Communication

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First Report of the Peach Leaf Spot Caused by *Nigrospora sphaerica* in China

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Abstract: Peach (*Prunus persica* L.) is a globally significant fruit valued for its high edible and ornamental qualities. Peach leaf spot disease has become increasingly prevalent in recent years, negatively affecting fruit quality and aesthetic appeal. During the summer of 2024, symptoms of leaf spots were observed on peach trees in an orchard in Bazhong City, Sichuan Province, China. Leaf samples displaying typical spot symptoms were collected from peach orchards, and the pathogenic agents were isolated. Based on their morphological characteristics and multi-locus phylogenetic analysis, the isolated and purified fungus SCBZPP6 was identified as *Nigrospora sphaerica*. Furthermore, the pathogenicity of the isolated fungus was verified via Koch's postulates. To our knowledge, this is the first report of *N. sphaerica* causing leaf spot on peach in China.

Keywords: peach; leaf spot; identification; *Nigrospora sphaerica*

1. Introduction

Peach (*Prunus persica L.*) ranks as the third most economically significant in temperate regions worldwide, valued for its ornamental blooms and nutritious fruits[1]. With a long history of cultivation, peaches are grown globally in countries including China, Spain, Italy, Turkey, Iran, USA, Egypt, Chile, and India. In China, the peach cultivation area reached 840 thousand hectares, with a production of approximately 15.8 million tons, as reported by the Food and Agriculture Organization of the United Nations (FAO) 2022 Report[2]. Beyond fresh consumption, peaches are employed on various forms such as dried fruit snacks, fruit juice, and canned fruit. However, the productivity and quality of peach fruits are greatly threatened by various diseases, resulting in a marked reduction in fruit yield and significantly limiting the development of peach production.

Approximately twenty diseases have been reported in peach, including brown rot caused by *Monilinia fructicola*, bacterial spot caused by *Xanthomonas arboricola* pv. *pruni* (*Xap*), bacterial canker caused by *Pseudomonas syringae*, and powdery mildew caused by *Podosphaera pannosa*, peach leaf curl caused by *Taphrina deformans*[3–6]. To date, compared with these peach diseases, less attention has been paid to the peach leaf spots caused by fungi. It has been reported that peach leaf spots occur frequently and affect fruit quality and yield[7–9]. However, until now, the identification of the peach leaf spot diseases caused by fungi have been rarely reported.

Nigrospora species are widely distributed in nature, with 45 species currently recorded in the MycoBank database[10]. This genus has a broad-host range and mainly infects plant leaves and stems[11]. Among them, *N. sphaerica* and *N. oryzae* are notable plant pathogens[12]. *N. sphaerica* typically infects plant leaves, causing leaf spot disease. In recent years, new hosts of this pathogen have been continuously discovered, such as blueberry plants, watermelon, *Rhododendron* simsii, cacao, passion fruit, and olive [10,12–16]. Furthermore, *N. sphaerica* can cause human infections, including onychomycosis and corneal ulcer[11].

In the summer of 2024, a leaf spot disease occurred in peach orchards in Bazhong City, Sichuan Province, China, with symptoms distinct from the bacterial spots caused by *Pseudomonas syringae*.

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This study aims to identify the causal agent of this disease based on morphological and molecular characterization.

2. Materials and Methods

2.1. Sample Collection

Peach leaves with typical lesions were harvested from an orchard in Bazhong City (106°44′35.97″ E, 31°52′12.27″ N), Sichuan Province, China. The collected leaves were wrapped in moistened cotton to retain moisture and placed in a labelled sterile sample bag. The samples were then immediately transported to the laboratory for pathogen isolation. After two weeks, the same-sized healthy leaves were collected from the orchard for pathogenicity testing.

2.2. Isolation and Purification of Pathogenic Fungi

To isolate pathogens, the boundary tissues of the leaf lesions were cut into 1 cm × 1 cm fragments, disinfected in 75% ethanol for 1 min, rinsed with sterile water for three times, then immersed in 3% sodium hypochlorite solution for 1 min, followed by rinsing with sterile water three times. The treated fragments were dried on filter paper and incubated on potato dextrose agar (PDA) plate at 25 °C for two days. The isolated strains were purified by picking the hyphal tip from the colonial margin two or three times[17].

