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Colletotrichum siamense causes anthracnose on the fruits of *Pongamia pinnata* in India

Dwarka DJ[1], Sharma G[2, 3,*] and Rajasab AH[1]

[1]*Mycology and Plant Pathology Laboratory, Department of P.G. Studies and Research in Botany, Gulbarga University, Gulbarga-585106, Karnataka, India*
[2]*Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology, Sector 39-A, Chandigarh - 160036, India*
[3]*Department of Plant Pathology and Weed Research, Agriculture Research Organization, The Volcani centre, Bet Dagan - 7528809, Israel*

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Abstract

Pongamia pinnata is an economically important biodiesel yielding plant. The growth and yield of this plant is inhibited by infections caused by fungal pathogens. In this study, we investigated the association and pathogenicity potentials of *Colletotrichum siamense* on fruits of *P. pinnata* collected from Gulbarga University Campus, Karnataka, India. The pathogen was isolated from pericarp (epicarp and endocarp) of *P. pinnata* by blotter and carpoplane method. Fungal characterization and identification was performed based on morphology and multi-locus phylogeny. Pathogenicity testing on *P. pinnata* fruits confirmed the pathogenic potential of the isolate. This is the first report of *C. siamense* as a causative agent of anthracnose on the fruits of *P. pinnata* from Karnataka, India.

Key words – Biofuel – disease – germination pattern – identification – morphology – multigene phylogeny

Introduction

Pongamia pinnata (L.) Pierre is a small evergreen tree species belonging to *Fabaceae*. This legume tree species is widely distributed in Australia, China, India and Pakistan (Kirtikar et al. 1994). The biofuel obtained from *P. pinnata* is used as a biodiesel in industries (De & Bhattacharya 1999, Azam et al. 2005, Karmee & Chadha 2005). This plant has long been used as a source of animal fodder, fuel, manure, fish poison, timber and traditional medicine for a long time. It is believed that the large scale cultivation of *P. pinnata* would fulfill the demands of biofuel in India (Punia et al. 2006).

Colletotrichum is one of the ten most important phytopathogenic fungal genera (Dean et al. 2012) which causes anthracnose in wide range of host plants, mainly in the tropical and subtropical regions (Sutton 1992, Hyde et al. 2009a, b, Cannon et al. 2012, Damm et al. 2012a, b, Weir et al. 2012). Identification of *Colletotrichum* species is difficult due to overlapping morphological characters and low genetic divergence within different species complexes (Cannon et al. 2012, Damm et al. 2012a, b, Sharma et al. 2013a, Hyde et al. 2014, Yan et al. 2015). *Colletotrichum* species can easily adapt to new environmental conditions, thus having the capacity to infect diverse hosts (Sanders & Korsten 2003, Photita et al. 2004, Yan et al. 2015). It is essential to identify the

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*Corresponding Author: Gunjan Sharma – e-mail – gunjansharma128@gmail.com

pathogen accurately to apply effective disease control strategies (Hyde et al. 2009 a, b, Hyde et al. 2014, Yan et al. 2015). Since October 2012 till June 2013, it was observed that the fruits and legumes of *P. pinnata* in almost 50% trees of Gulbarga University Campus (Karnataka, India) displayed anthracnose symptoms. Thus this study was initiated to characterize the pathogenic *Colletotrichum* species responsible for the anthracnose on fruits of *P. pinnata*, based on morphology, multi-locus sequence analysis and pathogenicity data.

Materials & Methods

Fungal isolation and DNA extraction

Infected (mature, symptomatic) *P. pinnata* fruit samples were collected from October 2012 till June 2013, at five locations within the Gulbarga University Campus, Gulbarga, Karnataka, India (17.33°N 76.83°E). Fungal isolation was performed using two methods: moist blotting technique and serial dilution (carpoplane) plate method (Aneja 2008). A total of 20 fungal isolates were obtained, and tentatively identified as *Colletotrichum* sp. based on morphology. The genomic DNA was isolated for a representative strain (MTCC 11766) using DNA isolation kit (Zymo Research, Catalogue number D6005, California, USA) and stored at -20 ºC till further processing.

PCR amplification and sequencing of genes

The representative *Colletotrichum* strain (MTCC 11766) was subjected to PCR amplification and sequencing of actin (*act*), calmodulin (*cal*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), β-tubulin (*tub2*) and internal transcribed spacer region (ITS)/ 5.8S rRNA genes as described in Sharma et al. (2013a). The resulting gene sequences have been submitted to NCBI-GenBank (accession nos. KX068699–KX068704).

