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Colletotrichum kahawae subsp. *cigarro* causing leaf spot disease on arecanut, *Areca catechu* L. in India: A first report

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ABSTRACT

Arecanut is a prominent cash crop in India, known for its potential to significantly boost per capita income. The presence of fungal diseases poses a significant constraint to arecanut cultivation. In October 2019, a severe outbreak of leaf spot disease with 90°% disease incidence was recorded in the farmer's arecanut gardens from Kalasa hobli, Chikkamangaluru, Karnataka, India. Leaf spot symptoms started as small, irregular, light-dark brown spots (3–10 mm in diameter) with a yellow halo on the leaf lamina, which later coalesced to form larger necrotic lesions and a blighted appearance. The symptoms were also observed on leaf sheaths and nuts. In the present study, we identified and characterized the pathogen causing leaf spot disease using cultural, morphological, molecular characterization using multi-gene phylogeny, and pathogenicity assays. Microscopic, and molecular identification of CPCRICkc7G isolate retrieved from the leaf spot infected sample established the association of *Colletotrichum kahawae* subsp. *cigarro* as the causal agent. Pathogenicity assays confirmed the Koch's postulate by reproducing the field observed symptoms. To best of our knowledge, this confirms the first report of *C. kahawae* subsp. *cigarro* causing leaf spot disease on arecanut in India. The findings from this current study play a pivotal role in enhancing the knowledge of arecanut diseases in India, and lay an essential foundation for devising effective disease management approaches to curtail the disease spread.

1. Introduction

'Arecanut' or 'betel nut' (*Areca catechu* L.) is a highly lucrative cash plantation crop extensively cultivated in India and various tropical regions of Asia. Arecanut cultivation is economically significant, providing a decent livelihood for over three million people and generating guaranteed employment for around 16 million individuals annually in India alone. It provides huge returns due to its high per capita income. India exported arecanut worth of 698.74 Crore Indian Rupees which indicates the economic significance of arecanut in India's export market (DASD, 2019–20). It is used mainly for masticatory purpose, and also it plays a vital role in various religious, social, and cultural celebrations in India. It is often offered during ceremonies, festivals, and traditional rituals in India. Arecanut has a history of use in traditional Ayurvedic medicine in India. It has been employed for its potential medicinal properties in India and broadly used in many clinical trials in China and other Southeast Asian countries (Bavappa et al., 1982).

The biotic factors especially diseases have a significant impact on palm productivity. Fruit rot (*Phytophthora meadii*), ganoderma wilt (*Ganoderma* spp.), and inflorescence dieback (*Colletotrichum gloeosporioides*) are the major fungal diseases responsible for major yield loss in arecanut (Chowdappa et al., 2016). In the context of climate change, the

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emergence of new pathogens, such as *Fusarium falciforme* causing root decay (Pandian et al., 2022), *Ganoderma ryverdinii*, and *G. causarinicola* leading to basal stem rot in arecanut (Palanna et al., 2021), and *Phytophthora palmivora* affecting *Areca triandra* (Pandian et al. 2021), has raised serious concerns.

During 2019 post-monsoon, arecanut palms (10–12 years old; recorded on Mangala variety during first survey) expressing severe outbreak of leaf spot disease was reported from the farmer's gardens located in Samse village, Kalasa hobli, Chikkamangaluru, Karnataka, India where, arecanut is cultivated along with coffee plantation (N 13° 11.420'; E 075° 20.023'; 792 MSL). In the field, palms affected by leaf spot disease displayed the following notable symptoms: (i) the presence of small, irregular light-dark brown spots, typically 3–10 mm in diameter, surrounded by a yellow halo on the leaf lamina (Fig. 1A), (ii) these spots often merged to form larger necrotic lesions, resulting in a blighted appearance of the leaves, (iii) similar irregularly shaped light-dark brown spots with yellow halos were also observed on nuts and leaf sheaths, (iv) on occasion, nuts affected by the disease would split open at these spots and fall off, and (v) in severe cases, a heavily infected arecanut garden looks like burnt in appearance (Fig. 1B). Furthermore, the incidence of leaf spot disease, affecting more than 90°% of the crops, has spread throughout the entire hobli, causing significant economic losses and anxiety among local farmers.

This current study is focused on identifying the pathogen responsible for the newly emerging leaf spot disease in arecanut. The pathogen's identity is confirmed through a comprehensive documentation of its symptoms, stringent morphological studies and molecular chacterization using multi-gene analysis.

