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Symptomatological, morphological and molecular validation of *Colletotrichum gloeosporioides* (Penz.) Sac. associated with leaf spot disease of arecanut

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ABSTRACT: The outbreak of leaf spot disease of arecanut caused by *Colletotrichum* spp. created havoc in hill and coastal zones of Karnataka. *Colletotrichum* is one among the top ten widespread plant-pathogenic fungi. The symptoms which are predominant on leaves were also noticed on nuts with circular or oblong to irregular brownish spots surrounded by larger yellow halo. Cultural characteristic of the isolated pathogen on potato dextrose agar produced dense, cottony, whitish to slight greyish mycelium with even margin without any zonation. Microscopic observation revealed that conidia with single celled, hyaline and cylindrical with rounded ends besides that length of the conidia varied from 12.06 to 12.30 µm and width 3.8 to 4 µm having 2-3 oil globules. The ITS based sequence analysis revealed that *Colletotrichum gloeosporioides* is associated with leaf spot disease of arecanut. It is concluded that the *C. gloeosporioides* was found predominant among the isolated cultures and sole responsible for epidemic of leaf spot disease.

Keywords: Arecanut, *Colletotrichum,* identification, leaf spot, symptoms

INTRODUCTION

Arecanut (*Areca catechu* L.) is an important plantation crop of India belongs to the family Arecaceae. Arecanut industry forms the economic backbone of a substantial number of farm families (Balasimha and Rajagopal, 2004). It is extensively used in India by all sections of the people as masticatory and in several social and religious ceremonies (Bhat *et al.,* 2021). Areca nut is extensively cultivated in the plains and foothills of Western Ghats and North Eastern regions of India. Presently it is cultivated in 9.38 lakh ha with a production of 13.68 lakh tones and average productivity is 1.46 MT/ha. In Karnataka it is grown in an area of 4.71 lakh hectares with a production of 7.03 lakh tonnes and 1.49 MT/ha. The major area under cultivation is confined to Karnataka, Kerala and Assam. Among the states Karnataka stands first in area, production and productivity (Anon., 2022). Arecanut palm is affected by a number of diseases at different stages of growth and development. About 20 diseases, causing varying degrees of damage to the palm have been recorded in India (Bavappa, 1982). Among the fungal disease, leaf spot cause more catastrophic yield loss up to 60 percent (Hedge, 2018). In recent year's leaf spot disease is epidemic in Karnataka and Kerala. Leaf spot of arecanut though a minor disease in the past, has nowbecome a major disease especially during rainy season. The disease began as circular to irregular spots which enlarged as the disease progressed. Later, the spots were light to dark brown in color having ash grey center, surrounded by dark brown margins and yellow halo. In severe cases, the adjacent spots eventually coalesced to form large irregular patches leading to blighted appearances and finally covered the entire leaf lamina turning the leaf color to pale yellow. Fungus produces the conidia within 3 to 5 days at 30 °C and at 90% relative humidity. Survival of conidia, and conidia in infected leaf debris was studied in soil maintained at different soil moisture levels. Survival of conidia declined rapidly under moist conditions (≥12% moisture, vol/wt.), but under dry conditions, viable conidia could be detected up to 12 months after incorporation into soil (Hartung *et al.*, 1981; Mohanan *et al.*, 1989; Salotti *et al.*, 2022). Sudden outbreak of disease in traditional growing areas made impact on socioeconomic status and livelihood of farming community. Hence, early detection based on field symptoms and prophylactic spray should be followed for effective management. Therefore, present investigation was more focused on symptomatology of leaf spot disease in different growth stages of arecanut and molecular confirmation of etiology based on ITS sequence analysis.

MATERIALS AND METHODS

Collection of disease samples: As result of epidemic of leaf spot disease in hill zone and coastal zones, the roving survey was conducted to know the disease severity. The diseased leaf samples of arecanut palm showing typical leaf spot symptoms were collected during survey from Shivamogga and Chikkamagaluru districts.

Isolation of pathogen: The samples were brought to the Arecanut Research Centre, Shivamogga. The standard

tissue isolation technique was used to isolate the fungus. The infected portion of the leaf bits were thoroughly washed and cut into small pieces (1 mm) in such a way that each piece consisted of infected along with surrounding portion of healthy green tissues. The pieces were surface sterilized with 1% sodium hypochlorite solution for 30 seconds followed by three successive washing with sterilized distilled water to remove the traces of chemical if any and then left for drying. After drying, the sterilized pieces were transferred to autoclaved Petri plates containing 20ml PDA media under aseptic conditions. These Petri plates were then placed in BOD incubator at 27±1°C for 9 days.

Morphological identification: A loopful of pure culture was taken from the nine days old culture and placed it on the slide and mixed thoroughly with lactophenol to obtain uniform spread. A cover slip was placed over it. Length and breadth of the conidia were measured under high power objective lens of Lawrence and Mayo binocular microscope (LM-52-1803-S) and TC capture 3.9.0 software and drivers were installed for image acquisition, managing and processing. Further, the morphological characters such as hyphae, conidial shape, size and oil globules were documented.