Phylogenetic analysis

The forward and reverse sequences obtained for each gene were aligned using Sequencher v.4.10.1 (Gene Codes Corp., Ann Arbor, Michigan, USA) to generate a consensus sequence. A concatenated multi-locus dataset comprising *act*, *cal*, *gapdh*, *tub2* and ITS was generated using Sequence Matrix v.1.7.8 (Vaidya et al. 2011). Maximum parsimony (MP) analysis was performed using PAUP version 4.0b10 (Swofford 2003), as described in (Sharma et al. 2013a).

Morphological characterization

Mycelial discs (4 mm diam.) were taken from actively sporulating areas near the growing edge, transferred to potato dextrose agar (PDA) plates, incubated at 28 °C and investigated after 7 days (Fig. 1a-c, g). The size and shape of 100 conidia harvested from MTCC 11766 were recorded. The colony size, color of the conidial mass and zonation were also recorded. Appressoria were studied using slide culture technique, in which 10 mm^2 piece of PDA was placed in an empty Petri dish. The edge of the agar was inoculated with spores taken from a sporulating culture and a sterile cover slip was placed over the inoculated agar (Johnston & Jones 1997). The shape and size of the appressoria formed across the underside of the cover slip were studied after 3–7 days (Fig. 1d-f).

Growth rate

A mycelial disc from the actively growing edge of 7 days old culture was transferred onto PDA plates. The culture plates were incubated at 28 ºC under constant fluorescent light. Colony diameter was measured daily for 7 days and growth rate was calculated as the average of growth for 7 days (mm/day).

Germination pattern

The conidial mass from the sporulating culture was taken with the help of sterilized needle and released into sterile distilled water. Conidial suspension of concentration 1×10^6 spores/ml was prepared using serial dilution. The conidial suspension was mixed with different concentrations

(1%, 2%, 5% and 10%) of sucrose solution and a drop was placed on a cavity slide. The cavity slide was incubated for 4–6 hours in a Petri plate lined with sterilized moist blotting sheet at room temperature. Simultaneously, a sterilized glass slide was coated with a thin layer of PDA medium and placed in Petri plate lined with sterilized moist blotting sheet having glass rods for support. The suspension was sprayed over PDA and incubated for 6–8 hours at 28 ºC under lab conditions. Ten such Petri plates were set separately and all of them were observed under microscope.

Pathogenicity test

A representative isolate, MTCC 11766 was used for pathogenicity testing. The spores were harvested from 7 day old pure culture by adding 10 ml of sterilized distilled water onto the Petri plate, followed by gentle swirling. Spore concentration was adjusted to 1×10^6 conidia/ml using haemocytometer. Healthy *P. pinnata* fruits were taken from the University campus, disinfected with 1% sodium hypochlorite for five minutes and washed three times with distilled water. Five fruits were blotted dry and inoculated using 6 µl of conidia suspension (10^6 conidia/ml) via wound/drop and non-wound/drop inoculation method (Cai et al. 2009) respectively. Five control fruits were inoculated with 6 µl of sterilized distilled water. The inoculated fruits were incubated at room temperature in a moist chamber. The appearances of infection were observed from 4–6 days on incubation (Fig. 1h-i). The pathogenicity assay was repeated thrice.

Results

Morphological and cultural characterization

The isolate MTCC 11766 initially produced white colony which later turned grayish (Fig. 1a), and the reverse was pale yellowish to orange (Fig. 1b). Aerial mycelia were grayish white and cottony. The growth rate was recorded as 5.0–12.5 mm/day (mean = 8 mm/day; n=30). Setae were absent. The conidia were cylindrical with smooth wall, obtuse to slightly rounded ends, 9.0–16.0 µm (mean 13.3±1.6 µm; n=100) in length and 4–7.0 µm (mean 5.7±0.7 µm; n=100) in width, L/W ratio = 2.3 (Fig. 1c). Sclerotia were present. Appressoria were irregular in shape, brown and ovoid in structure, 5.0–8.0 µm (mean 6.3±0.5 µm; n=50) in length and 3–5.0 µm (mean 3.7±0.3 µm; n=50) in width; observed in slide cultures, in all concentration (1%, 2%, 5% and 10%) of sucrose solutions, and mostly formed from mycelia. The sexual morph was not observed in culture. The morphological characteristics of MTCC 11766 are comparable to the type strain of *C. siamense* MFLU 090230 (Prihastuti et al. 2009).

