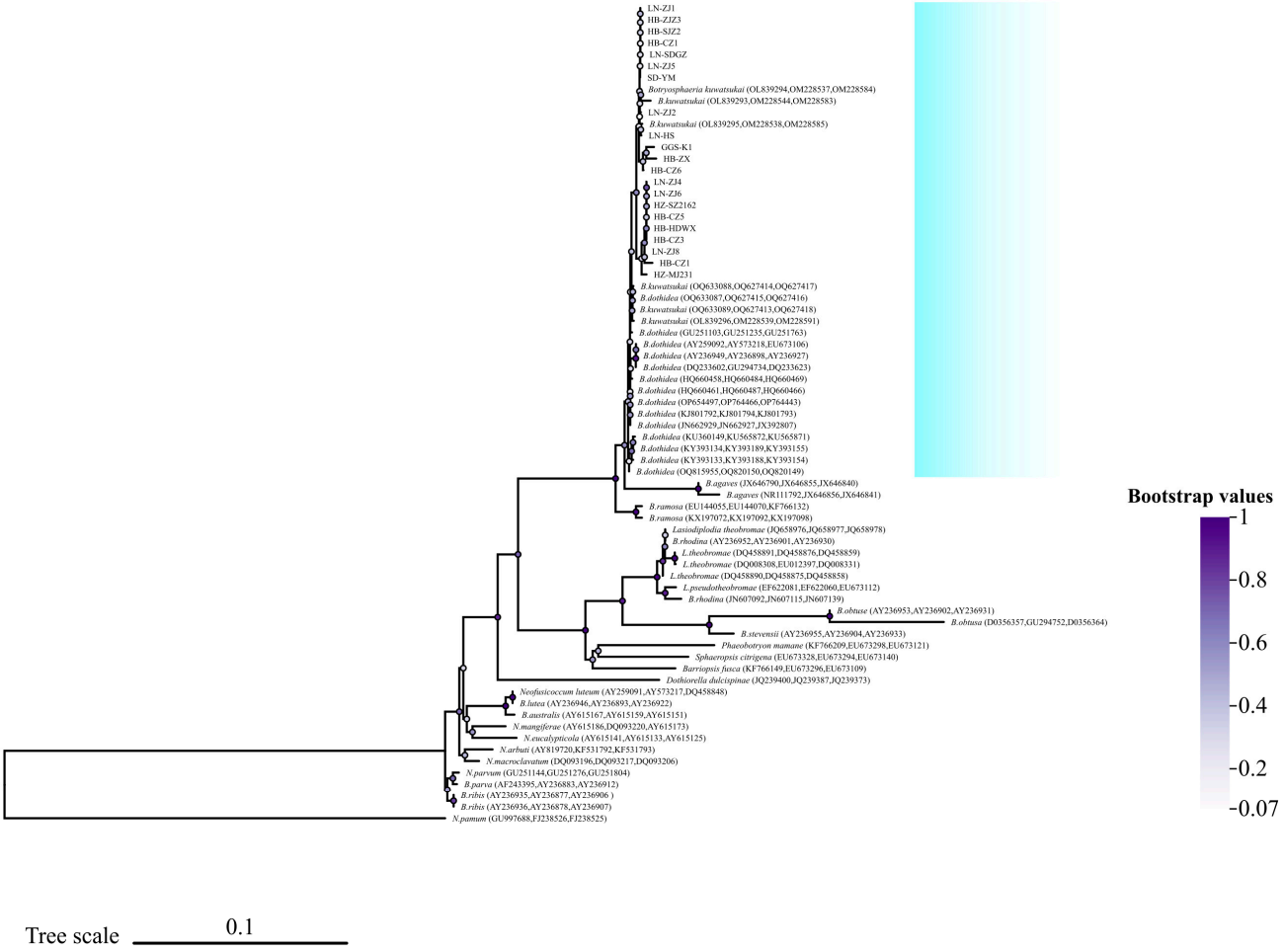

Figure 3. Phylogram of *Botryosphaeria* based on three combined genes: partial internal transcribed spacer (ITS), translation elongation factor (TEF), and β-tubulin (TUB). *Neofusicoccum parvum* was taken as the outgroup. The tree was constructed using concatenated sequences of ITS, TEF, and TUB genes. The node is marked with bootstrap values. The colored areas represent the branches that contain the isolates in this study.