Molecular identification of the pathogen: The genomic DNA was extracted using Cetyltrimethyl ammonium bromide (CTAB) protocol given by Doyle and Doyle (1987). ITS1/ITS4 5′-TCCGTAGGTGAACCTGCGG-3′ and 5’-TCCTCCGCTTATTGATATGC-3’ primers were used in the experiment and PCR conditions were followed as initial denaturation (94°C; 5 min.), denaturation (94°C; 1 min.), annealing (54°C; 1 min.), extension (72°C; 2 min.), final extension (72°C; 10 min.) for 35 cycles. Separation of amplified product on 1.5% agarose gel. PCR product were purified using protocol of QIAquick PCR Purification Kit. Purified PCR product sequenced by Barcode Biosciences Pvt. Ltd. Bengaluru. Homology of obtained sequence was accomplished through NCBI (National Centre for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov) and the sequences were submitted to NCBI database as OQ948330.

Phylogenetic analysis: A multiple-sequence alignment was carried out using comparable reference sequences of other *Colletotrichum* species which are retrieved from NCBI database and multiple sequence aligned using CLUSTAL W (Edgar and Batzoglou, 2006) algorithm of MEGA 6.0 software to check the genetic diversity

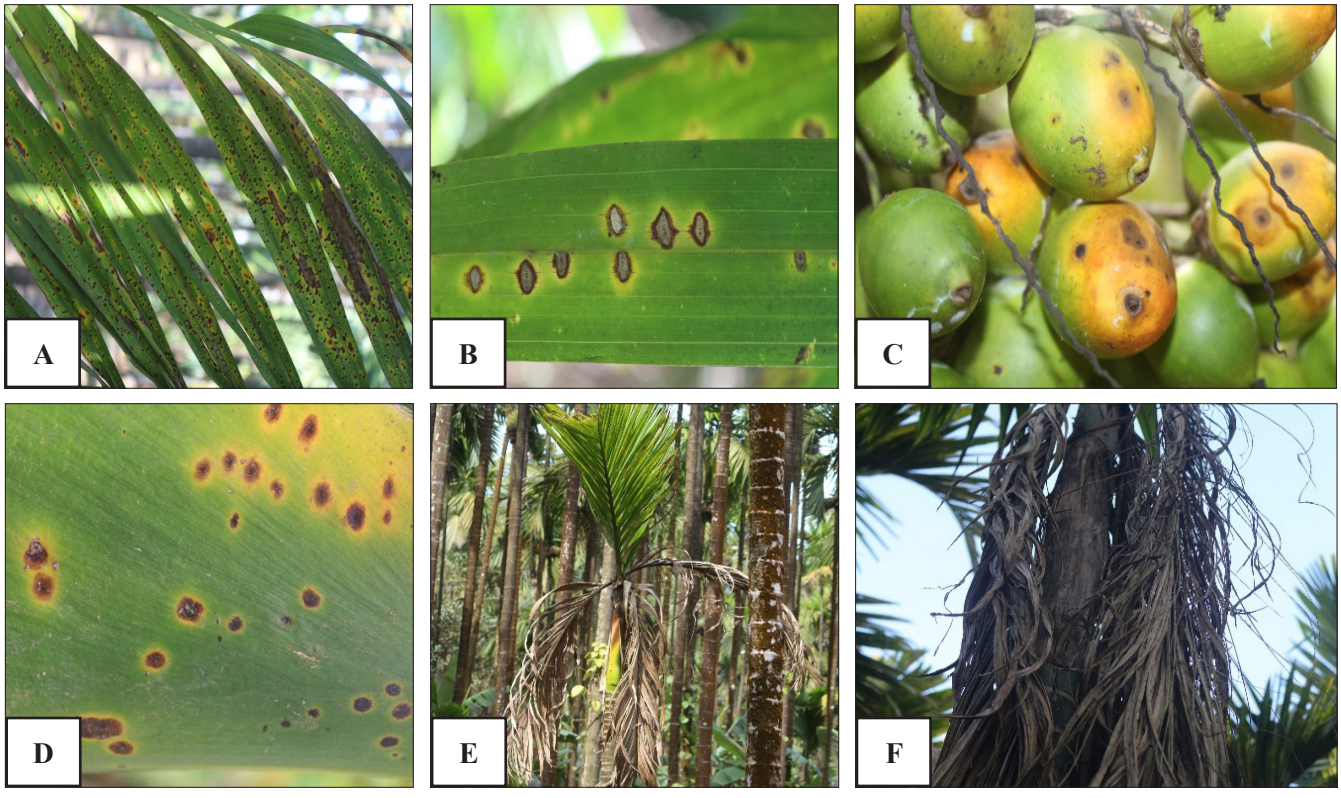

Fig. 1. Symptomatology of arecanut leaf spot disease incited by *C. gloeosporioides* A-B. Indicates small, circular or oblong to irregular brownish spots on leaves. C. Circular brown spots on thenuts. D. Brownish spot on leaf sheath. E-F. Severely blighted symptoms of leaves lead to drooping.

among different *Colletotrichum* isolates (Tamura *et al.* 2013). Phylogenetic relationships were analyzed by the distance methods. The distance matrix for the aligned sequences was calculated using Kimura's two parameter model (Kimura 1980), and analyzed with the neighbor-joining (NJ) method (Saitou and Nei 1987) using MEGA (Molecular Evolutionary Genetics Analysis program) version 6, excluding positions with gaps. The reliability of the inferred tree was estimated by bootstrap analysis (Felsenstein, 1985). The final trees were displayed using Clustal Omega multiple alignment package (Sievers and Higgins, 2021).

