Morpho-cultural, Pathogenicity and Molecular Characterization of *Phyllosticta capitalensis* Inciting Cavendish Banana Freckle Disease in Hainan

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Abstract [**Objectives**] The study was to identify the casual agent of freckle disease on Cavendish banana in Hainan Province, China. [**Methods**] Fungal isolates were isolated from affected leaf tissues and identified by the morphological features, molecular identification and pathogenicity test. [**Results**] The fungus isolated from affected leaf tissues was identified as *Phyllosticta capitalensis* based on the morphological properties of the colony and spore, coupled with sequence analyses of the internal transcribed spacer (ITS) region and the large subunit (LSU) rDNA gene. Koch's postulates were fulfilled by successfully re-isolating the pathogen from the artificial inoculated leaves. [**Conclusions**] *P. capitalensis* is a new pathogen responsible for Cavendish banana freckle disease in Hainan.

Key words *Phyllosticta capitalensis*; Cavendish banana; Freckle disease

1 Introduction

Banana (*Musa* sp.) is an economically significant crop in China, with a notable socioeconomic impact. The monitoring data of the National Banana Industry Technology System indicates that the annual production of banana approximates to around 1.21×10^7 t in 2021. The output value was more than 300 billion yuan, and the banana industry employs more than 2 million practitioners. Freckle disease, caused by *Phyllosticta* spp., represents a significant challenge for the banana industry, resulting in substantial economic losses[1-3]. During 2003 - 2023, symptoms consisting of scattered dark-black spots and long ellipse-shaped lesions with a yellow halo when being serious were observed on Cavendish banana plants (*Musa acuminate* AAA Cavendish, cv. Formosana) in Hainan, China. In the period between April and May 2023, the Tropical Fruit Tree Diseases Research Group of the Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences, reported a diseased leaf rate of over 80% across the field, with a moderate to severe level of severity. This study was carried out to identify the pathogen responsible for freckle disease on the Cavendish banana by morphological features, molecular identification and pathogenicity test.

2 Materials and methods

2.1 Pathogen isolation and morphological observation To obtain the pathogen, 25 tissue pieces (9 mm^2) each of symptomatic leaves were surface disinfected in 0.8% NaClO (1 min) and 70% ethanol (20 s), and rinsed in sterile water three times. The samples were then transferred to potato dextrose agar (PDA) amended with streptomycin sulfate (0.2 g/L). The plates were incubated at 28 ℃ in the dark for 5 d. Pure cultures were obtained by transferring the hyphal tips of the fungal colony to fresh PDA plates for 14 d, after which the colony and spore morphology were observed.

2.2 Molecular characterization and phylogenetic analysis Representative isolates, which exhibited morphological characteristics consistent with those described for the genus *Phyllosticta*, were grown on PDA at 28 ℃ for 14 d. Genomic DNA was extracted in accordance with the methodology described by Qi *et al*[4]. The internal transcribed spacer (ITS) region and the large subunit (LSU) rDNA gene were amplified using primers ITS1/ITS4 and LROR/LR5, respectively[5-6]. The PCR amplification was carried out in a 25 μL reaction mixture containing 0.5 μL of DNA sample, 12.5 μL of Premix *Taq*™ (TaKaRa Biotech, Dalian, China), 0.5 μL of each primer (20 μM), and 11 μL of nuclease-free sterile distilled water. The PCR conditions were as follows: pre-denaturating at 94 ℃ for 3 min; denaturating at 94 ℃ for 1 min, annealing for 1 min (55 ℃ for ITS, 53 ℃ for LSU), extension at 72 ℃ for 1 min, 35 cycles; final extension at 72 ℃ for 8 min. Amplified PCR products were gel-purified and sequenced directly by Huada Genomic Center (Shenzhen, China). The resulting sequences were submitted to GenBank and employed as queries in a Blastn search. The sequences of orthologs with high similarities to the queries were retrieved and aligned using Clustal W[7] and subsequently adjusted manually. Phylogenetic analyses were conducted using the maximum-likelihood method with MEGA 7.0 software[8].

