Identification and Biological Characteristic Analysis of *Pythium aristosporum* Causing Rice Seedling Blight in Northeast China

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Abstract

Background: Rice seedling blight caused by diverse pathogenic microorganism occurs worldwide, and it is currently the most important seedling disease affecting rice production in Northeast China. Understanding the biological characteristics, host range, and fungicide sensitivity of pathogens causing rice seedling blight has important theoretical and practical significance for further exploring occurrence of the disease and for making targeted prevention and control measures.

Results: 10 isolates of *Pythium* spp. were obtained from rice seedling blight, according to Koch's postulates, in Heilongjiang Province, China. Based on morphology, molecular biology, and phylogenetic tree analyses, the obtained fungal isolates were identified as *P. aristosporum*. The biological characteristics of *P. aristosporum* showed that the optimum fermentation conditions were as follows: temperature, 25°C; initial pH, 6; photoperiod, 24 h light; and carbon and nitrogen sources, starch and glycine, respectively. *P. aristosporum* causing rice seedling blight had a wide range of hosts and could infect tomato (*Solanum lycopersicum*), carrot (*Daucus carota* L.), sweetpotato (*Ipomoea batatas*), cucumber (*Cucumis sativus* L.), and zucchini (*Cucurbita pepo* L.), as well as seedlings of broad bean (*Vicia faba* L.), red bean (*Vigna angularis*), snailflower (*Lablab purpureus* (Linn.) Sweet), soybean (*Glycine max*), mung bean (*Vigna radiata*), kidney bean (*Phaseolus vulgaris*), and hyacinth bean (*Dolichos lablab* L.); however, it could not infect cabbage (*Brassica oleracea* L.) and maize (*Zea mays* L.) seedlings. We also evaluated *P. aristosporum* sensitivity to five commonly used fungicides and found that fludioxonil ($EC_{50} = 0.0731 \mu\text{g/ml}$) was the most effective against *P. aristosporum*.

Conclusion: To our knowledge, this is the first report of *P. aristosporum* causing rice seedling blight in China. Fludioxonil is a relatively sensitive chemical fungicide against *P. aristosporum*. *P. aristosporum* causing rice seedling blight has a good environmental adaptability and a wide range of hosts, which poses a great threat to rice seedling production. Occurrence of the new disease caused by *P. aristosporum* should give full consideration for the use of *proper disease management* to protect rice.

Background

Rice, wheat, and maize are the three major staple crops worldwide (Yu and Tian 2018; Shindell et al. 2019); among these, rice is the most important cultivated cereal and food crop in China, and it plays an important role in agriculture (Cao 2020; Liu 2017). Heilongjiang Province is the northernmost rice-growing region and is one of the largest commercial Japonica rice production bases in China (Meng et al. 2008). Therefore, ensuring the sustainability of rice production systems in this area is important to global food security and protecting economic livelihoods (Motschenbacher et al. 2014). Rice seedling blight is a serious disease that poses an emerging threat to global rice production. In China, the incidence of this disease, which delays growth of rice seedlings and reduces rice grain yield, has recently been increasing every year (Li et al. 2016). Rice seedling blight disease is caused by several pathogens and is greatly influenced by low temperature (Liu et al. 2004). Because Heilongjiang Province is located in the cold

northern region of China, incidence of rice seedling blight in this province is significantly higher than that in other rice planting areas.

The use of chemicals remains the most effective way to control rice diseases (Liu et al. 2020); however, it is relatively ineffective for protecting plants from seedling blight mainly because the pathogens responsible for rice seedling blight are complex and vary based on location (Riera-Ruiz et al. 2018). There are diverse rice seedling blight pathogens in different regions, soil types, and periods; these include *Fusarium* spp., *Pythium* spp., *Rhizopus* spp., *Curvularia lunata*, *Burkholderia glumae*, *Burkholderia plantarii*, and *Trichoderma* spp. (Aye et al. 2008; Rush 1977; Wang et al. 2016; Whitley 1982; Yoneyama et al. 1998; Ma et al. 2019; Verma et al. 2018). Thus, blindly selecting chemical agents while ignoring the pathogenic species is relatively ineffective for protecting plants from seedling blight. As such, clarifying species and biological character of pathogens causing rice seedling blight is essential for developing effective disease control measures (Gao et al. 2001) that ensure a high and stable yield of rice.

