



First report of *Boeremia exigua* causing leaf spot of tobacco (*Nicotiana tabacum*) in China

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Abstract

Tobacco (*Nicotiana tabacum* L.) is an economically important crop, widely cultivated in China. Since 2021, a new leaf spot disease has been seen frequently in Enshi, Xiangyang, Yichang, and Shiyan, all in Hubei Province, China. Diseased leaves exhibiting roundish lesions with greenish-yellow edges were collected to determine the causal agent in summer 2022. After isolation, morphological characterization, and molecular identification (ITS, LSU, and TUB), the pathogen of the new tobacco leaf spot disease was identified as *Boeremia exigua*. Inoculation tests showed that *B. exigua* strains could cause this disease on detached tobacco leaves, and fungi were re-isolated with morphology identical to the inoculated strain, confirming Koch's postulates. This is the first report of tobacco leaf spot caused by *B. exigua* worldwide.

Keywords Tobacco · *Boeremia exigua* · Leaf spot

Tobacco (*Nicotiana tabacum* L.) is an economically important crop, widely cultivated in various regions worldwide, including Argentina, Brazil, China, India, Indonesia, Malawi, the United States, and Zimbabwe. The tobacco industry provides one of the largest sources of tax revenue for many governments. In some countries, it also promotes employment and industrial development (Wang et al. 2022). China is the world's largest producer of tobacco, and it is grown in more than half of the provinces, with the planting area estimated to be over 1 million ha (Department of Rural Social and Economic Survey 2019). Since 2021, from June to September annually, a new leaf disease of tobacco was observed on cv. Yunyan87, commonly during collections in

Enshi, Xiangyang, Yichang, and Shiyan, all in Hubei Province, China. The disease incidence reached up to 90% in some fields. Leaf spot has become the main disease limiting production in Hubei province. The diseased tobacco leaves showed small, round, or irregularly shaped spots, with faded green and yellow edges. Sometimes, the inner part of the spots appeared fragile and disintegrated. As the disease progressed, the size of the lesions gradually enlarged, with the diameter of the lesions expanding from 1 to 2 centimeters to 3 centimeters or larger. The color deepened, and the edges turned brown or purple-brown. The center of the lesions became necrotic and some fell out, resulting in leaf damage (Fig. 1A).

To isolate the pathogen responsible for causing the disease, leaves with early symptoms were collected in summer 2021. One hundred leaf pieces (2 mm×2 mm) were cut from the border of leaf spots, and disinfected in 75% ethanol solution for 10 s and then 1% sodium hypochlorite for 30 s. After surface disinfection, the leaf pieces were rinsed five times in sterile water. The surface-sterilized leaf pieces were placed onto potato dextrose agar (PDA) and incubated at 28 °C. After five days, the hyphae on the edge of colonies were transferred to fresh PDA. *Boeremia* (in the *Phoma* species complex) were consistently recovered, and three strains, BZ2, HP4, and MJW1, isolated from Yichang and Enshi cities, were selected for further analysis. These strains were placed on PDA, malt extract agar

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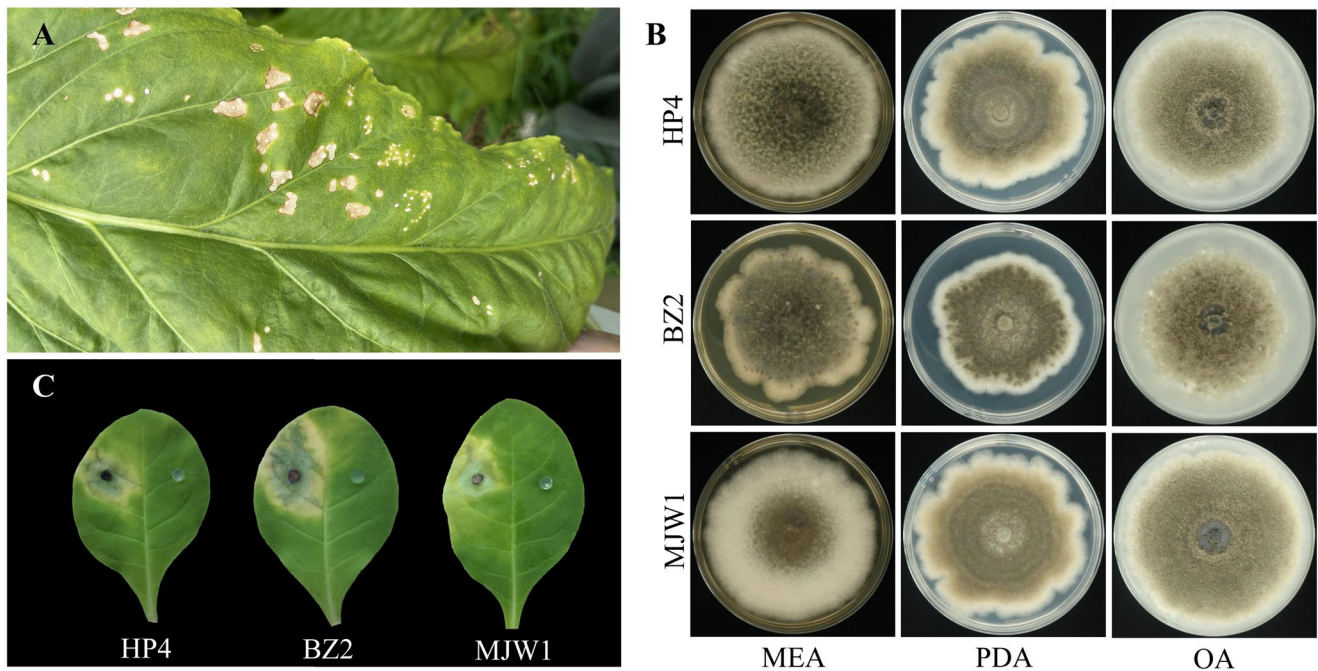