2.3 Pathogenicity tests To validate the Koch's postulates, a pathogenicity test was conducted on potted healthy banana plants at the 7-leaf stage by artificial inoculation. Two leaves were selected from each potted plant and stabbed with a sterilized needle. Then, 10 μL of conidial suspensions (1×10^6 conidia/mL) was

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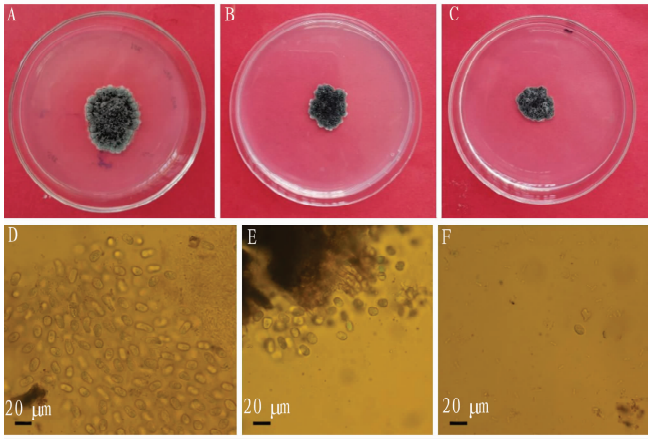
inoculated on the surface of wounded leaves, and sterile distilled water was inoculated to an equal number of leaves serving as a control. Inoculated plants were maintained within a plastic bag for 72 h, after which they were transformed to a room temperature environment with normal light conditions. The experiments were repeated twice. The fungi were re-isolated from the lesions on the leaves after inoculation in order to fulfill Koch's postulates.

3 Results and analysis

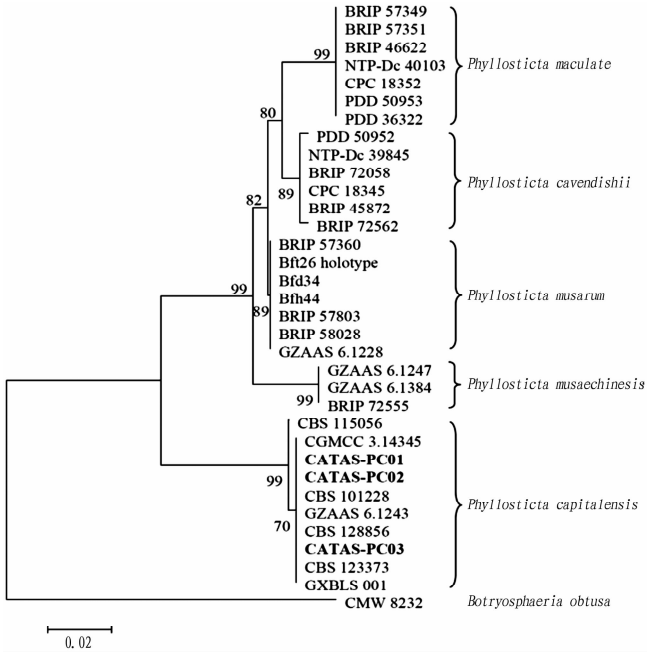
3.1 Fungal isolation and morphological observation Fifteen isolates obtained from the lesions of the infected leaves exhibited identical morphologies. Three representative isolates, designated CATAS-PC01, CATAS-PC02, and CATAS-PC03, were selected for further analyses. The colonies that formed on the PDA plates exhibited stromatic, coralloid, undulating, and extending characteristics. They were grey olive, black gray or black in color and exhibited very slow growth, lacking aerial mycelium (Fig. 1A-1C). Solitary, hyaline aseptate conidia (Fig. 1D-1F) were oblong or ellipsoid in shape, with dimensions of (7.95 – 20.87) μm × (4.92 – 10.18) μm. They were surrounded by a mucilaginous sheath 1 – 3 μm thick and had a straight to curved apical mucilaginous appendage with a length of 1.52 – 11.96 μm (Fig. 1D-1F). The dumbbell-shaped spermatia (Fig. 1D-1F) were hyaline, aseptate, biguttulate with dimensions of (4.97 – 8.18) μm × (1.12 – 2.01) μm. The morphological characteristics of the isolates matched the description of *Phyllosticta* spp.[2].