Our team found a novel pathogenic fungus in the process of studying the population structure of fungi causing rice seedling blight in Heilongjiang Province. In this study, we aimed to identify the pathogenic factors causing rice seedling blight and to analyze their biological characteristics, host range, and sensitivity to common fungicides. The results presented in this study may help researchers develop viable disease control strategies in the future.

Results

Disease symptoms and pathogenicity

In May 2019, diseased rice plants (cv. Suigeng 18) showing symptoms of withered tips, chlorosis, stunting, yellow leaves, leaf drop, crown rot, and inhibited root growth, were observed in Suihua City (46.63°N 126.98°E), Heilongjiang Province, China. In this city, the soil is mainly a black sandy clay, with a soil organic matter content of 4-5%. Disease incidence was 10-15% in the three fields sampled (~3 ha). Ten isolates different from known pathogens were obtained from this batch of samples and had similar characteristics. According to Koch's postulate, these 10 isolates were the pathogens of rice seedling blight.

Identification of isolates

The colonies of 10 isolates developed dense, aseptate, cottony, and white hyphae. The sporangia were either finger-like or lobe-like, germinating to produce germ tubes and produce zoospores at 10-15°C. The resting spore was spherical, with a diameter of 9-12 µm. The oogonium was subglobose, with a diameter of 19-35.5 µm (average 27.2 µm). The antheridium was clavate or curved neck, approximately 15 µm × 6 µm and in contact with the oogonium at the top. Each oogonium has 3-10 antheridia. The oospore was spherical, smooth and aplerotic, with a diameter of 13.5-26.3 µm (average 19.9 µm). Each oogonium has 1-2 oospores. Based on their cultural and micro-morphological characteristics, the 10 isolates were identified as *Pythium* spp. (van der Plaats-Niterink 1981).

Molecular identification

Genomic DNA from representative isolates JS22 and SH1 were extracted and the internal transcribed spacer regions (ITS) were amplified with the ITS1 and ITS4 primers.

PCR products were sequenced and deposited in GenBank (accession no. MT337429.1 and MT337436.1). BLAST analysis showed that the obtained sequence for the ITS amplicon shared 95% and 95% similarity with *P. aristosporum* voucher CBS_263.38 (accession no. AY598627.2) and *P. arrhenomanes* voucher CBS_324.62 (accession no. AY598628.1), respectively. They belonged to the same evolutionary branch as *P. aristosporum* and *P. arrhenomanes*, and the similarity was as high as 95% (Fig 1). The isolates were identified as *P. aristosporum* based on the fact that *P. aristosporum* has aplerotic oospores and less antheridia per oogonium than *P. arrhenomanes* (van der Plaats-Niterink 1981).

Based on morphological characteristics, molecular sequence data, and phylogenetic tree analysis, the 10 isolates were finally identified as *P. aristosporum*.

Biological characteristics

P. aristosporum can grow in the 4.0-10.0 pH range, and the differences were significant among the treatments ($P < 0.05$), with the optimum pH value for mycelial growth determined to be 6.0 (Fig. 2-A). *Paristosporum* can grow in the temperature range of 10-35°C, and the differences were significant among the treatments ($P < 0.05$), with the optimum temperature for mycelial growth determined to be 25°C (Fig. 2-B). *Paristosporum* mycelium can grow in various light conditions, and the differences were significant ($P < 0.05$), with the optimum photoperiod for mycelial growth determined to be 24 h light (Fig. 2-C). *Paristosporum* can grow on media with different nitrogen sources, and the differences were significant among the treatments ($P < 0.05$), with the optimal nitrogen source determined to be glycine (Fig. 2-D). *Paristosporum* can grow on media with different carbon sources, and the differences were significant among the treatments ($P < 0.05$), with the optimal carbon source determined to be starch medium (Fig. 2-E).