2.3. Morphological Identification

For morphological identification, the purified strains were cultured on PDA plates at 25 °C for 5–7 days. The colony morphology was observed when the fungal hyphae reached the plate edges. The characteristics of the mycelium and spores were observed under optical microscope (Olympus , Chongqing, China).

2.4. Molecular Identification and Phylogenetic Analysis

Approximately 0.5 g fresh mycelia were harvested from 7-day-old PDA cultures and ground into a fine powder in liquid nitrogen. Total genomic DNA was extracted using CTAB solution (2 M Tris-HCl, 5 M NaCl, 0.5 M EDTA, 2% (w/v) CTAB, and 0.2% (v/v) mercaptoethanol)[18]. DNA quantity and quality were assessed using 1.0% (w/v) agarose gel stained with GelRed nucleic acid stain (Vazyme, Nanjing, China). For molecular identification, primer pairs ITS1/ITS4[19], EF1-728F/EF1-986R[20], and Bt2a/Bt2b[21] were used to amplify the internal transcribed spacer (ITS), partial translation elongation factor 1-alpha (*TEF1-a*), and *β-tubulin* (TUB) sequences of all isolated fungal strains (Table 1). PCR was carried out with the following conditions: 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and elongation at 72 °C for 1 min, with an initial denaturation step at 95 °C for 5 min. PCR products were purified using Omega Bio-tek's E.Z.N.A.® Gel Extraction Kit and cloned into the pMD18-T vector (Takara, Shiga, Japan) for sequencing. The obtained sequences were compared with reference sequences using the BLAST tool on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The amplified sequences in this study were aligned with the reference sequences using ClustalW. The phylogenetic tree was constructed using the maximum-likelihood (ML) method via MEGA7.0 software, and the reliability of the tree was assessed using 1000 bootstrap replicates.

Table 1. List of primers used in this study.

Gene	Primer	Sequence (5′–3′)	Size
ITS	ITS1	TCCGTAGGTGAACCTGCGG	555 bp

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	ITS4	TCCTCCGCTTATTGATATGC	
	EF1-728F	CATCGAGAAGTTCGAGAAGG	
TEF1-a			279 bp
	EF1-986R	TACTTGAAGGAACCCTTACC	
	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	
β-tubulin			434 bp
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	

2.5. Pathogenicity Test

To assess the pathogenicity of the fungal isolates, healthy peach leaves were surface sterilized by immersion in 70% ethanol for 1 min, followed by rinsed in sterilized distilled water three times. The leaves were wounded by pin-pricking the surface with sterilized needle. Subsequently, 5 mm diameter mycelial plugs were obtained from the edges of a fresh colony and inoculated on the wounded leaves. Plugs of PDA were used as controls. The petioles of the inoculated leaves were wrapped with moistened cotton to retain moisture. The inoculated leaves were kept in a plastic box with high humidity at 28°C in the dark for disease development[22]. To fulfill the Koch's postulates, re-isolations were made from the diseased leaves. The experiment was repeated three times.

3. Results

In August 2024, leaf spot symptoms were observed on peach in an orchard in Ba zhong City, Sichuan Province, China. The peach plants with leaf spots accounted for 15% of the total plants. Initially, the lesions appeared small, irregular, and brown with indistinct edges. As the disease progressed, the lesions gradually expanded. Two or more lesions joined together, and the centers of lesions occasionally formed perforations. Additionally, the color of lesions became black or dark brown (Figure 1A).