Figure 4. Symptoms of disease spots produced by each isolated strain on pear branches.

In the virulence test of the branches inoculated on 'Hongxiangsu', HB-SJZ2 strains showed the strongest virulence, with an average spot diameter of 98.8 mm at 21 d, while HZ-MJ231 strains showed the weakest virulence, with an average spot diameter of 12.1 mm (Figure 5). There were significant differences in virulence between the 21 strains at 21 d (Figure 6), which indicated that there was a differentiation of virulence.

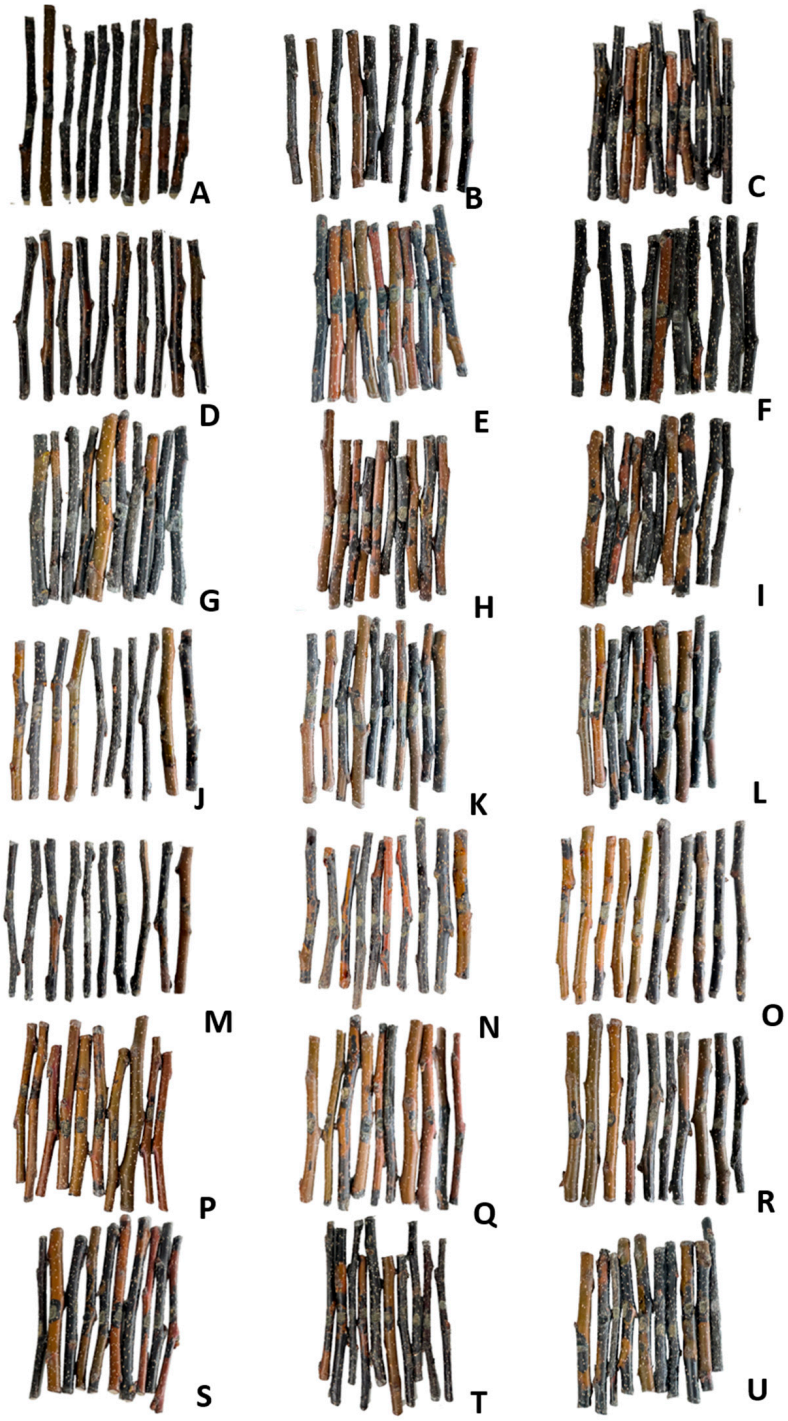

Figure 5. Symptoms of isolated fungi inoculated on 'Hongxiangsu' isolated branches. (**A**) LN-ZJ1, (**B**) LN-HS, (**C**) GGS-K1, (**D**) LN-ZJ2, (**E**) HB-CZ1, (**F**) HZ-MJ231, (**G**) LN-ZJ4, (**H**) HB-CZ2, (**I**) HZ-SZ2162, (**J**) LN-ZJ5, (**K**) HB-CZ5, (**L**) HZ-CZ3, (**M**) LN-ZJ6, (**N**) HB-CZ6, (**O**) HB-HDWX, (**P**) LN-ZJ8, (**Q**) HB-SJZ2, (**R**) HB-ZX, (**S**) LN-SDGZ, (**T**) HB-ZJZ3, and (**U**) SD-YM isolates.