Host range determination

P. aristosporum can infect tomato, carrot, sweet potato, cucumber, and zucchini, as well as seedlings of broad bean, red bean, snailflower, soybean, mung bean, kidney bean, and hyacinth bean; conversely, cabbage and maize seedlings showed no signs of infection. Moreover, the disease in mung bean was severe, whereas the control plant seedlings were asymptomatic (Fig. 3).

Sensitivity test

As shown in Table 1, the EC₅₀ value of fludioxonil was the lowest at 0.0731 µg/ml, followed by prochloraz and carbendazim, with EC₅₀ values of 0.2391 µg/ml and 1.0281 µg/ml, respectively. EC₅₀ values of hymexazol and azoxystrobin were higher, which were 8.0474 µg/ml and 11.0102 µg/ml, respectively.

Results showed that fludioxonil had the strongest inhibitory effect on *P. aristosporum*, whereas azoxystrobin had the weakest inhibitory effect.

Table 1
The EC₅₀ of common chemical agents to *Pythium aristosporum* in viro

Chemical agents	Virulence regression equation (y=)	Coefficient correlation (r)	EC ₅₀ (µg· mL ⁻¹)
Hymexazol	4.1829 + 0.9022x	0.9821	8.0474
Fludioxonil	6.2323 + 1.0847x	0.9686	0.0731
Prochloraz	5.3575 + 0.5753x	0.9852	0.2391
Carbendazim	4.9901 + 0.8243x	0.9078	1.0281
Azoxystrobin	3.6104 + 1.3338x	0.9949	11.0102

Discussion

Rice seedling blight has been responsible for severe decreases in rice yield and quality in many countries (Baek et al. 2019; Imolehin 1983). There are various complex pathogens that cause rice seedling blight, with previous studies identifying *Fusarium verticillioides* and *Xanthomonas oryzae* pv. *oryzae* in India (Ahuja et al. 2019; Verma et al. 2019); *Burkholderia glumae* in Guayaquil, Ecuador, and Korea (Riera-Ruiz et al. 2018; Nguyen et al. 2017); *Burkholderia plantarii* in China (Qian et al. 2016; Wang et al. 2016); and *P. arrhenomanes* in the Philippines (Van and Hofte 2013). In addition, Furuya et al (2003) found *P. aristosporum* could affect the growth of rice seedlings in the Japan. However, to the best of our knowledge, this is the first study describing *P. aristosporum* as a causal agent of rice seedling blight in Northeast China.

Historically, the key for identifying *Pythium* species is based on morphological characteristics (van der Plaats-Niterink 1981; Mostowfizadeh-Ghalamfarsa and Banihashemi 2005). However, this can be supplemented by molecular identification, which is more confirmative especially when there are overlapping morphological characteristics that make it difficult to determine species and often make identification time-consuming (Alaei and Rostami. 2013; Naima et al. 2020). The internal transcribed spacer (ITS) sequence is a widely used DNA region with good resolution and is commonly used for the molecular identification of *Pythium* spp. (Klemsdal et al. 2008; Robideau et al. 2011). In past studies, Rojas et al. (2016) used the ITS region of the rDNA to identify the *Pythium* genus and species associated with soybean seedlings; Lookabaugh et al. (2017) used the ITS region of the rDNA gene to identify three *Pythium* species isolated from stunted wheat; Ling et al (2018) used the universal primers ITS1/ITS4 to identify *P. arrhenomanes* isolated from rice in China. In this study, traditional morphological identification of the isolates were carried out, and then the ITS1/ITS4 primers were used to further verify the results.

Finally, a phylogenetic tree was constructed to ensure the accuracy and reliability of the identification results.

