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Isolation, identification and artificial inoculation of *Rhizoctonia solani* on pear during storage

*Xiaohui Jia, Jianing Xu, Yushuo Wu, Xinnan Zhang, Yanmin Du, and Wenhui Wang**

Research Institute of Pomology, Chinese Academy of Agricultural Sciences, Xingcheng, Liaoning 125100, China

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A B S T R A C T

Pear is a fruit crop of worldwide importance and cold storage is an integral part of the production and distribution of pears. An uncharacterized fungal disease has been observed on 'Huangguan' pear fruit during cold storage in Hebei Province. The fungus was consistently isolated from diseased fruit by routine tissue separation method, and shown to be the causal agent according to Koch postulates. Based on its morphology, molecular characteristics, pathogenicity and ITS sequence, the fungus was identified as *Rhizoctonia solani*. This study recorded postharvest fruit rot caused by *Rhizoctonia solani* on pear fruit in China.

Keywords: Pear; Pathogenicity; Postharvest; *Rhizoctonia solani*; Fruit rot

1. Introduction

Pear is widely harvested throughout the world, and by 2020, the total land usage for pear cultivation in China reached 940 700 hectares with an output of 16.078 million tons. Moreover, the annual cold storage capacity for pear fruit exceeds 5 million tons (Jia et al., 2020; Zhang et al., 2021). Hebei is the main pear producing area in China. 'Huangguan' pear fruit (*Pyrus bretschneideri* × *Pyrus pyrifolia*) is a well-known cultivar in Hebei Province, which is the main area of storage and export of pear fruit in China. The presence of microorganisms is one of the most important factors in the depreciation in quality of fresh pear fruit. The commercial value of fruit decreases due to different pathogens, especially fungi, which manifest their destructive action during storage (Puia et al., 2003).

In 2019, postharvest fruit rot was found on fresh pear in Hebei Province, caused by an uncharacterized disease, and accounting for significant potential economic losses to pear storage throughout the world. In order to understand this new pear fruit rot disease, we isolated and identified the suspected pathogenic strain. The results of our research can help guide future studies on methods for prediction and control of the disease.

2. Materials and methods

2.1. Observation of disease symptoms

In April 2019, ten fruit with typical symptoms of rot were collected from storage facilities within the main production areas of 'Huangguan' pear in Xinji, Hebei Province, China. The symptoms of the disease were observed and recorded.

2.2. Separation, purification, and preservation of fungal strains

The pathogen was readily isolated from diseased tissue onto potato dextrose agar (PDA, potatoes 200 g, glucose 20 g, agar 18 g, distilled water 1 000 mL) amended with 250 mg · L^{-1} chloramphenicol (Come from Solarbio Biotechnology Co., Ltd.). The marginal mycelia were picked out in order to obtain purified fungal strains, which were preserved at 4 °C (Fang, 2007).

* Corresponding author.
E-mail address: wangwenhui@caas.cn

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2.3. *Pathogenicity test*

A pathogenicity test was performed according to Koch's postulates (Fang, 2007). Healthy ripe 'Huangguan' pears were gently washed using 70% ethanol for 2 s. Then 1% sodium hypochlorite was used to wash them for 2 min. Next, the fruit were washed using sterilized distilled water.

Healthy pears (30 fruit) were then subjected to three treatments: artificial unwounded inoculation, artificial wounded inoculation, or inoculation of unwounded fruit with sterile PDA (sterile PDA of mycelial free was used to inoculate wounded fruit as controls). The artificial unwounded inoculation was performed by inoculating the equatorial surface of unwounded, healthy fruit with a mycelium 5 mm in diameter. The artificial wounded inoculation was performed by stabbing a small hole about 0.5 cm deep on the equatorial surface of every fruit with a sterile inoculation needle. These characteristics were used to morphologically identify purified strains according to Lu (2004). Each treatment required 10 fruit and each fruit was inoculated at 3 locations.

After inoculation, three treatment groups for pears were placed in a sterilized plastic container on a layer of moist filter paper at 25 °C for 10 days. The experiments were repeated twice. After pathogen fungi infected pear fruit, samples isolated from the lesion were cultured and then compared with the original strain HGRS0821.

2.4. *Morphological identification*

The purified strains were inoculated on PDA plates, and were cultivated in a biochemical incubator for 5 days at 25 °C. After growth from the 3rd to 7th day, we observed the colony, mycelium, and sclerotia. The morphological characteristics of cultured pathogenic mycelium were observed and recorded using a light microscope. We also used these characteristics to morphologically identify purified strains according to Lu (2004).