Fig. 1 Symptoms of tobacco leaf spot disease in Hubei Province, China. **(A)** Symptoms of tobacco leaf spot disease in fields, Xuan'en county, Hubei Province. **(B)** Colonies of the strains HP4, BZ2 and MJW1 on PDA (potato dextrose agar), MEA (malt extract agar) and OA (oat agar). These strains were cultured in 12 h light / 12 h dark at 28 °C for 7 days. **(C)** Tobacco leaf symptoms in the pathogenicity

(MEA), and oat agar (OA), and maintained in a 12 h/12 h light/dark photoperiod regime at 28 °C for 7 days (Fig. 1B). When grown on PDA, the colonies of *Boeremia* strains typically had irregular edges, with colony diameters reaching 8 cm. The center of the colonies were dark green, while the edges were covered with a grayish-white mycelium. On MEA, the mycelium initially appeared white, and then changed to dark green to gray-brown in the later stages, with colony diameters reaching 8.5 cm. On OA, the mycelium was initially grayish-white and dense, and later, the centers turned dark brown, with colony diameters reaching 9 cm. The DNA of each strain was extracted from 7-day-old PDA cultures using the CTAB method (Taylor et al. 1993). Sequencing was performed using the primer sets ITS1/ITS4, LR0R/LR7, and BT2Fw/BT4Rd, respectively for ITS, LSU and TUB loci (Aveskamp et al. 2010). Successful PCR reactions resulted in single bands observed on 1% agarose gels for each locus for the three isolates. The PCR products were sequenced by Beijing Tsingke Biotech Co., Ltd. The sequences for all three strains were deposited in GenBank: ITS (PP917553-PP917555), LSU (PP922345-PP922347) and TUB (PP932497-PP932499). The sequence similarity percentages of these isolates to *B. exigua* was up to 100% with between 0 and 15 base differences for each comparison. The sequences of the three loci for each isolate were concatenated and subjected to multiple sequence

tests. The 25-day-old tobacco leaves were inoculated with the strains HP4, BZ2 and MJW1. Fungal plugs were obtained from the edges of 5-day-old cultures of all three isolates with 5-mm-diameter cork borers. These were placed on the left half of each leaf, while a PDA plug was used as control and placed on the right half of that leaf. The leaves were maintained in a light/dark photoperiod regime at 28 °C for 4 days

alignment. A phylogenetic tree was constructed using Neighbor-Joining (NJ) analysis in MEGA (version 11.0). Along with four outgroup taxa, *Heterophoma novae verbas-cicola* (CBS 127.93), *Heterophoma sylvatica* (CBS 874.97), *Phoma herbarum* (CBS 127589 and CBS 502.91), and 1000 bootstrap replicates were used in the tree construction. After analysis, it was found that these three strains were closely related to *Boeremia exigua* species (Fig. 2). Based on the morphological characteristics and molecular identification, the three strains BZ2, HP4 and MJW1 were identified as *B. exigua*.

In pathogenicity tests, ten 25-day-old tobacco leaves (cv. Yunyan87) were inoculated with fungal plugs for each strain. Fungal plugs were obtained from the edges of 5-day-old cultures of all three isolates with 5-mm-diameter cork borers at the center of the left half of the leaf, while an agar plug of PDA medium was placed on the right half. The leaves were maintained in a light/dark photoperiod regime and covered with saran wrap at 28 °C for 4 days. All leaves began to show water-stained spots, which were initially faded green and then yellow (Fig. 1C). For mock inoculations with clean agar plugs, no symptoms appeared at inoculated sites. From three symptomatic spots, fungi were re-isolated and their morphology was identical to the inoculated strain, and hence Koch's postulates were confirmed. In previous studies, *B. exigua* has been reported to infect