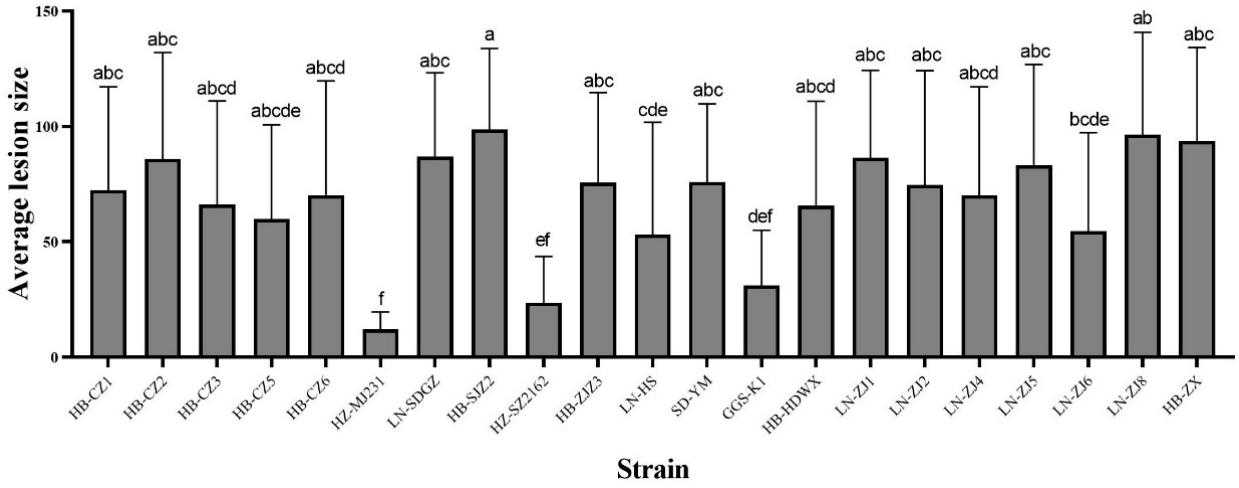

Figure 6. Differences in average damage length of isolated branches inoculated with different pathogenic strains on 'Hongxiangsu'. On the bar chart, the same letters indicate no significant difference, while different letters indicate a significant difference ($p < 0.05$).

The average diameter of lesions (ADLs) of the disease spots produced by each strain on the branches of 'Hongxiangsu' were selected for a systematic clustering analysis (Figure 7), and the virulence of the strains was determined according to the clustering results. The ADL scale was measured at 21 days of inoculation; when ADL $\geq$ 80 mm the strains were classified with strong virulence; when 40 mm $\leq$ ADL < 80 mm they were classified with medium virulence; and when ADL < 40 mm they were classified with weak virulence.