2.5. *Molecular identification*

To confirm the morphological identification, an isolate was randomly selected for DNA analysis. Mycelia were scraped from PDA plates, The genomic DNA was extracted and purified by the CTAB method, and the internal transcribed spacer (ITS) region of rDNA of the pathogen was amplified by PCR with the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) synthesized by Shanghai Bioengineering Technology Services Co., Ltd. The PCR reaction conditions were followed Jia's study with some modifications (Jia et al., 2018). The sequence was compared with homologous sequences in the GenBank nucleic acid sequence library of NCBI. A phylogenetic tree was constructed with the Neighbor-Joining method in MEGA 7.0.

3. Results

3.1. *Symptoms of infected pear fruit*

The initial symptoms appeared on fruit surfaces as a scatter of dark brown spots. The spots gradually enlarged and finally gathered together to form a large spot (Fig. 1, A). The lesion was depressed to form a cavity. The boundary between diseased and healthy flesh was not obvious (Fig. 1, B). Several lesions were likely to appear from one fruit and the gray-white mycelia covered the entire surface of a fruit over time (Fig. 1, A). The fruit became watery and eventually rotted.

3.2. *Pathogenicity test*

Pathogenicity test was conducted by inoculating surface-sterilized healthy fruit. The symptoms of the site of artificial wound inoculation began to show after twenty-four hours, and formed a visible diseased spot after 48 h (Fig. 1, C). The rate of lesion expansion was 2.21 mm · d^{-1}. However, control fruit remained healthy during the experimental period. Tissue isolation and cultures were again carried out to obtain new colonies. *R. solani* was successfully reisolated from the lesions with the same symptoms as the original strain. Fulfilling Koch's postulates, the original strain was a pathogen that could cause the same unknown disease on pear.

3.3. *Morphological characteristics of the pathogen*

R. solani was isolated from the artificially induced diseased tissue from each treated fruit (Fig. 2, A, B). Colonies radially expanded at an average rate of 42.76 mm · d^{-1} (Fig. 2, C). Conidia were not produced, but gray-black spherical sclerotia were produced (Fig. 2, D). Isolates formed white colonies of hyphae on the PDA plate, aerial mycelium and substrate mycelium were white to cream, slender with a width of 5.12–8.67 μm and separated (Fig. 2, E).

3.4. *Molecular identification of the original strain*

The ITS region of the pathogen was amplified and sequenced. BLAST search and phylogenetic analysis showed that the ITS sequence (GenBank Accession No. MT846141.1) had 99.00% similarity with sequences of two *R. solani* isolates (MH172561.1 and LC504760.1) (Fig. 3).

4. Discussion

R. solani is a typical soil-borne disease that is difficult to control and has a wide host range, including rice, corn, soybean, and potato (Baker et al., 1970). *R. solani* is divided into 14 anastomosis groups (AGs), including Ag-1 to Ag-13 and Ag-Bi, most of which are plant pathogenic fungi (Ogoshi et al., 1987; Stodart et al., 2007). The ITS sequence (MT846141.1) of the pathogen on pears showed high similarity with MH172561.1 and LC504760.1. These two isolates belong to AG-4 HGIII type *R. solani* (https://www.ncbi.nlm.nih.gov/nuccore/MH172561.1; https://www.ncbi.nlm.nih.gov/nuccore/LC504760.1), thus the pathogen isolated in this study should be *R. solani* AG-4 HGIII based on phylogenetic analysis.

R. solani has been reported to infect pear leaves, apple leaves, and peach leaves (Hideo et al., 1996; Liu et al., 1999; Zhang et al., 2010). *R. solani* mainly attacks the roots and lower stems of plants, causing serious diseases (Baker et al., 1970), such as rice sheath blight (Vidhyasekaran et al., 1997). The results of this study indicated that *R. solani* could lead to hollow formation in postharvest lesions of pear fruit. The boundary between diseased and healthy

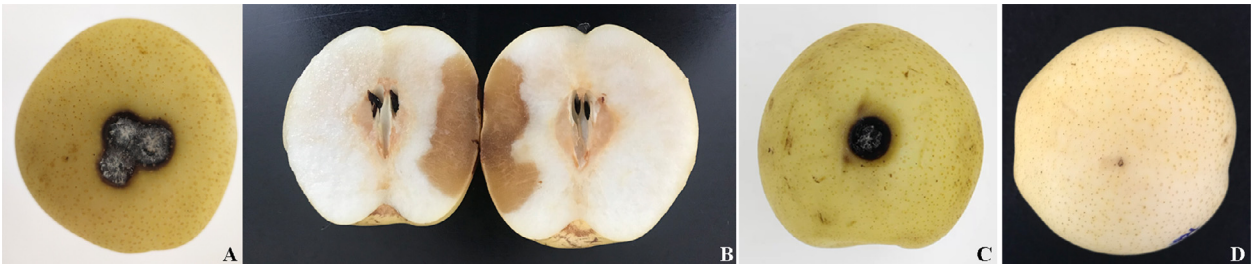


Fig. 1 Symptoms of postharvest fruit rot caused by *Rhizoctonia solani* on 'Huangguan' pear
A, Natural infection; B. Internal symptoms of the spot; C. Artificial wounded inoculation; D, Control.