NOTE A-C. The colony on PDA at 28 ℃ after inoculation for 14 d; D-E. Conidia and spermatia.

Fig. 1 Morphological characteristics of the fungal isolate

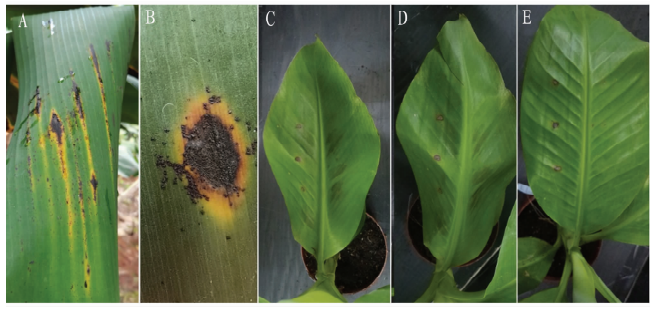
3.2 Molecular identification and phylogenetic analysis The partial regions of ITS region and LSU were generated from three representative isolates, CATAS-PC01, CATAS-PC02, and CATAS-PC03. These sequences were deposited in GenBank (ITS: MW412576, MW412577 and MW412578, LSU: OM301677, OM301678 and OM301679), and were found to be 100% identical to those of *P. capitalensis* ex-type strain CBS 128856 (OL957169 and NG 069995), respectively. A maximum likelihood phylogenetic tree was constructed based on the concatenated sequences ITS and LSU regions. Cluster analysis revealed that the representative isolates were placed within a clade comprising *P. capitalensis* (Fig. 2).



NOTE Isolates CATAS-PC01, CATAS-PC02, and CATAS-PC03 were obtained in the present study, and other *Phyllosticta* spp. were ex-type strains. *Botryosphaeria obtusa* (CMW 8232) was selected as an outgroup in this study. The bootstrap values (1 000 replicates) greater than 60% are shown at the nodes. The scale bar represents 0.02 nucleotide substitutions per site.

Fig. 2 Phylogenetic analysis of the concatenated sequences ITS region and LSU sequences alignment of *Phyllosticta* species based on maximum likelihood method

3.3 Pathogenicity test A pathogenicity test was performed on banana leaves through artificial inoculation. No necrotic lesions were observed on the leaves of control leaves during the observation period, whereas lesions were present on the leaves inoculated with 100% conidia after 14 d post-inoculation (Fig. 3). The fungus was recovered from inoculated leaves, and its taxonomy was confirmed morphologically and molecularly, thus fulfilling Koch's postulates.



NOTE A-B. Symptoms of leaf spot on banana leaves in the field; C-E. Symptoms on leaves artificially inoculated after 14 d with the *P. capitalensis* isolate CATAS-PC01, CATAS-PC02, and CATAS-PC03 (left). The negative controls inoculated with sterile distilled water were indicated as CK (right).

Fig. 3 Pathogenicity tests on banana leaves

4 Discussion

The genus *Phyllosticta* is widely distributed globally and comprises a diverse group of pathogenic and endophytic fungi associated with a broad range of plant hosts[9-10]. It is possible for endophytes to be transformed into pathogens, as evidenced by the case of *P. capitalensis*, which has been reported to cause a disease on the leaves and pseudobulbs of *Bifrenaria harrisoniae* in Brazil[11], on the leaves of *Musa* spp. in Guangxi of China[12], *Rubus chingii* in Guizhou of China[13] and *Ligustrum japonicum* in Iran[14], and on the fruits of *Diospyros kaki* in Taiwan of China[15] and *Psidium guajava* in Mexico[16]. *P. musarum* and *P. cavendishii* have been reported to be associated with banana freckle disease in Hainan, China[17-18]. To date, no reports have been available for *P. capitalensis* isolates associated with banana in Hainan, China. The prevalence of banana freckle disease was found to be above 80% at the field level. The pathogen *P. capitalensis* was mainly observed in samples of the disease collected from the banana orchards in Hainan, with a detection rate of 100%. The pathogen *P. capitalensis* may pose a significant threat to banana cultivation in the future. The identification of *P. capitalensis* as the causal agent of the observed freckle disease on Cavendish banana is crucial for the prevention and control of this disease in the future.

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