2. Materials and methods

2.1. Sample collection and pathogen isolation

The leaf spot infected leaves (n = 5) from five different arecanut palms (Cv. Mangala) were collected, washed under running tap water to remove any impurities and brought to laboratory conditions. Leaf spot infected tissues were excised at the margins, cut into small pieces (2 mm × 5 mm), surface sterilized in 2°% sodium hypochlorite (NaOCl) for 30 s, rinsed in distilled water for three times, and air dried on sterile filter paper. A small bit of dried infected tissue (∼1 cm) was transferred to potato dextrose agar (PDA) medium (HIMEDIA Laboratories Pvt. Ltd., India) and incubated for 5–7 days at 28 ± 2 °C under BOD incubator (Pandian et al., 2018). To obtain the pure culture, single hyphal tip method is followed where the tip of the single hyphae growing from a colony is cut and placed onto new PDA plates and incubated for morphological observations at 28 ± 2 °C.

2.2. Morphological identification

A pure culture, obtained through the hyphal tip method, was subjected to morphological identification. Macroscopic traits such as colony appearance, growth rate, and pigmentation, along with microscopic characteristics including conidial size, shape, and pigmentation, were systematically recorded (n = 50) using a compound microscope equipped with an image analyzer (Nikon Eclipse Ni–U, Tokyo, Japan).

2.3. Molecular identification and phylogenetic analysis

The pure culture of the pathogen was cultured on potato dextrose broth (PDB) and incubated for 7 days at 28 ± 2 °C. Fungal mycelium was harvested using Whatmann No. 1 filter paper and grounded using liquid nitrogen for total genomic DNA extraction using Cetyltrimethylammonium bromide (CTAB) method with minor modifications (Pandian et al., 2018). To confirm the species identity, polymerase chain reaction (PCR) was performed with multi-genes specific primers [Calmodulin, β-tubulin, actin, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), ribosomal DNA, glutamine synthetase (GS), and Apn2-Mat1-2 intergenic spacer and partial mating type (Mat1-2) gene (AP-Mat)] as given in Table 1.

PCR reactions were performed using 50 μl reaction volume containing 5.0 μl Taq A buffer (Thermo Fisher Scientific, USA), 2.0 μl each of the forward and reverse primers (10 pmol/μl), 2.0 μl of 10 mM dNTP's, 2.0 μl DNA, 1.0 μl Taq buffer and 36.0 μl sterile distilled water. PCR was performed in a Thermo cycler (Bio-Rad T100, Hercules, CA, USA) with the following conditions: For internal transcribed spacer (ITS) region of ribosomal DNA amplification, initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min; for other genes, initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The amplified product was evaluated using electrophoresis on a 1.0°% agarose gel (Sambrook and Russell, 2001). PCR amplified products were sent for Sanger sequencing (Biokart India Pvt. Ltd., Bengaluru, India).

The DNA sequences were manually aligned using BioEdit (Biological sequence alignment editor: http://www.mbio.ncsu.edu/BioEdit/bioedit.html). BLAST searches were performed in National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) to confirm the species identity. The DNA sequences of multi-genes for the representative isolate were deposited into GenBank. Reference sequences available in NCBI database were downloaded and included for the sequence similarity and phylogenetic analysis. Concatenated sequence was prepared for the fungal isolate using calmodulin,

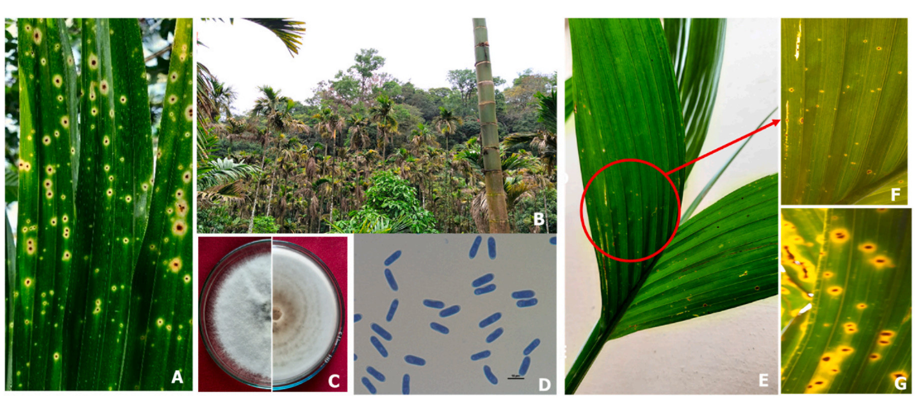

Fig. 1. Arecanut leaf spot disease symptoms and characterization A) Light to dark brown spots with yellow halo on leaves; B) Severe leaf spot affected garden shows burnt appearance; C) 7-days old fungus growth on PDA medium at 28 ± 2 °C; D) Conidial characteristics; E), F) & G) Leaf spot symptoms produced on symptomatic arecanut leaves during Pathogenicity test which resembles the field recorded symptoms.