Phylogenetic analysis

The multi-locus dataset included 2367 characters, including gaps. The gene boundaries in the dataset included: ITS: 1–601, *act*: 602–880, *cal*: 881–1628, *gapdh*: 1629–1936 and *tub2*: 1937–2367. The analysis involved 42 taxa. Seventy-one characters from the ambiguously-aligned regions were excluded from the analysis. Out of the remaining 2296 characters, 1735 characters were constant; 272 characters were parsimony-informative and 289 characters were parsimony-uninformative. The MP analysis resulted in 348 trees and based on the KH test, and these trees were not significantly different (details not shown). One of the 348 trees (TL = 875, CI = 0.747, RI = 0.861, RC = 0.644, HI = 0.253) generated from the MP analysis is shown in Fig. 2. The tree is rooted with *C. xanthorrhoeae* ICMP 17903. The bootstrap support values higher than 50% for the observed branches are shown. In the MP tree shown in Fig. 2, the isolate MTCC 11766 clustered with the ex-type strains of *C. siamense sensu stricto* with 60% bootstrap support.

Germination pattern

Conidia of MTCC 11766 were readily germinated within 12 hours of incubation at 28 ºC under lab conditions. During first 6 hours of incubation, the conidial germination was absent. After 6–10 hours incubation, 6–8% of conidia germinated with budding on both ends. The germination was observed in all the concentrations of sucrose. Conidia bud-off and produced one or two germ

tube to form mycelia and appressoria. Appressorial formation generally started after 12 hours of incubation. The appressoria were also formed from the mycelia (Fig. 1d–f). Some conidia produced a single germ tube at terminal or sub-terminal portion and later branched further. A few conidia produced germ tubes with horizontal or lateral branches. Generally, at 25–30º C, the percentage of conidial germination in *Colletotrichum* is highest and almost reaches 100% within 6 hours (Estrada et al. 2000).However, in our study, during first 6 hours of incubation the germination was absent. The germination started only after 6 hours of incubation and reached 6–10% after 12 hours.

Pathogenicity test

The *Pongamia* fruits inoculated with conidial suspension of strain MTCC 11766 developed typical anthracnose lesions (Fig. 1h), while the control fruits did not develop any symptoms (Fig. 1i). The inoculated pathogen was isolated again from the infected fruits on to the PDA medium to confirm Koch's postulates. With the wound/drop inoculation method, lesions appeared after 3 days of inoculation (Fig. 1h), while non/wound drop inoculation, lesions appeared after 7 days of inoculation. The identity of the pathogen isolated from artificially inoculated fruits was verified again by sequencing and morphology.

