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Research Article

Leaf disease caused by *Epicoccum thailandicum* on *Amomum villosum* in China

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Abstract

Amomum villosum is widely planted as a cash crop and medicinal plant. Recent reports of leaf diseases that result in yield reduction of *Amomum villosum* fruits raise concerns, which may play an important role in yield losses. Understanding the aetiology of current and new diseases of *Amomum villosum* is important to address production issues and to formulate effective disease control measures. An organizational separation method was used to isolate phytopathogen from leaves. Ten fungi were isolated from the diseased leaves. Among the selected microorganisms, fungi AP5 showed typical pathogenic symptoms and fulfilling Koch's requirements. The rDNA internal transcribed spacer region (ITS) with primers ITS1/ITS4, 28S ribosomal RNA gene (LSU) with primers NL1/NL4, RNA polymerase II second largest subunit (RPB II) with primers fRPB2-5f/fRPB2-7cR, and beta-tubulin (tub2) with primers BT2a/BT2b of pathogenic strain were amplified and sequenced. AP5 was identified as *Epicoccum thailandicum* based on morphological and phylogenetic analyses. This is the first report that *Epicoccum thailandicum* is the pathogenic fungi infecting *Amomum villosum* leaves.

Keywords: *Amomum villosum*; *Epicoccum thailandicum*; leaf disease; pathogenic fungi; phylogenetic analyses

Introduction

The *Amomum villosum* plant is a *Zingiberaceae* family member whose fruits are valuable medicinally. With the continuous expansion of *Amomum villosum* planting scale and the increase of planting years, various degrees of leaf diseases appearing in various regions, if failed to timely control, may lead to leaf and plant wilt, which cause considerable yield losses in many *Amomum villosum* growing areas. Leaf lesions usually start from the tip or margin. The lesions initially appear as light yellowish brown, then turn to grayish white, and finally become grayish brown. The plaques may expand and heal with each other, leading to leaf drying and death. When wet, there are many small black spots on the plaque, which is the conidia disc of the pathogen.

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Anthracnose is one of the most common and destructive diseases of *Amomum villosum*. The pathogens causing anthracnose on *Amomum villosum* reported in the existing literature, include *Colletotrichum gloeosporioides (Penz & Sacc.)* (Lu, 2001) and *Glomerella cingulata* (Callan and Carris, 2004). *Colletotrichum gloeosporioides* is the asexual form of *Glomerella cingulata*.

Diseased leaves with anthracnose symptoms have been found in a wide variety of *Amomum villosum* in Guangzhou, Guangdong Province (23.15°N, 113.27°E) on April, 2021. More than 50% of plants in the cultivation area were infected. Most anthracnose spots appear at the tip of the leaf, initially as small yellow spots, which later become slightly concave, dark brown patches with concentric wheel patterns. Symptoms were the same as anthracnose's symptoms reported from other hosts and locations, such as maize (*Zea mays* L.) in the United States (Belisário, 2022), chili (*Capsicum annuum* L.) in Malaysia (Ridzuan, 2018), and *Amomum villosum* L. in Guizhou, China (Sang, 2006).

Materials and Methods

Isolation of phytopathogen

Typical diseased leaves were collected from the planting base of *Amomum villosum*. In order to identify pathogenic fungi, observe the morphology of pathogenic fungi and culture plant pathogenicity inoculum, an organizational separation method (Azuddin *et al*., 2021) was used to isolate the fungi from leaves. As for one of the most basic operating techniques in plant pathology experiments, organizational separation method is to isolate pathogenic fungi from infected plant tissues of host plants, and purify the isolated pathogens by artificial culture. The samples taken from the junction of sick and healthy tissue were surface sterilized in 75% ethanol and 3.5% NaClO for 10 s, rinsed 3 times in sterile water, and air drying.

The lesion tissues were transferred to the ordinary Potato Dextrose Agar (PDA) medium, incubated at 25°C in constant temperature and dark. After the mycelium grew about 3-5 days, 3 x 3 mm fungus block at the edge of colony were cut to inoculate on a new PDA medium for separation and purification. The obtained isolates were successively passed on PDA until pure cultures were obtained.