Pythium activity is easily affected by environmental factors such as light, temperature, and pH (Huang and Su 2007; Guo et al. 2019; Li and Zhang 2020). Additionally, different nitrogen and carbon sources can also affect *Pythium* isolates growth (Foley and Deacon 1986; Temgot and Xu 1996). Therefore, determining *P. aristosporum* biological characteristics have contributed to understanding the reason behind its prevalence and has provided a theoretical basis for formulating rice seedling blight control measures. In this study, the biological characteristics of *P. aristosporum* showed that the optimum fermentation conditions were as follows: temperature, 25-30 °C, which is consistent with the farmland temperature in June and July; initial pH, 6.0–7.0, which is consistent with the pH value of the soil for planting rice; and photoperiod, 24 h. Moreover, *P. aristosporum* is easy to release zoospores, propagates and disperses at high temperature and humidity (Feng and Dernoeden 1999; Titone et al. 2009). Based on this, summer is the fastest growing period of *P. aristosporum* in Heilongjiang Province, we should prevent and control in advance to reduce the occurrence of the disease. Moreover, we found that the optimum carbon and nitrogen sources were starch and glycine, respectively. These results may help to develop effective strategy for the disease. These results deepen our understanding of *P. aristosporum* and lay a foundation for formulating effective measures in the future.

Pythium can cause pre- and/or post-emergence seedling damping off, as well as root, seed, or fruit rot on almost all cereals and grasses such as wheat, maize, and rice (Hendrix and Campbell 1973; van der Plaats-Niterink 1981; Santosh 2012). *P. aristosporum* can infect many plants including wheat (*Triticum aestivum* L.) (Lipps and Bruehl 1978), snap beans (*Phaseolus vulgaris* L.) (Kobriger and Hagedorn 1984), konnyaku (*Amorphophallus konjac* C. Koch) (Iwaizako 1987), turf grass (Titone et al. 2009), and maize (*Zea mays* L.) (Jin et al. 2019) and cause diseases. Moreover, improper crop rotation and farm practices may reduce crop yields. In this study, six common fruits and eight common crop seedlings in Northeast China were selected for host range determination. The results showed that *P. aristosporum* causing rice seedling blight can infect tomato, carrot, sweet potato, cucumber, and zucchini, as well as seedlings of broad bean, red bean, snailflower, soybean, mung bean, kidney bean, and hyacinth bean. Conversely, cabbage and maize seedling showed no signs of infection, which is a result different from that in Jin's study demonstrating that *P. aristosporum* can infect maize and show obvious signs. This may be due to the geographical differences that make our research environment different. Generally, maize diseases are mostly caused by multiple pathogens (Shan 2018), and single pathogens that cause disease may be affected by environmental conditions. So Jin and I get different results. Based on the above experimental results, farmers in Northeast China can potentially rotate cabbage or maize and rice to avoid large-scale outbreaks of diseases caused by *P. aristosporum* in the field.

P. aristosporum infects plants can result in serious yield loss (Kageyama 2014). At present, disease control depends mainly on the application of chemical fungicides. Mazzola and Cook (1991) found that metalaxyl can effectively control diseases caused by *P. aristosporum* in wheat. In recent years, mefenoxam, fosetyl-aluminum and propamocarb have also been used to control diseases caused by *P.*

aristosporum, but Titone et al. (2008) found that *P. aristosporum* strains had low sensitivity to the three fungicides. These results may be attributed to the repeated use of the same fungicides under high disease pressure conditions. In this study, we selected five common fungicides in Northeast China that were not used to control diseases caused by *P. aristosporum* to test sensitivity. The results showed that the EC₅₀ value of fludioxonil, prochloraz, carbendazim, hymexazol and azoxystrobin were 0.0731, 0.2391, 1.0281, 8.0474 and 11.0102 µg/ml, respectively. In other words, fludioxonil had the strong inhibitory effect on *P. aristosporum* and azoxystrobin had the weakest inhibitory effect. Therefore, we consider using fludioxonil to control rice seedling blight caused by *P. aristosporum* in Heilongjiang Province. In future studies, field trials should be conducted to determine the true effects of fludioxonil on rice seedling blight caused by *P. aristosporum*.