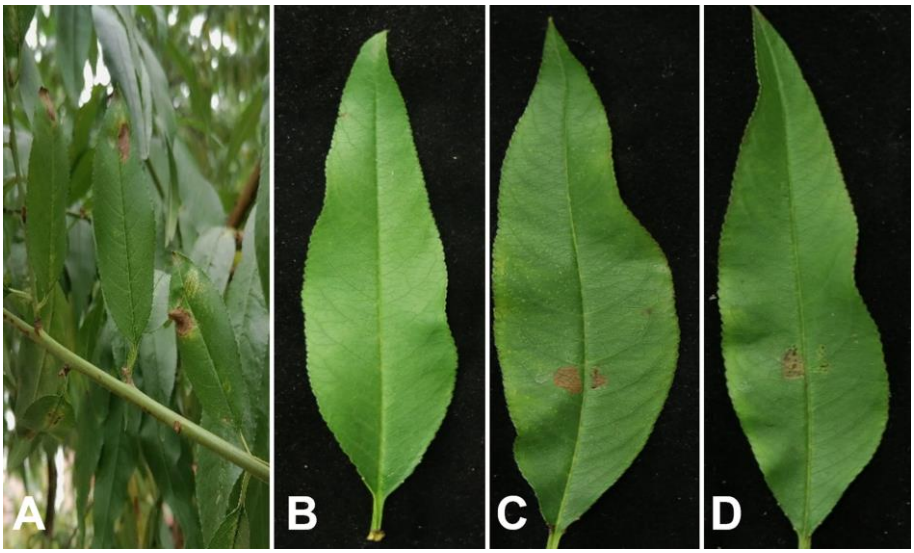

Figure 1. Symptoms of leaf spots on peach (A) peach leaf spots symptom in the field; (B) control peach leave inoculated with PDA; (C-D) peach leaves inoculated with SCBZPP6.

A total of six isolates were obtained from the diseased leaf samples and cultured on PDA plates for 5 days. These isolates exhibited consistent morphological features. Colonies on PDA were white

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and flocculent, but their reverse side was pale yellow. Under microscopic examination, conidiogenous cells were colorless and subglobose. The conidia were acrogenous, solitary, black and globose with diameters of 15-19 μm (Figure 2). Based on the morphological characteristics, these isolates were tentatively identified as belonging to the genus *Nigrospora* sp.

For precise identification, molecular methods were used to amplify the internal transcribed spacer (ITS) region, the partial translation elongation factor 1 alpha (*TEF1-a*) region, and the *β-tubulin* region of all isolates via PCR. The fragment sizes of PCR products were about 555 bp, 279 bp, and 434 bp, respectively. These fragments were cloned into the pMD18-T vector and then sequenced. Multiple sequence alignment revealed 100% identity in the ITS, *TEF1-a* or *β-tubulin* regions among the six isolates. The ITS, *TEF1-a*, and *β-tubulin* sequences of the representative isolate SCBZPP6 were further analyzed via NCBI Blast, which showed that the ITS region sequence of SCBZPP6 matched 100% with *N. sphaerica* strains (KU553345.1, KC519729.1, and PQ289163.1). The partial *TEF1-a* sequences of SCBZPP6 showed 99.2-100% identity with *N. sphaerica* (OM826971.1, MN053315.1, KY019393.1, and KY513872.1), and its β-tubulin region sequence was 99.5-99.7% identical to *N. sphaerica* (MK408565.1, OM826974.1, and MN719407.1).

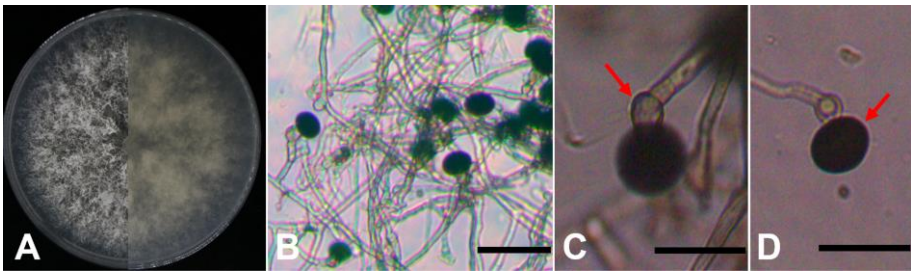

Figure 2. Morphological characteristics of the SCBZPP6 strain. (A) Front and reverse view of the colony on PDA after five days; (B) Colony on PDA; (C-D) Conidiogenous cell (red arrow) and conidia. Scale bars: B = 50 μm; C-D = 20 μm.

Then, the ITS, *TEF1-a*, and *β-tubulin* sequences were submitted to the NCBI, received accession numbers (PQ496816, PQ505108, and PQ505109). To analyze homologous relationships, a phylogenetic tree was constructed using the concatenated ITS, *TEF1-α*, and *β-tubulin* sequences of SCBZPP6 with reference sequences of related strains using the maximum-likelihood (ML) method. The phylogenetic results showed that SCBZPP6 was most closely related to *N. sphaerica* (Figure 3). Based on these findings, we conclude that the isolated pathogens were *N. sphaerica* based on morphological and molecular characteristics.