The morphological characteristics of colony, including growth status, colony color and texture were recorded.

Pathogenicity test

Pathogenicity tests (Koch, 1878) were carried out in 3 repetitions on the isolates. The above purified strains were inoculated on new PDA medium plates for activation 3-5 days separately. The leaves of healthy plants were wiped with distilled water and 75% ethanol successively. The fungus blocks, cut from the edge of the colony, was toward the leaves surface, avoiding the main leaf vein. At the same time, the PDA medium blocks with the same size were inoculated to the leaves of another healthy plant as a control. The disease was induced at room temperature with a daylight/dark cycle of 12/12 h. The symptoms of laboratory host disease were observed to verify whether they were consistent with those of field disease.

Microscopic observation of pathogen spores

The pathogenic fungus verified by Koch's postulate was inoculated in the center of the PDA medium and cultured in the dark at 25 °C. The sterilized glass slide was stick to the medium about 2 cm away from the inoculum. After fungal sporulation, the slide was removed and placed under sterile coverslip. The conidia and pycnidium morphology were observed and measured under the biological microscope (Olympus BX53, Japan).

Molecular biological tests

The TSINGKE Plant DNA Extraction Kit was used to extract the genome DNA of pathogenic strain. The rDNA internal transcribed spacer region (ITS) with primers ITS1/ITS4 (White *et al*., 1990), 28S ribosomal RNA gene (LSU) with primers NL1/NL4 (Boekhout *et al*., 1995), RNA polymerase II second largest subunit (RPB II) with primers fRPB2-5f/fRPB2-7cR (Liu *et al*., 1999), and beta-tubulin (tub2) with primers BT2a/BT2b (Glass and Donaldson, 1995) of pathogenic fungus were amplified via the PCR method respectively. 2 μL extracted DNA, 1 μL primer and 1 μL DNA polymerase were added in a centrifugal tube and put into the PCR machine. Steps of primer fragment amplification: pre-denaturation 98 °C 2 min; denaturing 98 °C 10 s; 56 °C 10 s, 72 °C 10 s, 35 cycles; extending 72 °C 5 min. The 2 μL amplified products were subjected to 1% agarose gel electrophoresis. The fragments were obtained based on the DL5000 marker and the sequences of the PCR products were tested. Maximum Likelihood (ML) analyses and Bayesian Inferences (BI) analyses based on concatenated ITS, LSU, RPB II and tub2 sequences were conducted with RAxML v. 7.2.6 and MrBayes v. 3.2.1, respectively.

Results and Discussion

Symptoms caused by phytopathogen

Ten fungi (AP1~AP10) were isolated from disease leaves of *Amomum villosum*. After inoculating the isolates 20 days, the leaves inoculated with the fungus AP5 showed typical symptoms (Figure 1), while the control leaves did not (Figure 1b). The strain producing typical symptoms was identified as pathogenic strain and this was verified using Koch's postulate.

Morphology of fungus and spores

AP5 has white hairy aerial hyphae which eventually turned to gray and orange-red pigmentation was visible when cultured on PDA medium (Figure 1c,1d). Conidia 6.74–8.52 × 4.48–4.56 μm ($\bar{x}$ = 7.30 × 4.52 μm, n = 20), oblong and smooth, while the pycnidium were dark, spherical (Figure 1e). According to the description by Km, the colony characteristics was consistent, while the conidia size was slightly smaller than Km's report (Km *et al*., 2017). Incidentally, *Epicoccum* species frequently mistaken as *Ustilaginales* and *Phoma* (Kirk *et al*., 2008; Hou *et al*., 2020).

