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First Report of Molecular characterization of *Colletotrichum gloeosporioides* L. causing Leaf Blight Disease of Arecanut in Karnataka, India

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ABSTRACT: Arecanut (*Areca catechu* L.) is one of the most profitable commercial plantation crop cultivated in hilly, maidan and coastal regions of Karnataka. The crop suffers from various biotic and abiotic stresses however, among the biotic stresses leaf spot or leaf blight diseases caused by *Colletotrichum gloeosporioides has* became very severe from lost few years resulting in huge financial loss to the growers. Initially the causal agent has been identified as *Colletotrichum gloeosporioides* based on morphological characters. However, pathogen identification was not done based on molecular characterization. Therefore in the present study effort was made to identify the organism through ITS rDNA sequencing and the obtained sequence was deposited in the Genbank. The present work is the first Indian report of ITS rDNA region amplification of *Colletotrichum gloeosporioides* causing leaf blight disease in arecanut.

Keywords: Arecanut, Leaf Blight, *Colletotrichum gloeosporioides*, Molecular characterization.

INTRODUCTION

Arecanut (*Areca catechu* L.) is an important palm belongs to family Arecaceae which is mostly grown in tropical Asia and East Africa. It serves as an important cash crop in the East, West and North Eastern regions of India. (Balasimha and Rajagopal, 2004). It is popularly known as betel nut or supari and is used even in ayurvedic and veterinary medicines (Bhat *et al.*, 2021). Areca palms are grown in many countries for their seeds. India is the largest producer of arecanut in the world. As per the second advanced estimates of 2019-20, arecanut is grown in an area of 730.82 lakh hectares with a production of 1208.93 MT, with the productivity of 1654 kg/ha. The major cultivated area under arecanut cultivation is confined to Karnataka, Kerala, and Assam. However, Karnataka stands first in area, production, and productivity, with an area of 500 lakh hectare accounting for more than half of the area grown in the country. The state alone produced 950 lakh MT with a productivity of 1900 kg/ha which is higher than the average country's productivity (Anon, 2020). From last one or two decades arecanut production is gradually decreasing due to the occurrence of various biotic and abiotic stresses. Among the biotic stresses leaf blight caused by *Colletotrichum gloeosporioides* is becoming more destructive. Colletotrichum is a cosmopolitan fungal genus comprised of more than 189 species distributed throughout tropical and temperate regions across the world. (Jayawarden, 2016). Colletotrichum species cause devastating diseases such as anthracnose, leaf spots and blights in wide range of plant hosts. Weir *et al.*, (2012). However, leaf blight disease incidence ranged from 12-62 per cent (Naik *et al*., 2021). Though the pathogen was earlier identified through cultural and morphological methods, but there is no reports showing its molecular identity. Therefore, an attempt was made to characterize the pathogen using molecular methods.

MATERIALS AND METHODS

A. Isolation and Identification of the pathogen

The leaf samples showing typical blighting symptoms were collected from the field and were subjected to isolation following standard tissue isolation method. The infected tissues were sterilized with sodium hypochlorite (0.1 per cent) for a minute followed by thorough rinsing in distilled water. The tissues were kept on potato dextrose agar medium under sterilized conditions and were incubated at 27°C for seven days upon observing the growth of mycelia in the Petri plates a loop full of fungal culture was picked and transferred onto a clean glass slide containing a drop of lacto phenol dye. Microscopic observation of the pathogen

mycelia and spores resembled to Colletotrichum spores. In order to confirm the pathogenicity of the organism. Koch postulates was proved.

B. Pathogenicity Test

Koch's postulates were employed for proving the pathogenicity of fungus by artificial inoculation method under glass house conditions. The fungal culture present in the potato dextrose broth was filtered through muslin cloth to get the pure conidial suspension. Three to four year old plants without leaf blight symptoms were selected for inoculation purpose, these leaves (one for water spray and another for inoculums spray) were sprayed with sterile distilled water (check) and with spore suspension (2×10^4 spores ml^{-1}) and was covered with polyethylene bags. Treated leaves were observed for the symptom expression. Initial symptoms like yellowing of leaves from the leaf tip followed by necrosis started appearing 25 days after inoculation and the pathogen was re-isolated from the infected plants. The symptoms and the colony of the pathogen was compared with original culture thus confirming the pathogen's identity by which the pathogenicity was proved.

C. Genomic DNA extraction

Pure culture obtained from the diseased tissue was used for genomic DNA extraction. The pathogen was grown on PDA medium for twelve days at 27°C. Thin mycelial mat in the culture plate was scraped from 12 days old culture using a sterile scalpel and it was added to a sterilized pestle and mortar. Later the fungus DNA was extracted by employing a modified cetyl trimethyl ammonium bromide (CTAB) protocol (Moller *et al*., 1992).