In summary, this is the first report of *P. aristosporum* causing rice seedling blight in China. We observed that *P. aristosporum* has good adaptability to the environment, and seedling blight caused by *P. aristosporum* can significantly reduce survival rates and healthy seedlings of rice; as such, its distribution needs to be investigated and effective disease management strategies need to be developed.

Conclusion

In this study, this is the first report of *P. aristosporum* causing rice seedling blight in China. Fludioxonil is a relatively sensitive chemical fungicide against *P. aristosporum*. *P. aristosporum* causing rice seedling blight has a good environmental adaptability and a wide range of hosts, which poses a great threat to rice seedling production. Occurrence of the new disease caused by *P. aristosporum* should give full consideration for the use of proper disease management to protect rice.

Materials And Methods

Isolation and pathogenicity of the pathogen

Rice seedling blight samples collected in Heilongjiang Province were used for pathogen isolation and identification. A published tissue isolation method (Li et al. 2019) was used to isolate and culture the pathogens on potato dextrose agar (PDA) at 25°C for 5 days.

The submerged roots method (Wang et al. 2019) was performed in pots and was used to assess the pathogenicity of the fungal cultures according to Koch's postulates (Shi et al. 2015). Fungi were then re-isolated from diseased rice plants and observed. Specifically, the roots of 15-day-old rice seedlings (cv. Longdao 18) were submerged for 10 min in a suspension containing 10⁶ conidia/ml (Li et al. 2019). Then, the treated seedlings were sown in pots, with five seedlings in each pot, repeated three times. Seedlings soaked in sterilized water were used as control. After 20 days, observed whether the symptoms of the inoculated seedlings were consistent with those of the original isolated.

Identification of the pathogen

Based on morphological characteristics (Wei 1979) and molecular identification, isolates causing rice seedling blight were identified. To further determine the genus and species of the isolates, genomic DNA was extracted from the mycelium of representative isolates using Tiangen Genome Extraction Kit (Tiangen Biotech, Beijing, China). Next, the internal transcribed spacer region (ITS) was amplified and sequenced using ITS1/ITS4 primers (White et al. 1990; [Lookabaugh et al. 2017](#)). PCR was carried out in a final volume of 50 µL, with 10 µM of each primer, 2 × Taq Master Mix and 10 ng of template DNA. PCR conditions were initial denaturation at 94 °C for 5 min, followed by 36 cycles of 94°C for 1 min, 58°C for 1 min, 72°C 1.5 min, and final extension at 72 °C for 10 min. PCR products were purified and sequenced by Shanghai Biological Engineering Co., Ltd (Shanghai, China). Phylogenetic trees of representative isolates were constructed using PhyML version 3.0 based on the maximum-likelihood principle (Guindon et al. 2010).

Biological characteristics

Using the mycelial growth rate method (Li et al. 2020), single factor testing was performed in PDA medium with different pH levels (4.0, 6.0, 8.0, and 10.0), temperatures (10°C, 20°C, 25°C, 28°C, 30°C, and 35°C) and photoperiods (light 24 h, light/dark = 12 h/12 h, and dark 24 h). Single-factor testing was performed using initial medium (agar powder 20 g, soluble starch 10 g, peptone 10 g, distilled water 1000 ml, pH 7.0-7.2) with a carbon source (an equal amount of lactose, glucose, mannitol, maltose, fructose, or sucrose) replacing soluble starch in the initial medium, and a nitrogen source (an equal amount of ammonium sulfate, ammonium nitrate, potassium nitrate, ammonium chloride, glycine, or sodium nitrate) replacing peptone in the initial medium. Each treatment was repeated thrice. Under the above conditions, the diameters of the colonies formed by the isolates were measured.

Host range determination

Fruits and plants, including tomato, carrot, sweet potato, cucumber, zucchini, and cabbage, as well as seedlings of broad bean, red bean, snailflower, soybean, mung bean, kidney bean, hyacinth bean, and maize, were selected to test the host range of the pathogenic fungi. The aforementioned fruits and plants (10 fruits or plants/each treatment) were inoculated with 1 ml conidial suspension of the isolates (1×10^6 spores/ml) by injection (Posada et al. 2007), respectively. Ten other fruits and plants were injected with 1 ml of sterile distilled water as control. After 5 days of inoculation, the disease incidence on the fruit and plant seedlings was analyzed.