Table 1
List of genes used for the molecular amplification of *Colletotrichum kahawae* subsp. *cigarro* CPCRICkc7G.

SN	Gene name	Sequence (5′-3′)	Reference
1	Internal transcribed spacer region of ribosomal DNA (ITS)	ITS1_F: TCCGTAGGTGAACCTGCGG ITS4_R-TCCTCCGCTTATTGATATGC	White et al. (1990)
2	Calmodulin	Cal1_F-GARTWCAAGGAGGCCTTCTC Cal2A_R-TTTTTGCATCATGAGTTGGAC	O'Donnell et al. (2000)
3	β-tubulin	Bt2a_F-GGTAACCAAATCGGTGCTGCTTTC Bt2b_R-ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson (1995)
4	Glyceraldehyde-3-phosphate dehydrogenase	GADPH_F-GCCGTCAACGACCCCTTCATTGA GADPH_R-GGGTGGAGTCGTACTTGAGCATGT	Templeton et al. (1992)
5	Actin	ACT512_F-ATGTGCAAGGCCGGTTTCGC ACT783_R-TACGAGTCCTTCTGGCCCAT	Carbone and Kohn (1999)
6	Apn2-Mat1-2-1 intergenic spacer and partial mating type (Mat1-2)	AM_F-TCATTCTACGTATGTGCCCG AM_R-CCAGAAATACACCGAACTTGC	Silva et al. (2012)
7	Glutamine synthetase	GS_F-ATGGCCGAGTACATCTGG GS_R-GAACCGTCGAAGTTCCAC	Weir et al. (2012)

β-tubulin, actin, GAPDH, ribosomal DNA, GS, and AP-Mat gene sequences. Phylogenetic analysis was performed with the concatenated sequences in MEGA-X software (https://www.megasoftware.net) (Tamura et al., 2013) using Maximum likelihood (ML) method with Kimura 2-parameter model for nucleotide substitutions with gamma distribution with a bootstrap of 5000 replicates. *Colletotrichum karstii* (a member of *Colletotrichum boninense* species complex) is used as out group taxon.

2.4. Pathogenicity test

In order to fulfil the Koch postulate, pathogenicity test was conducted with the representative isolate (CPCRICkc7G) on one year old healthy arecanut seedlings (Cv. Mangala; n = 5). The leaves were washed with running tap water and wiped with 70°% ethanol to remove impurities. A freshly prepared *C. kahawae* subsp. *cigarro* isolate CPCRICkc7G conidial suspension (2.4×10^6 conidia ml^{-1}) added with Tween-20 (2 μl) was sprayed over the arecanut leaves, whereas sterile distilled water spray served as control. Both treated and control palms were incubated at 28 ± 2 °C for 7 days and the symptom development was observed daily. Re-isolation of the pathogen from the symptomatic leaves was done after 7-days post inoculation (dpi). The re-isolated pathogen identity was confirmed using morphological analysis and molecular characterization using multi-gene amplification of calmodulin, β-tubulin, actin, GAPDH, ribosomal DNA, GS, and AP-Mat as described earlier. The pathogenicity experiment was repeated thrice for better reproducibility.

