# *Colletotrichum gloeosporioides*, the causal agent of citrus anthracnose in Guizhou Province

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Ten *Colletotrichum* strains were isolated from leaf spots of *Citrus* in six different locations of Guizhou Province. Koch's postulates were performed to prove these strains were the causal agents of the disease. Disease symptoms consist of small and irregular, yellow, brown or dark brown spots on the leaves. The fungal strains were identified as *Colletotrichum gloeosporioides* on the basis of morphology and ITS sequence data analyses.

**Key words** – Coelomycetes – ITS – plant pathogen

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## Introduction

In Guizhou Province, the total orange (*Citrus reticulata* Blanco) acreage is up to 57, 000 $hm^2$, and the total yield is 150, 000 tonnes. Orange production is therefore very important for the continuous development of agriculture in Guizhou, however, *Citrus* production is also affected by various fungal diseases. Among them, anthracnose is the most highly destructive disease of orange in this region.

During 2008–2010, we investigated the fungal diseases of orange in Guizhou Province, and ten locations were selected, which were mainly orange growing regions. We discovered obvious orange anthracnose in six sites and isolated ten fungal isolates from diseased orange leaves. We proved these strains were the causal agent of orange anthracnose by performing Koch's postulates, and examined their morphological characteristics and species identification using ITS sequence data analysis. Orange anthracnose in Guizhou Province was found to be caused by *Colletotrichum gloeosporioides* (Penz.) Sacc.

## Material and Methods

### Isolates and morphology

Ten strains of *Colletotrichum* were isolated from lesions of orange anthracnose from six locations within Guizhou Province (Shiqian, Huishui, Tianzhu, Jinping, Rongjiang and Tongzi). Five *ca.* 5 × 5 mm pieces of tissue were taken from the margin of diseased tissue, surface sterilized by dipping in 10% sodium hypochlorite for 3–5 minutes and washing in two or three changes of sterile water, and placed on potato dextrose agar (PDA). Plates were incubated at room temperature (28–30°C) and observed periodically. The growing tips of any fungal hyphae developing from the leaf disks were then transferred aseptically to PDA slants. Pure cultures were stored at 4°C on PDA slants. The fungi were identified following sporulation. Size and shape of conidia were recorded from the colonies grown on PDA plates at room temperature (28–30°C). Conidia were taken from actively growing colonies mounted in lactic acid, and examined


**Fig. 1** – Disease symptoms on *Citrus reticulata* caused by *Colletotrichum gloeosporioides*. **A** Symptoms on leaves. **B** Symptoms on fruit.

for size and shape.

**Pathogenicity testing**

To verify the pathogenicity of the fungi isolated, ten orange leaves were surface-sterilized, as outlined above, and then dipped for 10 min in conidial suspensions ($10^7$ conidia $ml^{-1}$). The conidia were collected from 10-days-old cultures on PDA medium and suspended in sterile distilled water. Inoculated leaves were kept in a humid chamber at 25°C for 10 days. Orange leaves dipped in sterile distilled water were used as the control. Three replicates were carried out and the assay was repeated twice.

**DNA extraction**

Each culture was derived from a single conidium from the original isolate and was subsequently maintained in potato dextrose broth. Cultures were incubated without shaking at room temperature for 3–5 days. After rinsing with sterile water, approximately 0.5 g of mycelium for each sample was frozen in liquid nitrogen and ground in a sterile mortar. Nucleic acid was extracted using the adapted CTAB method of Ford et al. (2000). The total genomic DNA concentration was estimated by absorbance at 260 nm.