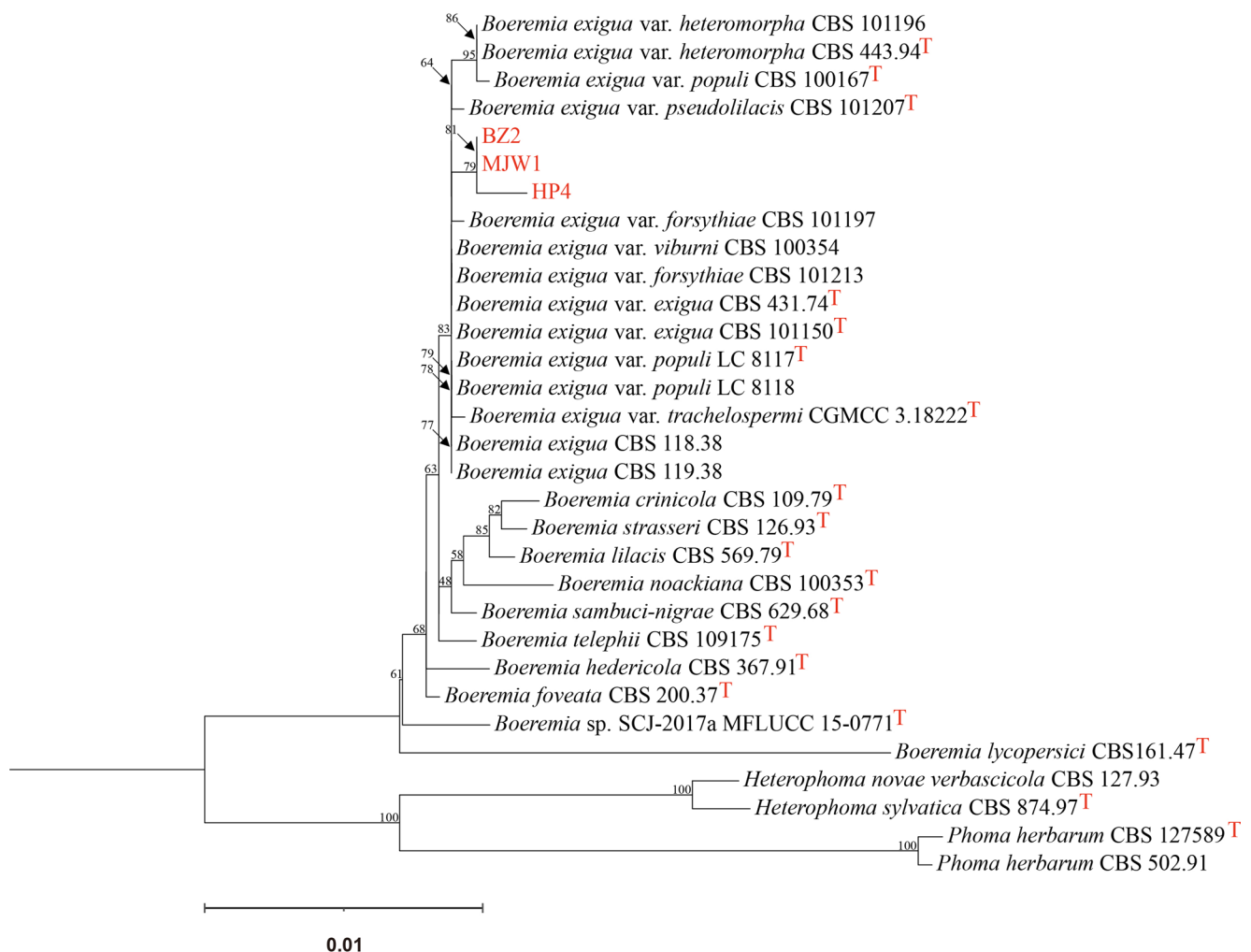


Fig. 2 Analysis of the concatenated ITS-LSU-tub2 sequences of *Boeremia* strains HP4, BZ2, MJW1. Ex-type and reference strains are indicated with ^T. The phylogenetic tree was constructed using the Neighbors-joining algorithm in MEGA 11.0, with 1000 bootstrap replicates.

pyrethrum (*Tanacetum cinerariifolium*), okra (*Abelmoschus esculentus*) and soybean (*Glycine max*) (Jones et al. 2011; Zhao et al. 2016; Schaffrath et al. 2020). On tobacco, there is no record of any species of *Boeremia* causing tobacco leaf disease. To our knowledge, this is the first report of tobacco leaf spot caused by *B. exigua* anywhere in the world.

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Data availability The sequences were deposited in the GenBank database (ITS: PP917553-PP917555; LSU: PP922345-PP922347; TUB: PP932497-PP932499).

The outgroup is *Heterophoma novae verbasicola* (CBS 127.93), *Heterophoma sylvatica* (CBS 874.97), *Phoma herbarum* (CBS 127589 and CBS 502.91). Isolates obtained in this study are in red. The scale bar indicates expected changes per site per branch

Declarations

Ethics approval Not applicable.

Conflict of interest The authors declare that there is no conflict of interest.

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