To assess the pathogenicity of SCBZPP6, mycelial plugs of the strain were inoculated onto detached peach leaves. Ten days post inoculation, visible brown spots formed on the leaves inoculated with SCBZPP6, similar to those observed on infected plants in the field, compared with the healthy control leaves (Figure 1C-1D). To fulfill Koch's postulates, the pathogen was re-isolated from the diseased leaf.

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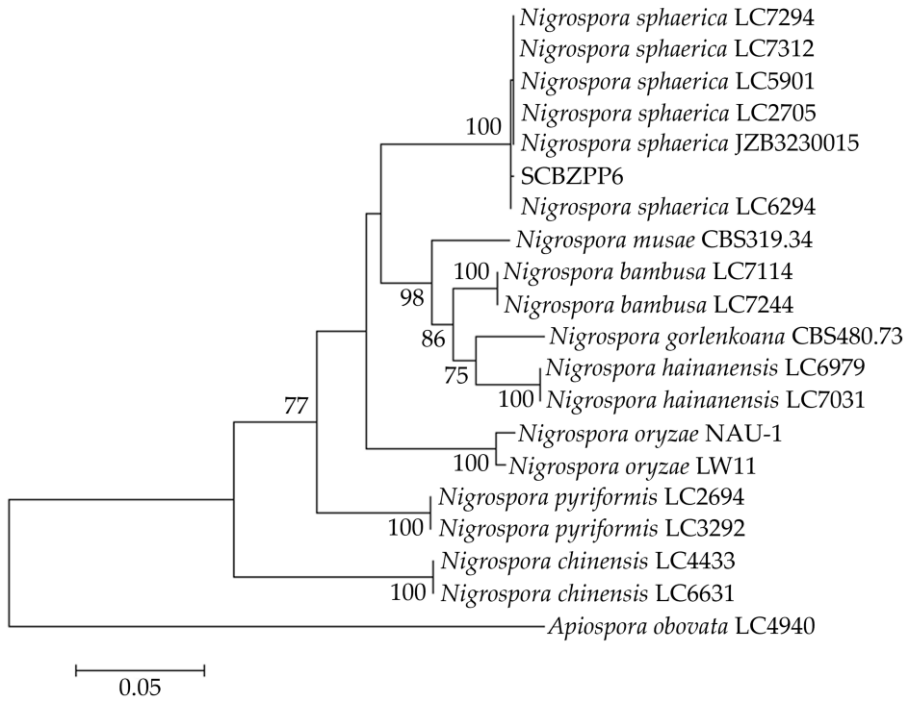

Figure 3. Phylogenetic tree generated from the maximum likelihood analysis based on ITS, *TEF1-a* and *β*-tubulin sequences of the isolate SCBZPP6 and 20 strains representing 9 species of the genus *Nigrospora*. Bootstrap values greater than 70% are shown at the nodes. *Apiospora obovata* was chosen as an outgroup.

4. Discussion

In recent years, climate changes with rising temperatures and increasing precipitation are believed to contribute to an increased occurrence of leaf diseases. The bacterial leaf spot diseases of peach caused by *Xanthomonas arboricola* pv. *pruni* (*Xap*) is well-studied, typically presenting as small, brown and water-soaked lesions. As the lesions enlarge, the centers become surrounded by yellow halos and eventually fall off, forming irregular perforations. In this study, the symptoms of the peach leaf spot were distinguishable from those caused by bacteria. Additionally, the isolates were identified as *N. sphaerica*. To our knowledge, this is the first report of peach leaf spot disease caused by *N. sphaerica*.

Nigrospora species have a wide host range, and *N. sphaerica* is one of the most reported *Nigrospora* species. So far, *N. sphaerica* is able to cause diseases in over 40 plants[10,15,16,23–29], including vegetables, fruits, flowers, herbs and ornamental trees. The fungus mainly causes leaf spot diseases but also infects twigs, roots, and fruits. It is imperative to identify *Nigrospora* species for future study. Historically, identification of *Nigrospora* species has relied on morphological characteristics, especially conidial dimensions. However, this approach can lack accuracy, so we used both morphological and molecular characterization to identify the isolates at the species level. Our study provides a foundational understanding for developing effective control measures against this new fungal disease in peach.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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