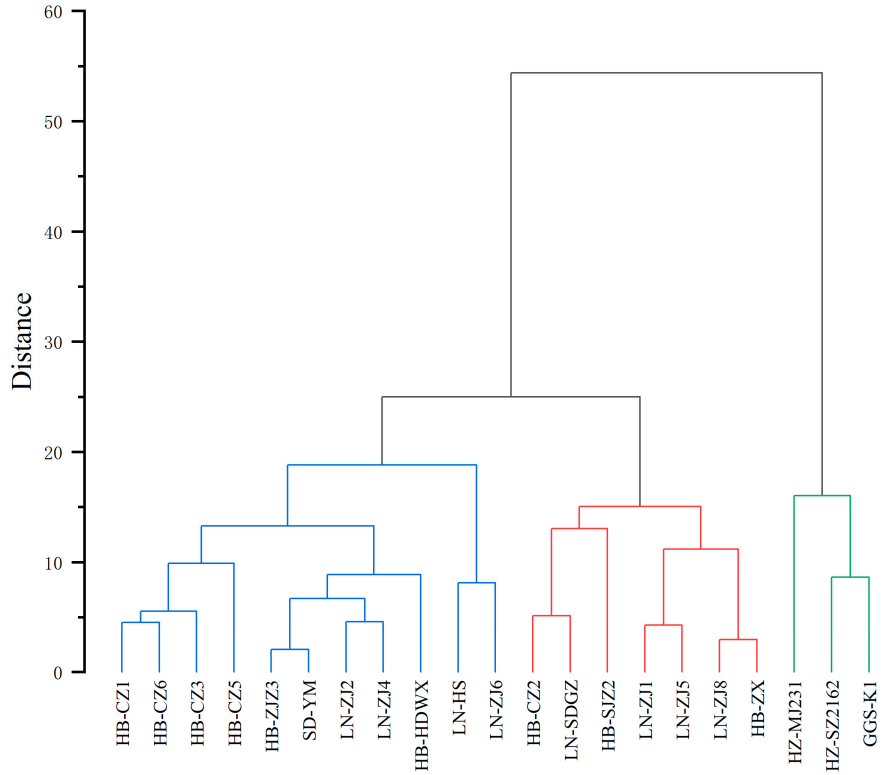

Figure 7. The average lesion length of each strain on the red fragrant crisp branches was analyzed by systematic clustering. All isolates were grouped into three categories. In the figure, the red category is the strong-virulence strain, the blue category is the moderate-virulence strain, and the green category is the weak-virulence strain.

Twenty-one strains were inoculated on the annual isolated branches of thirty germplasm resources, and according to the above criteria for the division of strong- and weak-virulence strains, the number of strains exhibiting strong and weak virulence in different germplasm resources was counted (Table 2).

Table 2. Each strain shows a different degree of virulence in the tested strains.

	Strong Virulence	Medium Virulence	Weak Virulence
GGS-K1	0	2	28
HB-CZ1	0	10	20
HB-CZ2	1	5	24
HB-CZ3	1	13	16
HB-CZ5	1	10	19
HB-CZ6	3	6	21
HB-HDWX	3	8	19
HB-SJZ2	4	8	18
HB-ZJZ3	1	12	17
HB-ZX	3	10	17
HZ-MJ231	0	1	29
HZ-SZ2162	0	2	28
LN-HS	0	8	22
LN-SDGZ	2	5	23
LN-ZJ1	4	7	19
LN-ZJ2	3	10	17
LN-ZJ4	1	9	20
LN-ZJ5	3	2	22
LN-ZJ6	2	8	20
LN-ZJ8	1	7	22
SD-YM	1	7	22

Most strains showed a weak virulence, among which HZ-MJ231 showed the weakest virulence. HB-CZ6, HB-HDWX, HB-SJZ2, HB-ZX, LN-ZJ1, LN-ZJ2, and LN-ZJ5 showed a strong virulence to three or more germplasm resources, and HB-CZ3 and HB-ZJZ3 showed a moderate virulence to more than ten germplasm resources.

3.5. Resistance Evaluation of Different Pear Germplasm Resources to Ring Rot Disease

By cluster analysis of the lesion size of different germplasm resources inoculated with HB-SJZ2, the resistance evaluation system of isolated branches was established as follows: after 21 days of trauma inoculation, ADL = 5 mm was an immune individual plant; 5 mm < ADL $\leq$ 15 mm was a high-resistance individual plant; 15 mm < ADL $\leq$ 50 mm was a medium-resistance individual plant; and 50 mm < ADL $\leq$ 80 mm was a moderately susceptible single plant. The single plant with ADL > 80 mm was highly susceptible (Figure 8).

