Australasian Plant Disease Notes (2021) 16: 11
https://doi.org/10.1007/s13314-021-00420-4

First report of leaf spot caused by *Colletotrichum siamense* on *Sophora tonkinensis*

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Received: 3 May 2020 / Accepted: 1 February 2021 / Published online: 9 April 2021

Abstract
A leaf spot, found on *Sophora tonkinensis* in Hechi city, Guangxi province, China was identified as *Colletotrichum siamense* based on morphological and molecular phylogenetic analysis of the internal transcribed spacer (ITS) of ribosomal DNA, β-tublin (TUB2), the translation elongation factor 1-alpha (TEF1-α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin (ACT), chitin synthase 1 (CHS-1), calmodulin (CAL) and glutamine synthetase (GS) genes. Koch's postulates were satisfied by successful reisolation of *C. siamense* only from plants inoculated with the pathogen. This is the first report of leaf spot caused by *C. siamense* on *Sophora tonkinensis*.

Keyword *Sophora tonkinensis* · *Colletotrichum siamense* · leaf spot

Sophora tonkinensis (Fabaceae) is known as Shān dòu gēn ("Mountain bean root") and is a well known leguminous medicinal plant in China. It grows in the tropical zone of Guangxi, Guangdong and Yunnan provinces in southern China (Wang et al. 2011). In May 2018, severe leaf spot symptoms were observed on 30–40% of *S. tonkinensis* plants growing in commercial plantations at Hechi city, Guangxi province, China.

On infected leaves there were circular brown spots, 1–2 mm in diameter, reaching 3–5 mm in diameter after 8 days. These leaves gradually changed from green to yellow before defoliating. The infected leaves were surface-sterilised in 75% ethanol for 30 s then in 2% sodium hypochlorite for 3 min, and finally 75% ethanol for 30 s. Then samples were rinsed three times with sterile water, dried on sterile filter paper, cut into small pieces and placed on potato dextrose agar (PDA) in 9 cm-diameter petri dishes which were incubated in an incubator at 28 °C under a 12 h light/12 h dark cycle.

Twenty-seven of the resulting 30 fungal isolates showed similar morphological characteristics. A single-spore isolate, s-8–2, was selected as representative for molecular identification, and deposited as ACCC 39,685 in Agricultural Culture Collection of China. Colonies of ACCC 39,685 grown on PDA for up to 15 days at 28 °C were initially light grey and later becoming darker, with cottony mycelium. Acervuli became evident initially at the centre of 10-day-old colonies, then developed radially outward from that point. Conidia produced in orange acevuloid masses were straight, aseptate, cylindrical, with obtuse apices and were 10-(15)-20 × 3-(4)-5 µm (n = 50). Based on these morphological characteristics, those isolates correspond to the asexual morphs of species belonging to *C. gloeosporioides* complex (Cannon et 1al. 2012; Weir et al. 2012; Hyde et al. 2009; Photita et al. 2005; Wang et al. 2016).

The single-spore isolate ACCC 39,685 was used to make a molecular identification (Fig. 1). For precise identification of the fungus, genomic DNA of ACCC 39,685 was extracted from the mycelium of a 7-day-old colony growing on PDA at 25 °C using a MightyAmp DNA Polymerase Ver.3 (1.25 U/50 µl) (Takara Bio, Kusatsu, Japan R076A) kit following the manufacturer's instructions. The internal transcribed spacer (ITS) of ribosomal DNA (White et al. 1990), β-tubulin (Weir et al. 2012), the translation elongation factor 1-alpha (EF-1α) (Carbone and Kohn 1999), glyceraldehyde -3-phosphate dehydrogenase (Weir et al. 2012), actin (ACT) (Weir et al. 2012), chitin synthase 1 (CHS-1) (Weir et al. 2012), calmodulin (CAL) (Weir et al. 2012) and glutamine synthetase (GS) (Stephenson et al. 1997) genes were

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Fig. 1 Symptoms on leaves 8 days after inoculation with a conidium suspension of isolate ACCC 39,685

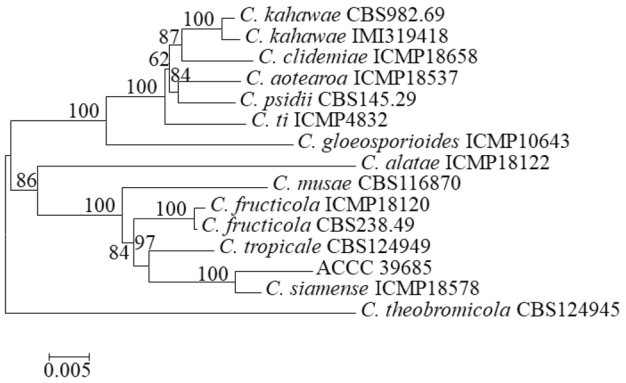

Fig. 3 Maximum Parsinomy phylogenetic tree using combined ITS, β-tubulin, TEF 1-α, glyceraldehyde-3-phosphate dehydrogenase, ACT, CS-1, CAL and GS genes. Bootstrap values > 50% (1,000 replication) are given at the nodes. Colletotrichum theobromicola CBS124945 was used as the outgroup isolate. NOTE: CBS: CBS-KNAW, Fungal Biodiversity Center, Utrecht, the Netherlands. ICMP: International Collection of Microorganisms from Plant, Landcare Research, Auckland, New Zealand