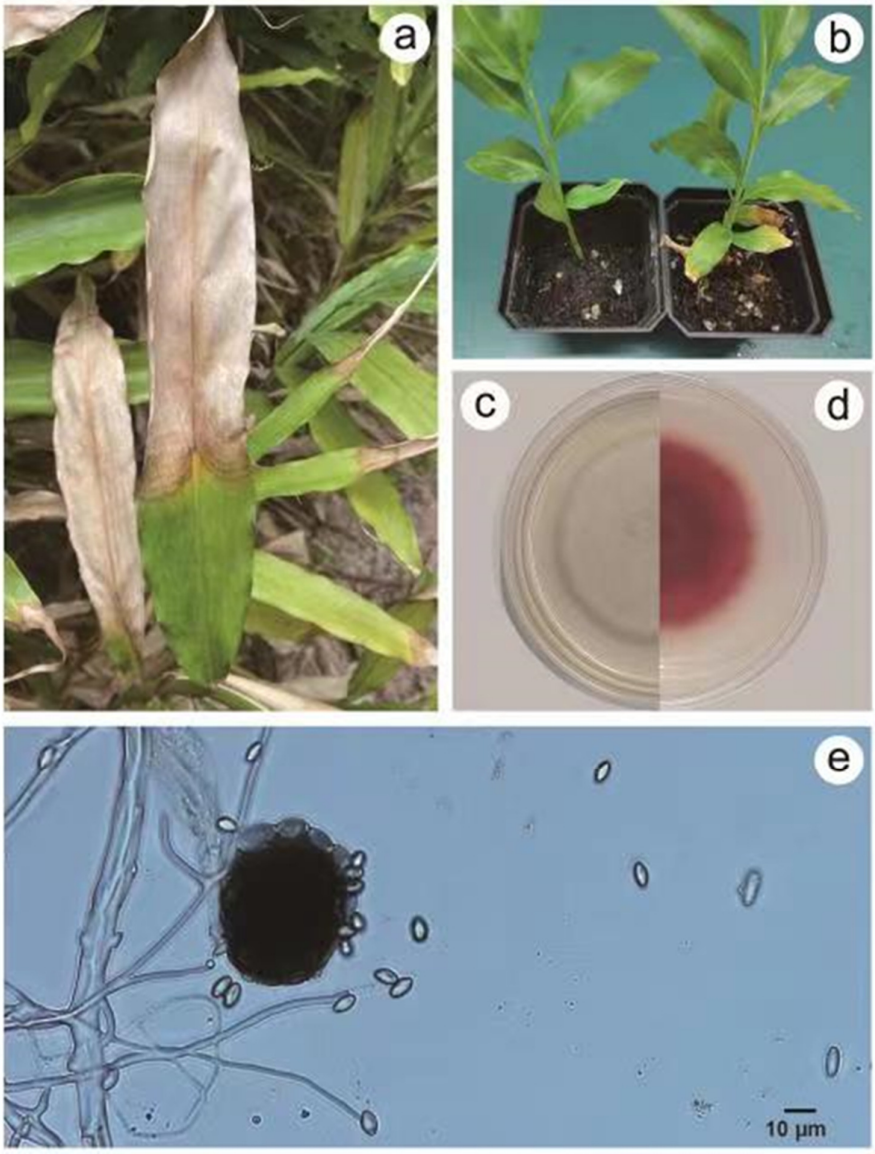

Figure 1. Pictures of infected plants, conidia of pathogenic fungi, *et al.* (a)Disease leaves of anthracnose; (b)Symptomatic leaves after inoculation of PDA medium served as control (left) and AP5 (right) for 20 days; (c) (d)Top and bottom colony morphology of *Epicoccum thailandicum* grown on PDA medium after 5 days of incubation at 25 °C; (e) Curved appearance of *E. thailandicum* conidia and pycnidium at ×400 magnification

Molecular test

Obtained sequences of AP5 were deposited into GenBank under accession numbers NR152926.1 (ITS), NG069435.1 (LSU), OM751594.1 (RPB II) and MH824403.1 (tub2). Maximum likelihood bootstrap analyses were carried out with 1,000 replicates. A phylogenetic tree showed that AP5 comprised a clade with *Epicoccum thailandicum* (MFLUCC 16-0892) (ML/BI = 97/0.99.Based on ML analysis, the bootstrap support value of this branch is 97, and based on BI analysis, the bootstrap support value is 0.99) (Figure 2).