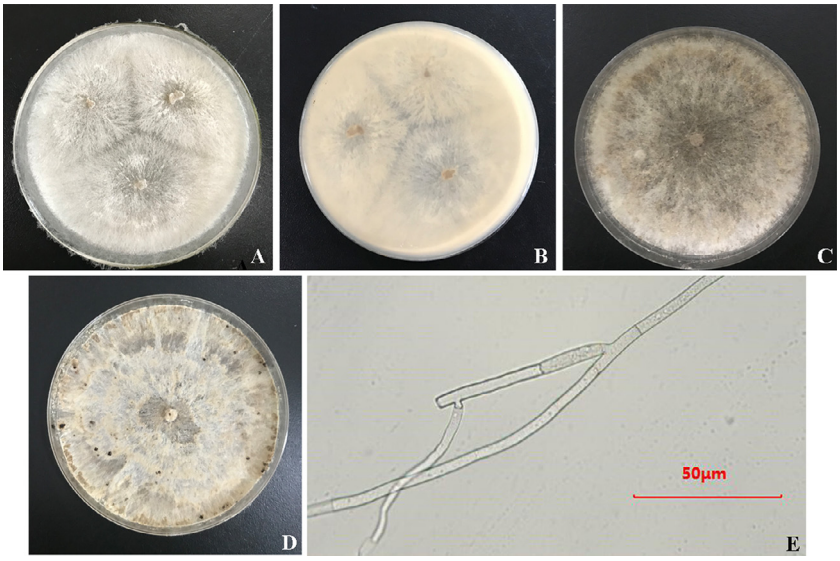


Fig. 2 *Rhizoctonia solani*
A. Colony on potato dextrose agar medium (PDA); B. Reverse colony on PDA; C. Colony on PDA for 3 days; D. Sclerotium; E. Mycelial morphology.

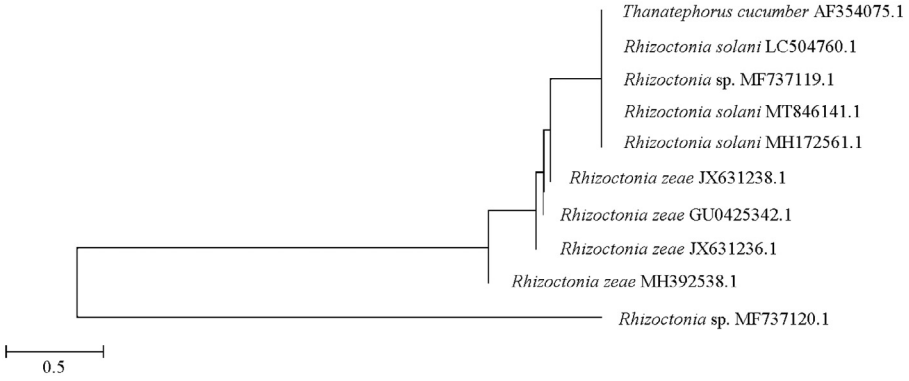


Fig. 3 Phylogenetic tree of *Rhizoctonia solani* (MT846141.1) based on rDNA-ITS (internal transcribed spacer) sequences

flesh was not clear. Eventually, the entire surface of the fruit developed pale hyphae, and the fruit became wet and rotted. *R. solani* was a host selective toxin and a determinant of pathogenicity, which meant that it induces only characteristic symptoms of the host (Kankam et al., 2016).

The pathogenic mechanism of *R. solani* has become a research hotspot, and its cell wall degrading enzyme is an important pathogenic factor. Artificially cultured cell wall-degrading enzymes could cause changes in membrane permeability and tissue leaching of rice leaf sheath, damaging tissue structure, resulting in broken cell walls and deformed organelles, and were essential in the formation and expansion of disease spots (Chen et al., 1992; Zhang et al., 2005). Other studies showed that pectinase was the pathogenic factor of *R. solani*, and the amount of pectinase and cellulase were closely related to the pathogenicity (Akter et al., 2016). The experimental results showed that *R.*

solani had an important damage effect on rice leaf. In addition to causing typical symptoms, *R. solani* also damaged cell membranes and caused electrolyte leakage (Yang et al., 2011). Research on the pathogenic mechanism of *R. solani* has been conducted, but no consistent conclusion had been reached. It was believed that the pathogenic mechanism of *R. solani* to pear fruit during storage was complex, and the function of cell wall degrading enzymes in the process of pathogen infection needs further study.

5. Conclusion

This study isolated a pathogen from 'Huangguan' pear in Hebei Province of China, and identified it as *R. solani* using both traditional morphological classification and rDNA-ITS sequence analysis. Our research can provide the basis for studies of this pathogen on pear in the future. It is urgent to carry out research on the biological characteristics and pathogenesis of the fungus, in order to prevent the disease.

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