Sensitivity test of pathogenic fungi to fungicides

The mycelial growth rate method (Jiang et al. 2015) was used to determine the pathogenic fungi sensitivity to the following fungicides: hymexazol (80% WP) [North American Nongda Conglomerate, Shanghai, China], fludioxonil (50% WP) [Syngenta (Suzhou) Crop Protection Co., Ltd., Suzhou, China], prochloraz (45% EW) [Donghe Biological, Shandong, China], carbendazim (50% WP) [Jiansu Pesticide Chemical Co., Ltd, Zhenjiang, China], and azoxystrobin (19% Suspension Emulsion) [Adama (Beijing) Agricultural Technology Co., Ltd, Beijing, China].

Through a pilot study, hymexazol was added to PDA at final concentrations of 0.5 µg/ml, 1 µg/ml, 3 µg/ml, 5 µg/ml, and 10 µg/ml; Fludioxonil at 0.05 µg/ml, 0.1 µg/ml, 0.3 µg/ml, 0.5 µg/ml, and 1 µg/ml; Prochloraz at 0.5 µg/ml, 1 µg/ml, 3 µg/ml, 5 µg/ml, and 10 µg/ml; Carbendazim at 0.5 µg/ml, 1 µg/ml, 3 µg/ml, 5 µg/ml, and 10 µg/ml; Azoxystrobin at 4 µg/ml, 5 µg/ml, 6 µg/ml, 8 µg/ml, and 10 µg/ml. Fungicide-containing PDA plates were made according to the above concentrations. Three plates were used for each treatment, and the experiment was conducted twice. PDA plates with an equal volume of sterile water were used as control. Each treatment was repeated thrice. A mycelial plug (0.7 cm diameter) with the pathogenic fungi was placed at the center of a PDA plate and was incubated in the dark at 25°C for 120 h. The colony diameter was then measured to calculate the growth inhibition rate.

Data analysis

Analysis of variance (ANOVA) was performed using SPSS Statistics 20.0 (IBM/SPSS, Chicago, IL, USA). Treatment means were assessed using Duncan's multiple range test ($P = 0.05$).

Declarations

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Authors' Contributions

Conceived and designed experiments: LJX, LYG. Performed experiments: LJX, XCZ, HWB; Analyzed data: LJX, LYG. Wrote and revised manuscript: LJX and LYG. All authors read and approved the final manuscript.

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Phylogenetic tree of *Pythium aristosporum* strains JS22 and SH1 of Rice seedling blight based on internal transcribed spacer regions (ITS) gene. The bootstrap values on the branching nodes were calculated on 1000 replications. The scale bar indicated 0.08 substitutions per nucleotide position.

Figure 2

Mycelium growth diameter of *Pythium aristosporum* strain JS22 at different pH values, temperatures, illuminations, nitrogen sources and carbon sources. Different letters above the bars indicate significant difference according to Duncan's multiple range test ($P = 0.05$).

Figure 3

Host range of *Pythium aristosporum* determined. A: tomato (*Solanum lycopersicum*). B: carrot (*Daucus carota* L.). C: sweet potato (*Ipomoea batatas*). D: cucumber (*Cucumis sativus* L.). E: zucchini (*Cucurbita pepo* L.). F: cabbage (*Brassica oleracea* L.). G: broad bean (*Vicia faba* L.) seedlings. H: red bean (*Vigna angularis*) seedlings. I: snailflower (*Lablab purpureus* (L.) Sweet) seedlings. J: soybean (*Glycine max*) seedlings. K: mung bean (*Vigna radiata*) seedlings. L: kidney bean (*Phaseolus vulgaris*) seedlings. M: hyacinth bean (*Dolichos lablab* L.) seedlings. N: maize (*Zea mays* L.) seedlings.