C. PCR amplification

The ITS/5.8S regions of the fungus were amplified using primer pair ITS1 (5' - TCCGTAGGTGAACCTGCGG-3') which hybridizes at the end of 18S recombinant deoxyribonucleic acid (rDNA) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and at the beginning of 28S rDNA (Ferrer *et al*., 2001). The DNA amplification was carried out in 25 µL reaction mixtures containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.6 mM of each dNTPs, 0.25 µM of each primer, 1.25 U *Taq* polymerase (Promega) and 4 nggDNA using PCR machine. The PCR cycling profile includes : Hot start for 5 min at 95°C, 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 10 min. (Rojas, 2010). PCR products were resolved on 1% (w/v) agarose gel (with ethidium bromide, 10 ng/100 ml).

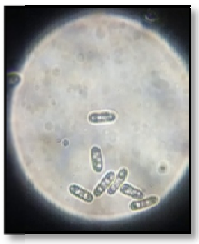
D. Sequencing and in silico analysis

The ITS rDNA region was sequenced for the obtained isolate. Thus the obtained PCR product was sequenced with forward and reverse primers at Biokart, India. Bengaluru. Homology search was done using BLAST algorithm available at the www.ncbi.nlm.nih.gov.

RESULTS AND DISCUSSION

Upon culturing the pathogen on PDA medium the pathogen grew rapidly covering the Petri dishes within 10-12 days of inoculation typically producing a grayish to cremish colored cottony mycelium. When the culture were examined microscopically, profuse cylindrical, straight with smooth round end spores were observed exhibiting the typical characters of *Colletotrichum gloeosporioides*. The obtained results were in line with (Zakaria and Bailey, 2000; Chowdappa *et al*., 2012; Khodadadi *et al*., 2020).

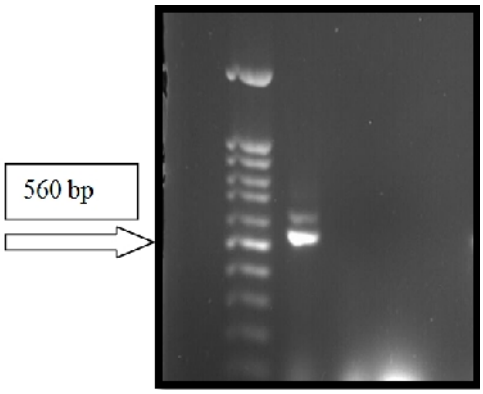
Pure culture of the pathogen



Conidia at 40X

A. Amplification of ITS1 and ITS4 region

Genomic DNA of the fungus observed by gel electrophoresis containing 1.2 per cent agarose gel with 7-8 Kb with approximate concentration of 91ηg μg^{-1}. PCR amplification was observed with the amplicon size of 560 bp and with a concentration of around 150 ηg μg^{-1}. The present work is the first Indian report of ITS rDNA region amplification of *Colletotrichum gloeosporioides* infecting arecanut. Hence the sequence of ITS rDNA region of *Colletotrichum gloeosporioides* was deposited in the Genbank and the accession number MW599823 was obtained. Genus *Colletotrichum* is reported as one of the ten most important plant pathogens in the world based on economic importance (Dean *et al*., 2012). PCR-based method is considered to be more authenticated and highly sensitive technique compared to the morphological identification. The Internal Transcribed Spacer (ITS) region of the nuclear ribosomal repeat unit has become the primary genetic marker for molecular identification and other species-level pursuits in many groups of fungi (Seifert, 2009) in this regard the present work is considered to be atmost important in the recent days.



CONCLUSION

Probably due to the change in scenario all the minor or neglected diseases/ pathogens are taking upper hands in

the recent years one such neglected pathogen is *Colletotrichum gloeosporioides*. Identifying the causal agent facilitates resistance breeding programs and determines the best control strategies against leaf blight disease. Identification of Colletotrichum to the species level was traditionally reliant on host, cultural and morphological descriptions such as size and shape of conidia, colony growth rate and pigmentation, but nowadays comparison of nuclear rDNA internal transcribed spacer is becoming predominant so one such attempt was made to determine the nature of the pathogen through ITS technique. To the best of our knowledge the present work is the first report of ITS rDNA region amplification of *Colletotrichum gloeosporioides* infecting arecanut in India. However in the future days the multi-locus phylogenetic analyses of the pathogen will be studied by which the pathogen would be identified much reliably.

FUTURE SCOPE

The multi-locus phylogenetic analyses of the pathogen from various location will be studied by which the pathogen would be identified much reliably.

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Conflict of Interest. None.

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