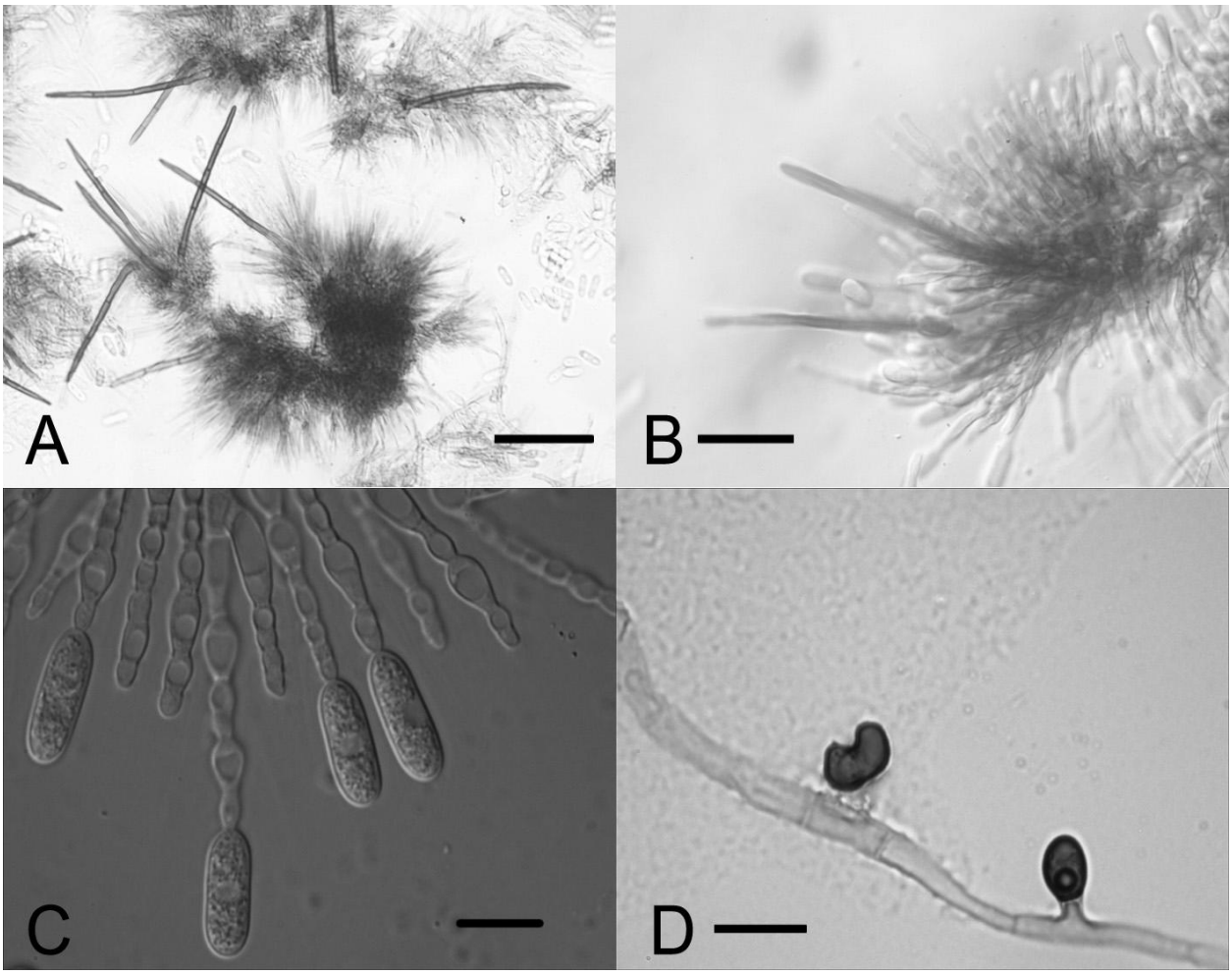


**Fig. 2** – *Colletotrichum gloeosporioides* (HGUP0012). **A** Conidial masses and setose acervuli on PDA, bar = 50 µm. **B** Acervulus with brown setae and falcate conidia on PDA, bar = 25 µm **C** Conidia and conidiophores, bar = 10 µm. **D** Appressoria, bar = 10 µm.

**Polymerase chain reaction**

The ITS gene region was amplified by PCR using the primer combinations ITS1 and ITS4 (White et al. 1990). Reaction mixtures contained 5 µL of 10 × ThermoPol reaction buffer [200 mM Tris-HCl, pH 8.3, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$ and 1 % Triton X-100], 5 µL of 10 mM $MgSO_4$, 20 mg template genomic DNA, 4 pM of each primer, 4 µL of 2.5 mM dNTPs, 2.5 U of AmpliTaq polymerase, and total volume was adjusted to 50 µL with deionized water. The PCR amplified DNA fragments were fractionated in 1 % agarose gels in 0.5 × TBE buffer, and DNA was visualized by ethidium bromide staining and UV illumination. Sequencing was performed with an ABI PRISM 3730 DNA autosequencer using either dRhodamine terminator or Big Dye Terminator chemistry (Applied Biosystems Foster 19 City, California). The DNA sequence of ITS region generated in this study was submitted to GenBank (Accession No. JN887341–JN887350).