RESULTS AND DISCUSSION

Symptomatology of leaf spot disease of arecanut: The initial symptoms of the leaf spot disease appeared as small, circular or oblong to irregular brownish spots (Figure 1). The center of the spot was grey or straw color surrounded by yellow halo. In the advanced stages, spots coalesced to give a blighted appearance to the leaves.Similar symptoms were observed by Hegdeand Hegde(1986) in anthracnose disease of arecanut. Based on survey it was observed that fungal infection was also noticed even on nuts.

Morphological characterization of isolated culture on potato dextrose agar: The cultural characteristic of the isolated pathogen on Potato dextrose agar produced dense, cottony, whitish to slight greyish mycelium with even margin without any zonation (Figure 2). Microscopic observation (40X Microscopic field) revealed that acoenocytic, hyaline hyphae with profuse branching habit. Conidia with single celled, hyaline and cylindrical with rounded ends besides that length of the conidia varied from 12.06 to 12.30 µm and width 3.8 to 4 µm having 2-3 oil globules. Microscopic observations are in confirmatory with literatures of Weir *et al.* (2012) and Hassan *et al.* (2018) in citrus anthracnose from New Zealand and persimmon anthracnose from South Korea, respectively.

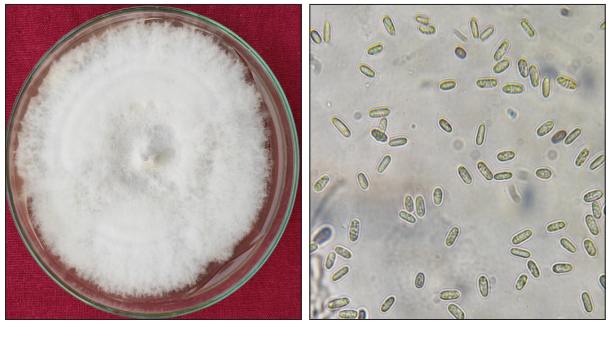

Fig. 2. Pathogen isolated on PDA media and microscopic view of conidia (40X)

DNA sequences of obtained isolates were compared using bioinformatics tool like NCBI (National Centre for Biotechnological Information) blast programme. Based on sequence comparison, nucleotide sequences of the ITS1/ITS4 region of the ribosomal DNA of isolates had 100% homology with *C. gloeosporioides* isolates available in the NCBI. Thus, obtained isolates were confirmed as *Colletotrichum gloeosporioides*.

Phylogenetic analysis: The Cladogram (Figure 3) obtained from MEGA 6.0 software showed that obtained sequence grouped with *Colletotrichum gloeosporioides* and compared with reference sequences of different species which fell in different group when it was rooted with *Colletotrichum xanthorrhoeae.* The results obtained are in agreement with the results obtained by earlier workers Serra *et al*. (2011) and Zivkovic (2017).

CONCLUSION

The pathogen causing leaf spot disease of arecanut was isolated and identified based on symptomatology and ITS based molecular conformation as *Colletotrichum gloeosporioides*. However, on the basis of available literature, this is the recent report from Karnataka.

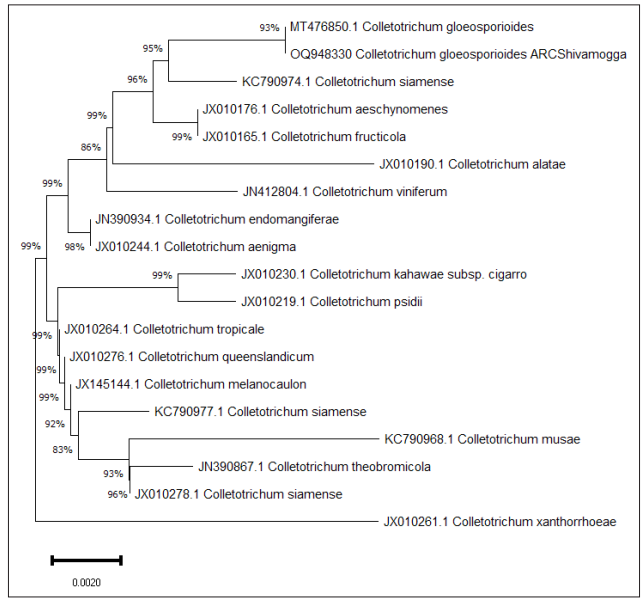

Fig. 3. Phylogenetic analysis of different isolates of *C. gloeosporioides*

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