Similarly, 21 strains were inoculated on the annual isolated branches of 30 germplasm resources, and the resistance performance of each germplasm was calculated according to the number of germplasm resources that showed resistance to different strains according to the above criteria for dividing resistant individual strains (Table 3).

The results of the resistance identification showed that most of the germplasm resources were resistant to the fungus. Among them, 12 pear germplasm resources, including 'Zaohong Comice', 'Pingguoli', 'Jinxiang', 'Wujiuxiang', 'Zaosuhong', 'Bali', 'Zaosu', 'Duli', 'Jinhua', 'Conference', 'Zhongjia 1', and 'Zhongaihongli', showed resistance to all strains. They were called ring-rot-resistant germplasm resources. In addition, 'Jingbai', 'Hongxiangsu', 'Huobali', and 'Yuluxiang' were all susceptible to the vast majority of strains, so they were defined as susceptible germplasm resources.

From the point of view of different pear series, *P. communis* showed the best resistance to ring rot disease, followed by *P. bretschneideri*, *P. ussuriensis*, *P. pyrifolia*, and *P. sinkiangensis*.

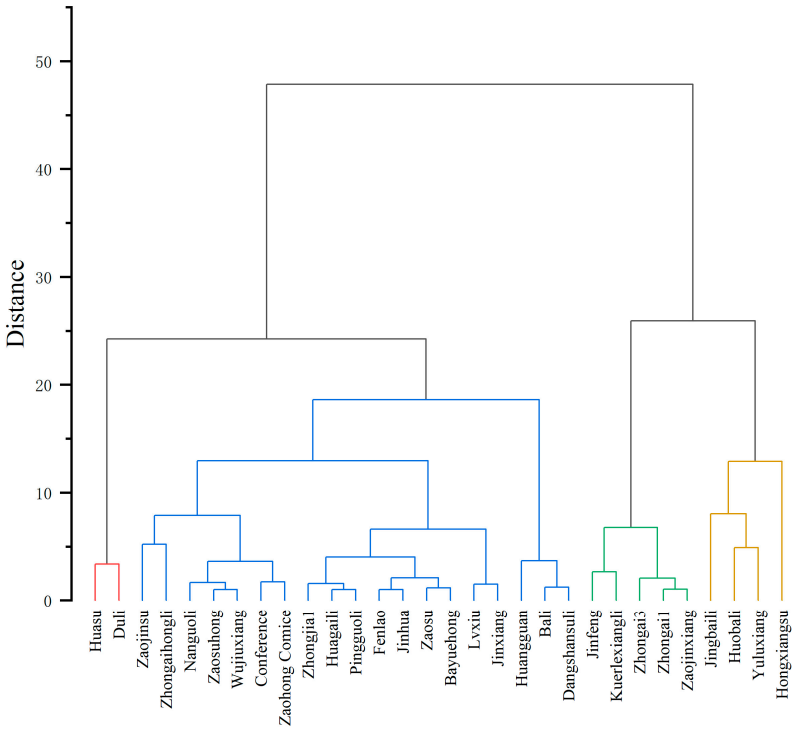

Figure 8. The average lesion size of HB-SJZ2 inoculated on different germplasm resources was analyzed utilizing systematic clustering. All germplasm resources are divided into 4 categories. In the figure, the red category is highly resistant, the blue category is moderately resistant, the green category is moderately susceptible, and the yellow category is highly susceptible.

Table 3. Resistance evaluation of different germplasm resources to strains.