3. Results

3.1. Pathogen identification and phylogenetic analysis

The fungus consistently isolated from the leaf spot infected leaves on PDA plates exhibited fluffy, whitish-grey aerial mycelium on both the upper and lower sides (Fig. 1C). Microscopic examination revealed numerous single-celled, hyaline, cylindrical shaped conidia with dimensions averaging 12.78 ± 1.54 μm in length and 4.75 ± 0.75 μm in width (n = 50) (Fig. 1D). Based on cultural and microscopic characteristics, the isolated pathogen was identified as belonging to the *Colletotrichum* genus. A reference isolate labelled as 'CPCRICkc7G′ is deposited at ICAR-National Bureau of Agriculturally Important Microorganism Culture Collection (NBAIMCC), Mau Nath Bhanjan, Uttar Pradesh, India with accession no. 'TF-3388'. GenBank accession numbers have been assigned to the partial gene sequences of *Colletotrichum* spp. CPCRICkc7G, specifically for calmodulin, β-tubulin, actin, GAPDH, ribosomal DNA, GS, and AP-Mat, which are as follows: OQ920260, OQ920258, OR031766, OQ920266, OR019096, OQ920264, and OQ920268, respectively. Additionally, a BLAST homology sequence analysis indicated a high degree of nucleotide similarity, ranging from 97°% to 99°%, with *Colletotrichum kahawae* subsp. *cigarro* ICMP18534 (GenBank ID: JX009634 for Calmodulin, JX010427 for β-tubulin, JX009473 for Actin; JX009904 for GAPDH, JX010227 for ITS, JX010116 for GS, and HE655657 for AP-Mat). Furthermore, a multi-gene phylogeny analysis with the closely related species in *Colletotrichum gloeosporioides* species complex (Cgsc) revealed that the *Colletotrichum kahawae* subsp. *cigarro* CPCRICkc7G isolate, obtained in this study, forms a distinct cluster closely related to the *Colletotrichum kahawae* subsp. *cigarro* ICMP18534 sequence (Fig. 2), whereas *C. karstii* MANE189 formed a separate cluster. Collectively, these findings, along with cultural, microscopic, and molecular characterizations, provide strong evidence and confirming the association of *C. kahawae* subsp. *cigarro* with emerging leaf spot disease in arecanut.

3.2. Pathogenicity test

In the pathogenicity test, symptomatic palms exhibited light-dark brown spots with yellow halos seven days after inoculation (Fig. 1E, F & 1G), mirroring the field observations, which confirmed *C. kahawae* subsp. *cigarro* CPCRICkc7G as pathogenic while the control palms remained symptom-free. The re-isolation of the fungal pathogen from the symptomatic leaves, showing consistent cultural, microscopy, and molecular characteristics with the initially isolated culture from infected seedlings, confirmed its identity as *C. kahawae* subsp. *cigarro* and fulfilled Koch's postulates. Furthermore, multi-gene amplification using calmodulin, β-tubulin, actin, GAPDH, ribosomal DNA, GS, and AP-Mat genes provided additional evidence supporting the pathogen's identification.

4. Discussion

Colletotrichum, a fungal genus of great economic significance, is renowned for its role in causing anthracnose disease, which has a wide host range, predominantly affecting tropical and subtropical crops as well as fruit trees (Sutton, 1992). This pathogen can afflict above-ground plant parts at all stages of growth, including stems, leaves, flowers, and fruits. Advancement of molecular tools over the last decade lead to a tremendous shift in the taxonomy of the genus *Colletotrichum.* A total of 257 different species reported and grouped into 15 species complexes so far. *Colletotrichum gloeosporioides* species complex (Cgsc) is one of the unique, morphologically, and phylogenetically diverse species complex, which contains majorly polyphagous to some extent host-specific (Weir et al., 2012; Talhinhas and Baroncelli, 2021).

The present study explicitly reports the association of *C. kahawae* subsp. *cigarro* with emerging leaf spot disease on arecanut palms. The symptoms documented during the present investigations are in complementary with the symptoms reported by Zhang et al. (2020) on arecanut. In addition, *C. kahawae* subsp. *cigarro* has been reported to cause anthracnose on *Olea europaea* (Weir et al., 2012), *Mangifera indica* (Ismail et al., 2015), *Citrus reticulata* (Perrone et al., 2016) and *Lonicera macranthoides* (Xiao et al., 2023). Being an airborne pathogen, once established in Indian arecanut plantations, managing this pathogen becomes exceedingly challenging. Moreover, it has been observed to infect a wide range of arecanut varieties, encompassing both high-yielding and local cultivars such as Mangala, Mohitnagar, Shatamangala, Sreemangala, Ratnagiri local, Sirsi local, and SK local (Data