amplified with the eight primer pairs. The ITS, β-tublin, TEF1-α, GADPH, actin, CHS-1, CAL and GS sequences, which were deposited in the GenBank database (accession numbers MK371784, MK976658, MK350298, MK952143, MK976660, MK976659, MT263723, MT263724, respectively). A BLAST search of GenBank showed that ITS and EF-1α were 100% identical to *C. gloeosporioides*, respectively, but, the other six sequences of the strain ACCC 39,685 were 99% identified as *C. siamense*. The multilocus analysis (Mo et al. 2018) carried out with 14 other isolates of *Colletotrichum* revealed that ACCC 39,685 was a 100% match with isolate ICMP 18,578, the type of *C. siamense* (Fig. 2). Based on morphology and molecular results, isolate ACCC 39,685 was identified as *C. siamense*.

Eight leaves on each of two healthy 3-year-old trees of *S. tonkinensis* growing in non-sterile soil in 13.4 L pots were surface-sterilized with 75% ethanol, rinsed in sterile water, then spray-inoculated with 20 µL per leaf of an aqueous conidium suspension (1×10^6 conidia/mL). Eight leaves on a control plant were surface-sterilized with 75% ethanol, rinsed with sterile water afterwards, then sprayed with 20 µL of sterilized water. All plants were covered with a plastic bag for 48 h to maintain high humidity. After 8 days incubation at 28 °C and 90% relative humidity in a greenhouse, the area around the inoculation site had symptoms that were identical to those initially observed, whereas the control leaves on healthy seedlings inoculated with sterile water free remained symptomless. The pathogen *C. siamense* was reisolated from the lesions, fulfilling Koch's postulates (Fig. 3). The same pathogenicity assay was performed twice, with the same results.

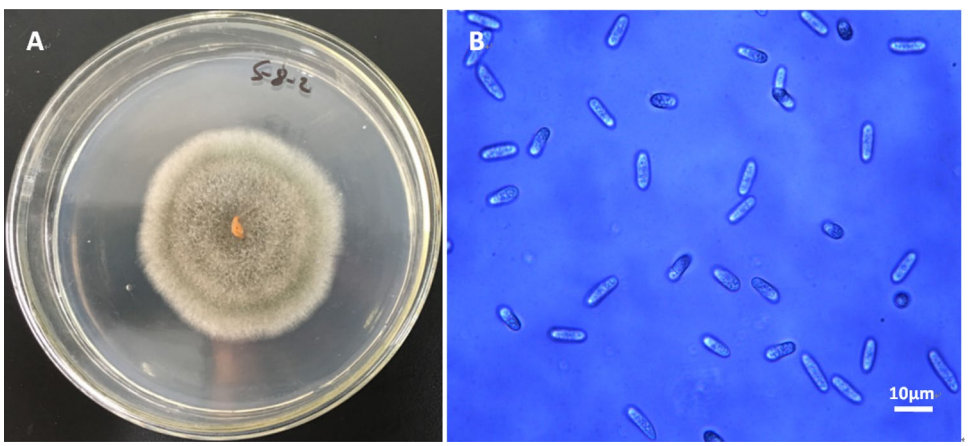

Fig. 2 Morphological characteristics of a *Colletotrichum siamense* colony (isolate ACCC 39,685) (A) and microconidia (B) on potato dextrose agar after incubation for 7 days at 25 °C and a 12 h light/ 12 h dark regime

The pathogen responsible for leaf spot on *S. tonkinensis* in China has been identified in this study as *C. siamense* based on morphological and molecular studies. *Colletotrichum siamense* can infect more than 60 species of plants worldwide (Ji et al. 2019), but there have been no previous reports of the pathogen on *S. tonkinensis*. In China, various diseases caused by *C. siamense* can infect other hosts include *Sterculia nobilis* (Zhang et al. 2020a, b), *Sterculia lanceolata* (Zhang et al. 2020a, b), *Hevea brasiliensis* (rubber tree) (Cao et al. 2019), *Mallotus oblongifolius* (Partridge tea) (Liu et al. 2018), *Macadamia* sp. (Qiu et al. 2020), *Litchi chinensis* (Ling et al. 2019), and *Camellia sinensis* (Wang et al. 2016). Although there have been other reports of *Colletotrichum* species on *S. tonkinensis*, namely *C. gloeosporioides* (Shivas and Alcorn 1996) and *C. simmondsii* (as an endophyte from roots) (Yao et al. 2017), ours is the first known record of *C. siamense* on the plant of *S. tonkinensis*.

Acknowledgements This study was supported by the Special Fund for Innovation-driven Development in Guangxi, P. R. China (AA17204056-4), and the Guangxi Science and Technology Base and Talent Special Project (Gui ke AD16380013), Guangxi science and technology plan project (Gui ke AD17292004), Organic medicine cultivation and evaluation research team, (Gui yao chuang 2019007), Youth Science Fundation of Guangxi Botanical Garden of Medicinal Plants (Gui yao ji 201801).

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