**Results and Discussions**

**Symptoms**

Anthracnose disease attacks leaves and fruits at any growth stage. The symptoms are most visible on leaves. At first, *C. gloeosporioides* generally appears on leaves as small and irregular yellow, brown or dark brown spots (Fig. 1 A). The spots can expand and merge to cover the whole affected area. The colour of the infected part darkens. Infected fruit has small, water soaked, sunken, circular spots that may increase in size (Fig. 1 B).

**Koch's postulates**

Following mycelial inoculation, *Citrus reticulata* leaves exhibited typical anthracnose spots after 4 days, which were very similar to

those in the field. *C. gloeosporioides* was successfully re-isolated from the artificially inoculated leaves of *Ci. reticulata*, thus establishing proof of pathogenicity. The controls remained healthy and leaves did not yield any other microorganisms.

***Colletotrichum gloeosporioides*** (Penz.) Sacc., Atti Inst. Veneto Sci. lett., ed Arti, Sér. 3 2: 670, 1884. Fig. 2 (A–D)

Colonies on PDA extremely variable, effuse, grey to brown, with pinkish patches; reverse dark brown with vinaceous stains. Black acervuli with orange conidial masses, sometimes felted with aerial mycelium. Setae abundant or not in acervulus, black, 2–4-septate, 80–100 µm long. Conidia borne on elongated phialides in acervular conidiomata. Conidia straight, cylindrical, obtuse at the apex, 8.4–16.8 × 3.5–4.2 (av. 14 × 3.9) µm. Appressoria 7–12 × 5.5–8 (av. 9 × 6.5) µm, clavate or irregular.

Known distribution – cosmopolitan.

Material examined – China, Guizhou province, Tianzhu, on leaf of *Citrus reticulata*, September 2009, X.L. Hou (GT214), Culture (HGUP0011); Tianzhu, on leaf of *Citrus reticulata*, September 2009, X.L. Hou (GT224), Culture (HGUP0012); Huishui, on leaf of *Citrus reticulata*, September 2009, X.L. Hou (GHS2), Culture (HGUP0013); Huishui, on leaf of *Citrus reticulata*, September 2009, X.Y. Zhou (GHS6), Culture (HGUP0014); Shiqian, on leaf of *Citrus reticulata*, September 2009, P. Tan (GSQ2), Culture (HGUP0015); Jinping, on leaf of *Citrus reticulata*, September 2009, X.L. Hou (GJP05), Culture (HGUP0016); Huishui, on leaf of *Citrus reticulata*, September 2009, P. Tan (GHS2), Culture (HGUP0017); Rongjiang, on leaf of *Citrus reticulata*, September 2009, X.Y. Zhou (GRJ03), Culture (HGUP0018); Tongzi, on leaf of *Citrus reticulata*, September 2009, X.L. Hou (Gtg), Culture (HGUP0019); Tianzhu, on leaf of *Citrus reticulata*, September 2009, P. Tan (GT203), Culture (HGUP0020).

The present species is morphologically very close to *C. gloeosporioides* as epitypified by Cannon et al. (2008). Conidia from Guizhou oranges were similar in length to those described by Cannon et al. (2008), although some are narrower. Appressoria (7–12 × 5.5–8 µm) are slightly wider than those of the epitype (7.2–12 × 4.7–6.0 µm). The morphological identification indicated that this pathogen is *C. gloeosporioides*.

Additionally, we amplified a partial sequence of the internal transcribed spacer (ITS) region of the present *Colletotrichum* strains, and then aligned them with the ITS sequences of the epitype of *C. gloeosporioides* (EU371022) in GenBank, which originated from IMI 356878 (Cai et al. 2009, Hyde et al. 2009). The phylogenetic analysis of Cai et al. (2009) based on the ITS sequences from specimens including 42 ex-type strains provided important criteria for our identification. The DNA alignment result (473 characters) supported these isolates as belonging to *C. gloeosporioides* with little sequence divergence (less than 3 positions). Among them, two isolates (HGUP0014 and HGUP0012) have only one base difference with the epitype, isolates (HGUP0011, HGUP0017 and HGUP0015) have two base differences with the epitype, and the other five have three base differences with the epitype. Combining morphology and sequence analysis, we conclude that the citrus anthracnose in Guizhou Province is commonly caused by *C. gloeosporioides*. ITS sequence data can be useful for phylogenetic study in future work.

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