	Highly Resistant	Moderately Resistant	Moderately Susceptible	Highly Susceptible
Huasu	8	10	3	0
Zaohong Comice	3	18	0	0
Pingguoli	1	20	0	0
Huangguan	1	13	7	0
Jinxiang	4	17	0	0
Zaojinsu	4	16	1	0
Nanguoli	1	19	1	0
Wujiuxiang	4	17	0	0
Bali	6	15	0	0
Zaosuhong	12	9	0	0
Jingbaili	1	6	6	8
Kuerlexiangli	1	10	10	0
Hongxiangsu	1	2	9	9
Zaosu	10	11	0	0
Duli	20	1	0	0
Jinfeng	2	18	1	0
Huagaili	4	16	1	1
Jinhua	2	19	0	0
Conference	2	19	0	0
Huobali	1	8	4	8
Lvxiu	1	12	6	2
Zhongjia 1	11	10	0	0
Bayuehong	7	13	1	0
Yuluxiang	1	2	9	9
Zhongaihongli	7	14	0	0
Dangshansuli	3	11	6	1
Zhongai 1	1	17	3	0
Fenlao	7	13	1	0
Zhongai 3	3	17	1	0
Zaojinxiang	4	15	1	1

4. Discussion

In this research, leaf, fruit, and branch samples were collected from pear-producing regions across various provinces in northern China, and the isolated strains were subjected to morphological and polygenic molecular identification [25]. The results showed that there were two pathogenic species of ring rot disease collected, *B. dothidea* and *B. kuwatsukai* [26,27]. There exists a debate among researchers regarding the etiology of ring rot disease; some posit that it results from the synergistic action of both pathogens, while others argue that one species is predominant, leading to a distinct symptomatology associated with each pathogen [8]. They believed that different species of pathogens are isolated from different plaque samples, but in this study, all of the pathogens were isolated from the samples with pear wheel pattern symptoms.

Furthermore, this study compared the cultural characteristics and virulence of the isolated *Botryosphaeria* spp. across three different culture media, revealing that the choice of medium significantly influences the colony morphology.

B. dothidea exhibits growth across various media, although the rate of growth varies significantly. PDA serves as a standard medium for fungal cultivation, with each strain demonstrating consistent and vigorous growth on this substrate. At 5 d, the mycelium typically covers the entire surface of a 90 mm Petri dish. Conversely, while strains exhibit accelerated mycelial development on OA, the aging of the mycelium occurs at an expedited rate. The growth on WA is comparatively slower for all strains, and this medium appears to inhibit mycelial proliferation, which could theoretically facilitate the germination of conidia. However, the current study did not observe any induction of conidium production, suggesting that the conditions provided were not conducive to sporulation in this fungus. Consequently, alternative strategies to enhance conidia production were deemed necessary, as the methods employed were ineffective for this particular fungal species. Further research is warranted to identify more effective sporulation techniques.

The virulence of strains isolated from various geographical regions exhibits a degree of molecular differentiation [28]. Consequently, a classification system for the 21 strains was established based on virulence standards, specifically for pear ring rot disease. This classification was determined by measuring the ADL following a 21-day trauma inoculation period. Strains were categorized as follows: those with an ADL of 80 mm or greater were classified as exhibiting strong virulence; between 40 and 80 mm were classified as exhibiting medium virulence; and less than 40 mm were classified as weak virulence. Among the strains analyzed, seven, including HB-CZ2, LN-SDGZ, HB-SJZ2, LN-ZJ1, LN-ZJ5, LN-ZJ8, and HB-ZX, were identified as exhibiting strong virulence, while three strains, namely HZ-SZ2162, HZ-MJ231, and GGS-K1, were classified with weak virulence.

In terms of the growth state, most *B. dothidea* grew close to the medium in PDA medium, while the strains belonging to *B. kuwatsukai* tended to grow upright against the lid of the Petri dish. In terms of virulence, the combination analysis of different virulence strains and phylogeny showed that there was no obvious correlation between the strength of virulence and the type of fungi. There were both strong virulence strains and weak virulence strains in *B. dothidea*. This is also the case in *B. kuwatsukai*.

The assessment of virulence across different strains significantly influences our understanding of their virulence. Given the observed differentiation in virulence among the isolates and the absence of published physiological subspecies for *B. othidea*, inoculating various strains onto different plant varieties can elucidate the resistance of those varieties to specific differentiated strains, thereby providing clearer insights into their resistance profiles. Ideally, resistant germplasm resources should demonstrate resilience against the majority of strains. Therefore, employing a diverse array of strains for inoculation can yield a more accurate representation of a variety's resistance level, mitigating the risk of misjudging resistance based on a variety's performance against a singular strain. This approach will facilitate the selection of more targeted strains for subsequent resistance evaluations.

