

Isolation and identification of *Nigrospora oryzae* causing Leaf Spot of *Buxus megistophylla* in China

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Abstract: In a survey conducted from 2015 to 2016, a leaf spot of unknown etiology has been observed on young leaves of *Buxus megistophylla* grown in Wangjiang County, Anhui Province of China. The fungus was isolated from symptomatic infected leaves, and its pathogenicity was confirmed. Based on morphological characteristics and molecular analysis, the pathogen was identified as *Nigrospora oryzae*. This report is the first on *B. megistophylla* leaf spot caused by *N. oryzae* in China.

Introduction

Buxus megistophylla is shrub or small tree. Which is a plant species native to China, Japan, and Korea and it is a ornamental plant, popular, woody in China (Xie *et al.*, 2016). In a survey conducted from June 2015 to July 2016, a leaf spot of unknown etiology has been observed on young leaves of *B. megistophylla* grown in this region. Resulting in severely blighted and eventually the dried tissue falls.

There has been no prior report on leaf spot of *B. megistophylla* in China. The initial symptoms of this disease appear as dark spots surrounded by a yellow to rust-brown zone that gradually increases from 5 to 10 mm in diameter, changing from circular to elliptical lesions on the leaves. Lesions enlarge and coalesce, causing diseased leaves to become blighted (Fig. 1 A, B). The aim of this study was to determine the etiological agent of the leaf spot.

Material and methods

Sample collection and fungal isolation

In September 2015, leaves of *B. megistophylla* with typical symptoms were collected from ten orchards in Wangjiang county, Anhui Province, China. For fungal isolation, surface disinfection method was performed by cutting the infected leaf tissues (5 mm² sections) from healthy and margin of disease tissues. The tissues surface-sterilized with 1.5% sodium hypochlorite for 2 min. After rinsing in sterile distilled water three times, segments were plated on potato dextrose agar (PDA) and incubated at 25°C for 7 days. Single-spore isolates were obtained from fungal cultures incubated at 25°C with a 12-h photoperiod for 6 days and stored on PDA slant tubes at 4°C (Ben *et al.*, 2015).

Fungal identification

Conventional morphological characters tentatively identified the fungal isolates collected in this study to genus and species (Ellis 1971). The morphological data of anatomical features were based on at least 50 measurements of each structure under a light microscope (Olympus BX51, Japan).

Isolate WJ-10 was selected as a representative for molecular identification. Genomic DNA of the fungus was extracted using the SDS- CTAB method described by Suwannarach *et al.*, (2010). The primer pairs ITS4 - 5' – TCCTCCGCTTATTGATATGC - 3' and ITS5 - 5'- GGAAGTAAA AGTCGTAACAAGG - 3' were used for r-DNA amplification (Carbone and Kohn 1999). PCR was

performed using a DNA Engine System S1000 (BIO-RAD, Singapore). The PCR amplification was carried out in 25 µl reaction mixture containing 1 µl of DNA sample with 2.5 µl of 10×PCR buffer (contain MgCl₂), 0.5 µl of 2 mM dNTPs, 20 pmol of each forward and reverse primer (1.0 µl) and 0.125 µl of Taq DNA Polymerase and made up to 25µl with 18.875 µl of nuclease-free water. The PCR conditions include initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 52°C for 30 s, 72°C for 1 min and a final cycle of 72°C for 10 min. The amplified products were separated on 1.0% agarose gel containing 4 µl of ethidium bromide and visualized in a Biorad UV transilluminator. The PCR products were directly sequenced with the same primers. Sequences obtained in this study were compared with those from GenBank database using the BLAST software on the NCBI website: ([http:// www. ncbi. nlm. nih. gov/ BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)).

Pathogenicity test

Pathogenicity tests were conducted using the isolate WJ-10 on ten leaves of 2-year-old *B. megistophylla* plants, asymptomatic leaves that were disinfested with 70% ethanol for 1 min (Liu *et al.*, 2016). Leaves were pushpin-wounded, and placed in a greenhouse with constant humidity at 25°C and a 12-h photoperiod of fluorescent light. Pathogenicity tests were conducted by placing agar pieces (6 mm in diameter) from 7-day-old cultures on wounded leaves. An equal number of control plants were wounded and inoculated with noncolonized PDA agar pieces. The experiment was repeated three times. After inoculation, the development of symptoms was observed and recorded. The fungus was re-isolated from the disease symptoms, confirming Koch's postulates.