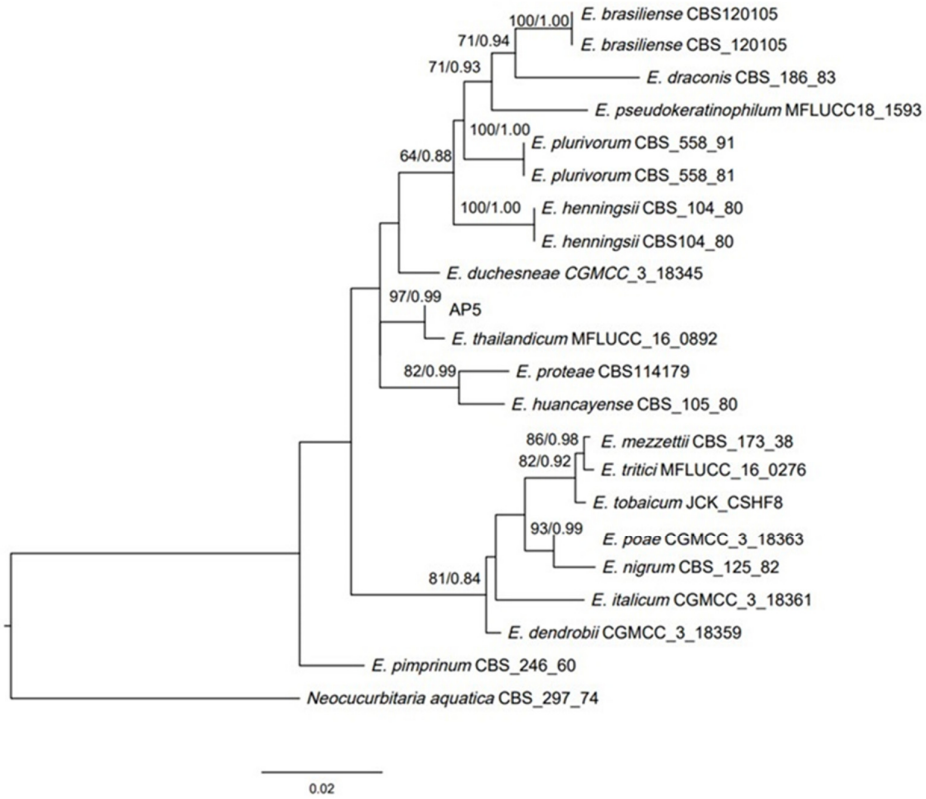

Figure 2. A phylogenetic tree inferred from combined sequences of internal transcribed spacer (ITS), partial 28S large subunit rDNA (LSU), RNA polymerase II second largest subunit (RPB II) and beta-tubulin (tub2) of isolate AP5 from *Amomum* leaves

Based on morphological and multigene molecular data, AP5 was identified as *Epicoccum thailandicum*. *Epicoccum* sp. was named by Link in 1815, anamorphic *Pleosporaceae* (Kirk *et al*., 2008). In existing reports, fungi of *Epicoccum* sp. are capable of acting as pathogen for plant diseases of tea [*Camellia sinensis* (L.) Kuntze] leaves (Yin *et al*., 2021), *Weigela florida* (Bunge) A. DC. (Tian *et al*., 2021), and flowering cherry (*Prunus* × *yedoensis* Matsumura; Somei-yoshino cherry) (Han *et al*., 2021).

Conclusions

To our knowledge, this is the first report of *Epicoccum thailandicum* identified as the cause of leaf disease on *Amomum villosum* in China. There are also reports of leaf diseases caused by *Pyricularia costina* on *Amomum villosum* (cardamom) in China with similar symptoms except concentric wheel patterns (Li *et al*., 2010). Further studies should be conducted on this pathogen to determine its pathogenic patterns, and disease prevention measures must be taken to reduce losses.

Authors' Contributions

SW, ZL: experimentation, investigation, processing of results, data analysis, writing, editing; WF, YC: plant collection, processing, editing; YZ: principal investigator, conceptualization, funding resources, proofing, data analysis;

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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