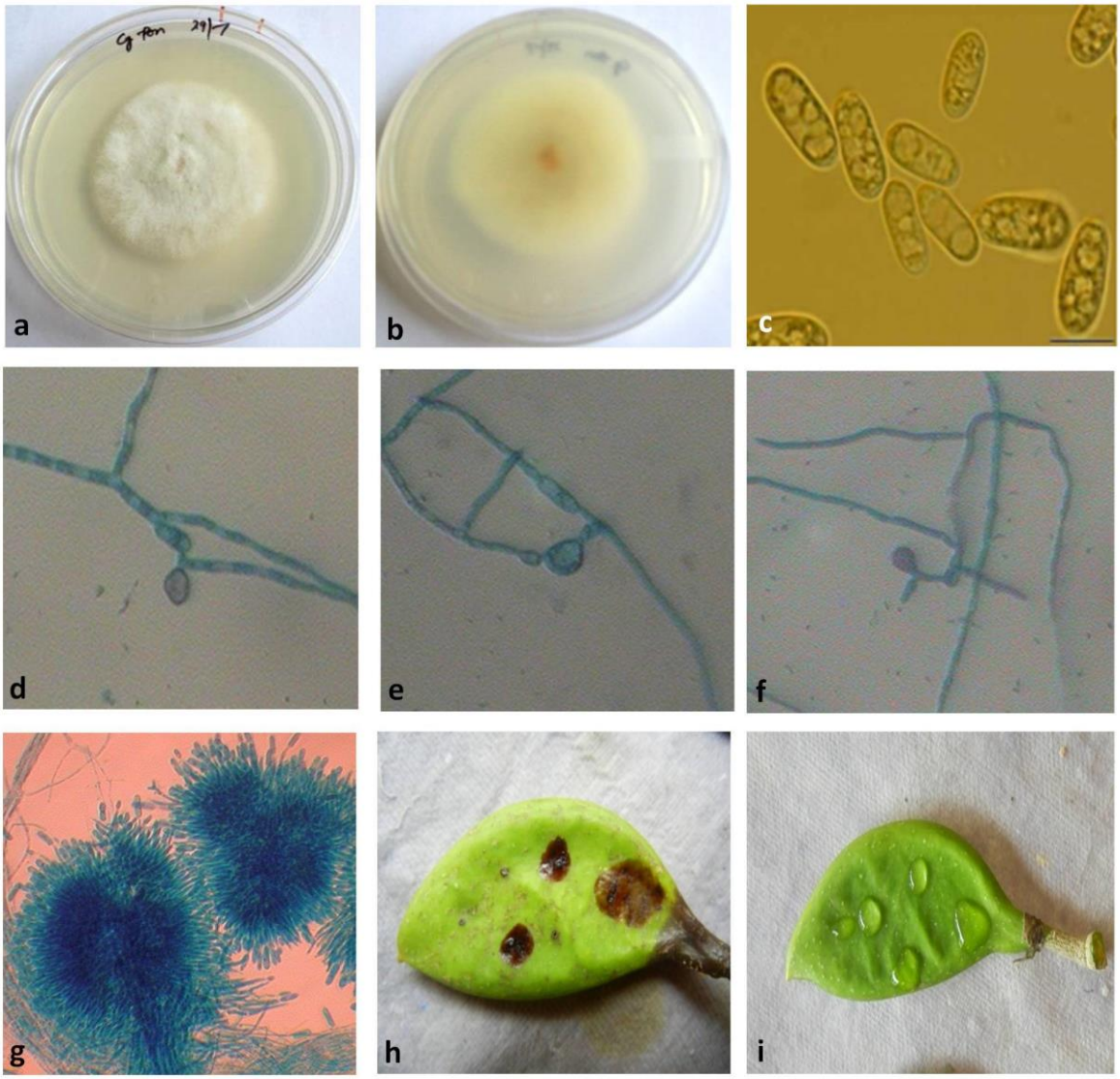

Fig. 1 – **a**. Colony morphology on PDA (front), **b**. Colony morphology on PDA (reverse), **c**. Conidia (Scale bar = 10 µm), **d**–**f**. Appressoria, **g**. Conidiogenous cells, **h**. Infection symptoms on *P. pinnata* fruits after 5 days of inoculation, **i**. control fruit