The findings from the resistance assessment indicated that the majority of the evaluated germplasm resources exhibited resistance to ring rot disease. Notably, the germplasm

resources 'Zaohong Comice', 'Pingguoli', 'Jinxiang', 'Wujiuxiang', 'Zaosuhong', 'Bali', 'Zaosu', 'Duli', 'Jinhua', 'Conference', 'Zhongjia 1', and 'Zhongaihongli' demonstrated resistance to all tested strains. Conversely, the varieties 'Yuluxiang', 'Jingbai', 'Huobali', and 'Hongxiangsu' displayed a high degree of susceptibility, being affected by nine, eight, eight, and nine strains, respectively, and exhibited greater vulnerability to *B. dothidea* in comparison to other varieties. In terms of the different pear species examined, *P. communis* emerged as the most resistant to ring rot disease, with the *P. communis* varieties in this study showing significant resistance. In contrast, certain varieties of *P. sinkiangensis* exhibited heightened susceptibility to the disease.

Currently, there is no consensus regarding the identification of resistance to pear ring rot disease, and various inoculation sites present distinct advantages and disadvantages depending on the experimental context. While leaves are convenient for use as identification materials, it is noteworthy that pear ring rot predominantly affects fruits and branches rather than leaves, raising questions about the representativeness of the results obtained from leaf-based assessments. In contrast, using fruits as inoculation materials allows for rapid disease onset and shorter experimental durations; however, the presence of latent diseases and pests within the fruits, which may not be detectable at the outset of the experiment, can significantly influence the outcomes. Furthermore, the inconsistency in fruit maturity among different individual plants, as well as the variability in the maturity of individual fruits, can also impact the experimental results. Utilizing branches as inoculation materials may provide a more accurate reflection of the field conditions, yet this approach is characterized by longer experimental durations, with disease symptoms in most individual plants typically beginning to manifest after 14 days, and significant differences becoming apparent only after 21 to 28 days. This extended timeline poses a challenge for rapid resistance identification. Overall, a comprehensive evaluation suggests that branches are more suitable for resistance identification. If an inoculation method can be developed to expedite disease progression in branches, the resistance evaluation framework could be further enhanced.

5. Conclusions

In this study, 21 strains were characterized using morphological and molecular biological techniques, with phylogenetic analysis revealing that all strains clustered closely with *B. kuwatsukai*. Examination of the colony morphology across three distinct media indicated that colonies grown on the PDA medium exhibited relative stability, with moderate rates of mycelial growth and aging. In contrast, mycelial growth and disappearance rates were more rapid on the OA medium, while growth was inhibited on the WA medium. The 21 strains were categorized into groups of strong, moderate, and weak virulence based on inoculation experiments conducted on isolated branches from various cultivars. There was no significant relationship between virulence and the geographical location of the pathogenic fungi. Additionally, the resistance of thirty cultivars were assessed, resulting in the identification of twelve resistant germplasm resources and four susceptible germplasm resources.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10111152/s1, Figure S1: Morphological characteristics of each strain on different media; Table S1: All culture media involved in this study; Table S2: All primer sequences involved in this study; Table S3: Gene bank used in constructing the phylogenetic tree.

Author Contributions: Conceptualization, C.W. (Chen Wang) and Y.Z.; methodology, C.W. (Chen Wang); software, C.W. (Chen Wang); validation, C.W. (Chen Wang), Y.Z. and C.O.; formal analysis, C.W. (Chen Wang); investigation, F.W. and L.M.; resources, C.W. (Chen Wang); data curation, C.W. (Chen Wang); writing—original draft preparation, C.W. (Caihong Wang) and Y.Z.; writing—review and editing, S.J. and C.W. (Caihong Wang); visualization, Y.Z.; supervision, S.J.; project administration, Y.Z.; and funding acquisition, S.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Special talents support program of the Chinese Academy of Agricultural Sciences—Leading Talents Program; China Agriculture Research System—Pear (CARS-28); and the Agricultural Science and Technology Innovation Program of Chinese Academy of Agricultural Sciences (CAAS-ASTIP-2021-RIP-01).

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials; further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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