Result and Discussion

20 fungal isolates were recovered from symptomatic infected leaves. These fungal colonies were initially white, becoming light to dark gray and produced black (Fig. 1 C, D), spherical to subspherical, single-celled conidia (10.9 to 13.8 × 11.8 to 14.5 µm) (Fig. 1 E, F), which were borne on a hyaline vesicle at the tip of the conidiophores. On the basis of these morphological features, the isolates appeared to be similar to *Nigrospora oryzae* (Hudson 1963).

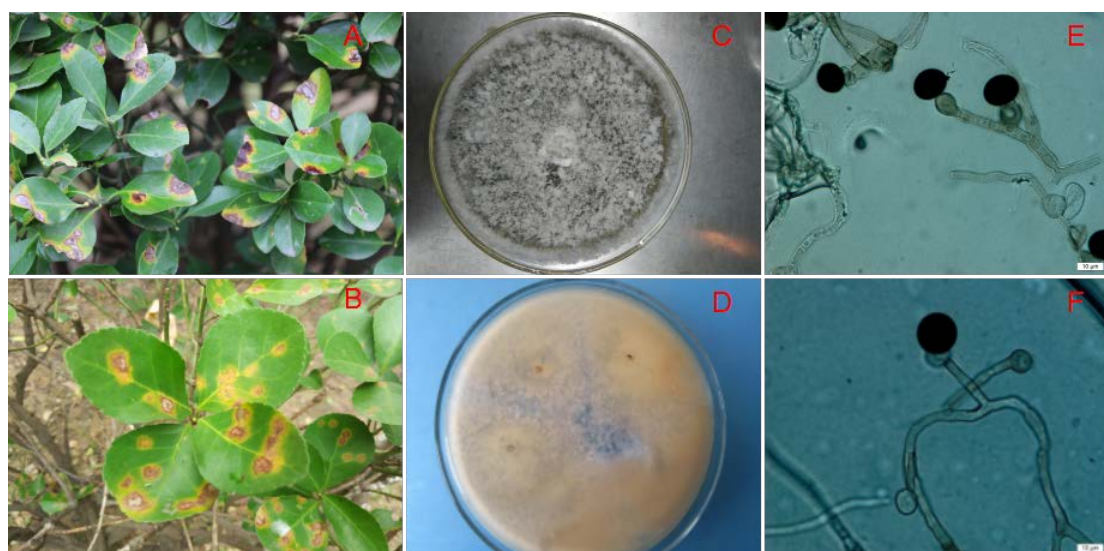


Fig. 1 Natural symptoms of leaf spot and morphological characteristics of *Nigrospora oryzae*. (A, B) Typical symptom on leaves; (C, D) Colonies on PDA culture from both the top side (upper) and bottom side (lower); (E, F) conidia (Scale bars= 10 µm).

The universal rDNA-ITS primer pair ITS4/ITS5 was used to amplify a DNA fragment of approximately 579 bp. The amplified PCR products was sequenced and compared through BLASTn analysis. Sequences of the isolate was 99% similar to (GenBank Accession Nos. MH087476. 1 and MH619723. 1). The sequence of isolate WJ-10 was deposited in GenBank (MK131325). Morphological features best match *N. oryzae* and with the supporting molecular data, the pathogen

was identified as *N. oryzae*.

For pathogenicity test, the isolate WJ-10 started to develop brown-to-black lesions on wounded leaves after 6 days and expanded to an average of 15 × 25 mm 15 days after inoculation. Inoculated leaves developed spot symptoms similar to those observed on naturally infected leaves in the field, with the incidence 100%, while control leaves remained symptomless. The pathogen was reisolated from the margins of necrotic tissues but not from the controls, thus satisfying Koch's postulates. Based on the above results, it can be concluded that *N. oryzae* is the causal agent of leaf spot of *B. megistophylla* in China.

Although *N. oryzae* is frequently encountered as a secondary invader or as a saprophyte on many plant species, this fungal agent is also known as a leaf pathogen on several hosts worldwide (Paul 1982; Khodke *et al.*, 2010; Mohammed *et al.*, 2013; Moshrefi Zarandi *et al.*, 2014; Begum *et al.*, 2018). To our best knowledge, this is the first report of *N. oryzae* as a leaf pathogen on *B. megistophylla* in China. Since the pathogen infections pose a serious threat to *B. megistophylla*, this disease needs to be considered for appropriate management measures in this region.

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