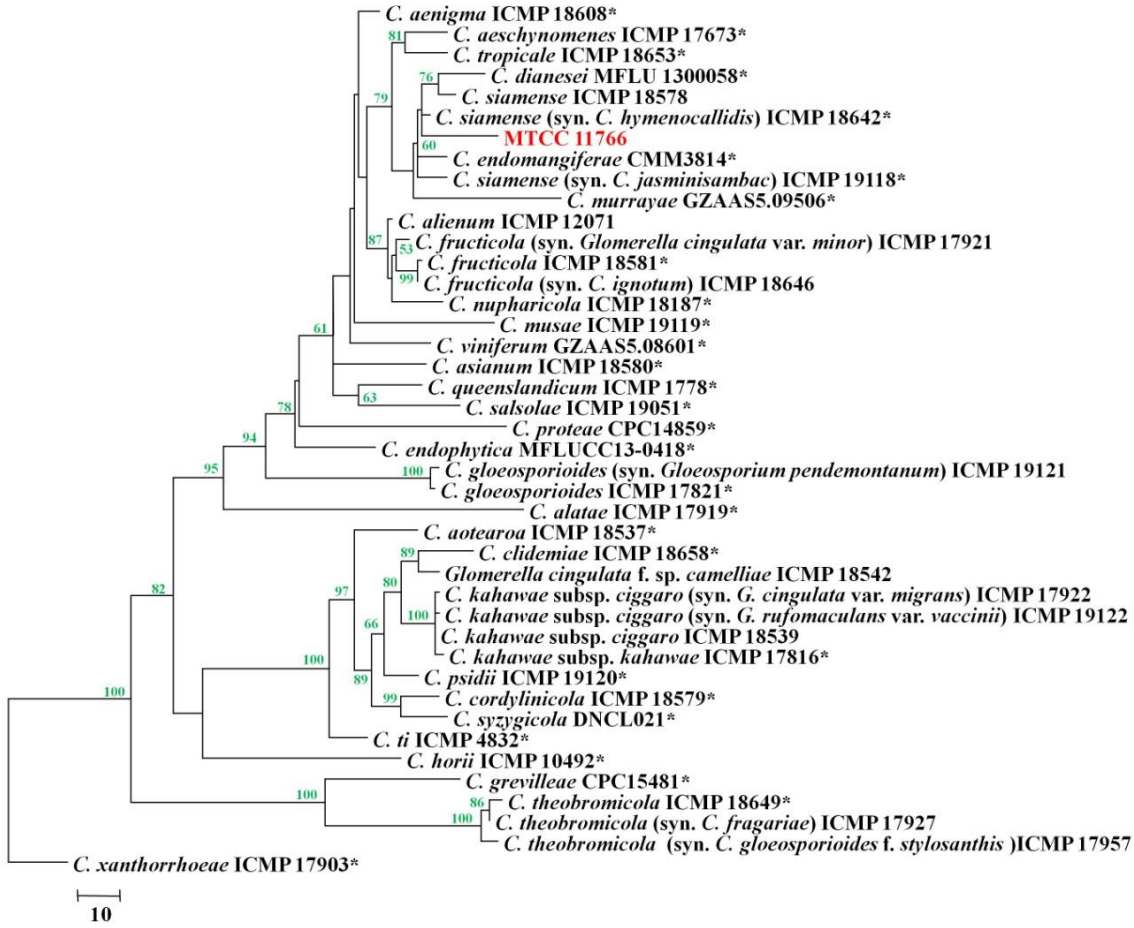

Fig. 2 – One of the 348 most parsimonious trees showing phylogenetic affinities of *Colletotrichum* isolate MTCC 11766 from India (highlighted in red). *Colletotrichum xanthorrhoeae* strain ICMP 17903 is the designated outgroup, and bootstrap support values higher than 50% are shown at the nodes. Ex-type strains are marked with an asterisk.

Discussion

In developing countries such as India, there is an increasing demand for fuel to meet everyday transportation needs. Concurrently, there is a gradual and frequent hike in the cost of fossil fuel in the international market. To minimize the import of crude oil, there is a need to increase the usage of renewable and eco-friendly biofuels. *Pongamia pinnata* is one of the promising candidates for bio-diesel production which are renewable, safe, non-polluting and eco-friendly. The seeds of *P. pinnata* contain around 30–40% of oil which has been used as a source of biofuel (Azam et al. 2005, Karmee & Chadha 2005). Various fungal species can infect *P. pinnata* fruits and seeds, on tree and during storage; which affects seed germination and oil production (Arya et al. 2015). This may threat the growth of this plant in fields and cause severe yield loss. So far, there have been limited anthracnose reports from this plant worldwide (Farr and Rossman 2016). In this study, *C. siamense* has been identified from the diseased fruits of *P. pinnata* on the basis of morphological characters, multi-locus phylogeny and pathogenicity test. The development of disease symptoms was faster (3 days) via wound inoculation as compared to non-wound inoculation (5–7 days). In addition, the germination pattern of the conidia was also investigated. Interestingly, no spore-germination was observed after first 6 hours of incubation, as opposed to previous report by Estrada et al. (2000). Germination of spores was initiated only after 6 hours of incubation, inferring that the rate of germination of the pathogen is less on fruits. More studies are required to obtain insights into germination rate of the pathogen on leaves and shoot.

Colletotrichum siamense was first described as a pathogen associated with anthracnose of coffee berries (Prihastuti et al. 2009) in northern Thailand. This species has been so far reported from various hosts such as apple, chilli, coffee, jasmine, mango, papaya and strawberry (Phoulivong et al. 2010, Weir et al. 2012, Sharma et al. 2013 a, b). It is also a dominant species on tropical fruits (Sharma et al. 2013a, Udayanga et al. 2013). The status of *C. siamense* as a species was under debate. Some of the research articles hint at the presence of cryptic species within *C. siamense* (Sharma et al. 2013a, 2015, Udayanga et al. 2013, Vieira et al. 2014), whereas a recent study by Liu et al. (2016) characterized *C. siamense* as a single species based on statistical analysis using multi-locus sequence data, as well as cross-mating and genetic recombination tests. Thus, in this report we have followed the recent approach by Liu et al. (2016) to avoid further confusion, and used the name "*Colletotrichum siamense*". This is the first report of *C. siamense* as a causative agent of anthracnose on the fruits of *P. pinnata* from Karnataka, India. This information of host and pathogen will be useful to plant pathologists to design disease control strategies for *P. pinnata*. *Pongamia pinnata* is a perennial plant; thus by inhibiting pathogens, healthy plants can be cultivated round the year and sufficient fuel and fodder can be obtained for the cattle.

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