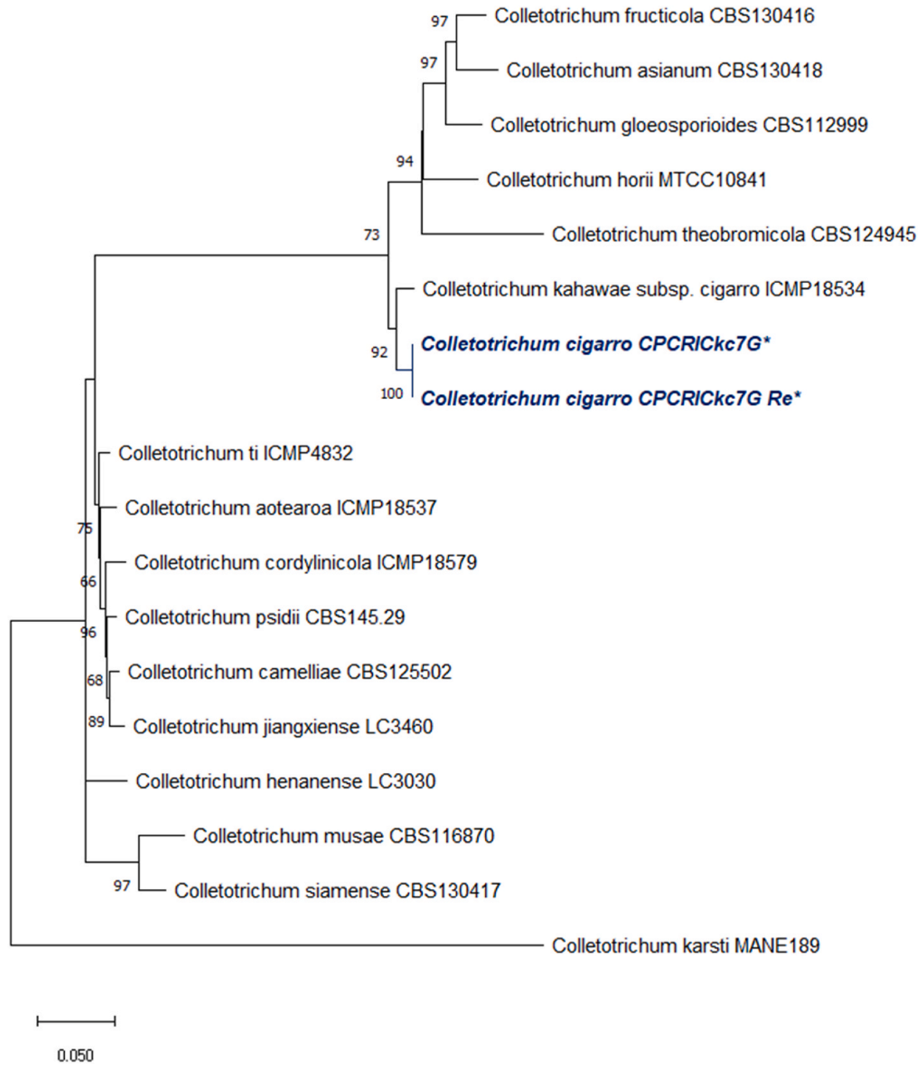

Fig. 2. Phylogenetic analysis of isolate CPCRICkc7G. Maximum-Likelihood (ML) tree generated from actin, calmodulin, β-tubulin, rDNA, GAPDH, GS, and AP-Mat gene sequences of isolate CPCRICkc7G (Bold with asterisk mark) in the present study and reference strains in GenBank. Bootstrap values (1000 replicates) of >60°% are shown at the nodes. *Colletotrichum karstii* was the outgroup.

not presented). This broad preference across different arecanut varieties highlights the alarming potential of *C. kahawae* subsp. *cigarro* to infect arecanut plants. In this context, *C. kahawae* subsp. *cigarro* is poised to serve as a significant limiting factor for both area and production of arecanut cultivation in India. Therefore, the development of integrated strategies for the sustainable management of *C. kahawae* subsp. *cigarro* in arecanut cultivation is of paramount importance. To our knowledge, this is the first report of *Colletotrichum kahawae* subsp. *cigarro* causing leaf spot disease in India. The insights gained from the current investigation can serve as valuable tools in formulating novel approaches to combat this devastating disease in arecanut plantations.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

CRediT authorship contribution statement

R. Thava Prakasa Pandian: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shivaji Hausrao Thube:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bhavishya:** Writing – review & editing, Writing – original draft, Investigation. **Merin babu:** Writing – review & editing, Writing – original draft, Formal analysis. **H. Rajashekara:** Writing – review & editing, Writing – original draft, Supervision. **T.N. Madhu:** Writing – review & editing, Writing – original draft, Data curation. **Y. Diwakar:** Writing – review & editing, Writing – original draft, Resources. **P. Santhoshkumar:** Writing – original draft, Supervision, Methodology. **B. J. Nirmalkumar:** Writing – original draft, Supervision, Methodology. **Balanagouda Patil:** Writing – review & editing, Writing – original draft, Formal analysis. **V. Hegde:** Writing – review & editing, Writing – original draft, Validation, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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