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Morphological and Molecular Characterization of C*ollectotrichum gloeosporioides* Causing Anthracnose Disease on Carambola in India

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ABSTRACT

Filamentous fungi of the genus Colletotrichum and its teleomorph Glomerella are considered major plant pathogens worldwide. The fungi cause disease symptoms that are generally known as anthracnose in a wide range of vegetables, fruits and other crops. The fruit anthracnose of different crops was relatively well studied in India but the information about anthracnose on Carambola is still very scarce and it causes extensive yield losses at both the pre- and post-harvest stages during warm and rainy seasons. The anthracnose infected samples of Carambola was collected from Central Horticultural Experiment Station, Chettalli, Karnataka state of India. The fungus was isolated from the lesions of infected leaves and fruits. The pathogenicity of fungus was confirmed by re-inoculating on the same host. Further the fungus was identified based on pathogenic, morphological and amplification of internal transcribed spacer (ITS) region by PCR using ITS specific primers. The amplified PCR product were cloned and sequenced. The sequence analysis of Carambola isolate showed maximum nucleotide identity of 100% with Colletotrichum gloeosporioides *(Penz.) Penz. & Sacc infecting tobacco crop.*

Key words: *Anthracnose,* Colletotrichum gloeosporioides*, Internal transcribed spacer (ITS) region, PCR*

INTRODUCTION

Herbal medicine is the oldest form of healthcare known to mankind. India has a rich heritage of traditional medicine. Over 50% of all modern drugs are of natural product origin and they play an important role in drug development programs of the pharmaceutical industry[13]. Population living in developing countries depends exclusively on traditional medicines for primary health care needs. One such plant to full fill the present day needs is Carambola (*Averrhoa carambola* L.) a multipurpose, drought resistant evergreen tree commonly known as "kamrakh" belonging to family Oxalidaceae, is gaining lot of importance for its therapeutic potentials and widely used in Ayurvedic preparations.

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The various parts of the plant have been explored as antioxidant, analgesic, anti-inflammatory, hypoglycemic, hepato-protective, anti-microbial and anti-ulcer activity[8]. The phyto-constituents are reported to be present in the plant are mainly flavonoids, alkaloids, tannins and saponins, which are responsible for pharmacological effects[19]. In countries like India, China and Brazil various parts of the tree is used as a broad spectrum medicine on several aliments. Carambola fruit is also rich in antioxidants, Potassium, Copper folate, Panthothenic acid, Vitamin C, Sodium and low in sugar and acids. It is also a potent source of both primary and secondary polyphenolic antioxidants. It is believed to have originated in Ceylon and the Moluccas, but it has been cultivated in Southeast Asia and Malaysia from ancient times[12]. The perennial herb is commonly grown in Malaysia, Taiwan, Thailand, Israel, Florida, Brazil, Philippines, China, Australia, Indonesia, sub-tropical regions of India and Bangladesh[7]. The tree is around 10 -12 m in height producing two types of fruit, which are ellipsoid light green or yellow colour fruits one with sour and acidic taste and another one is sweet and less acidic[11,20]. Several phyto-chemical studies are in progress to investigate individual bioactive compounds responsible for pharmacological effects. Recently Carambola fruit has also gain importance as exotic fruit in international markets. Therefore to promote Carambola cultivation commercially, selected improved line of Carambola germplasm is being grown and evaluated at experimental plot of Central Horticultural Experiment Station, Chettalli, Karnataka, India. In September 2016, anthracnose disease was observed on different selected improved line of Carambola germplasm, due to high rainfall and change climate, the level of percent disease incidence differed significantly and occur in severe farm on leaves and fruit and it was ranged from 40 to 60%. Therefore the present study was aimed to identify the causal agent of anthracnose associated with leaf and fruit disease of Carambola.

MATERIAL AND METHODS

Collection of disease samples: The infected leaves and fruit showing leaf spot symptoms was collected from Carambola germplasm maintained at experimental plot in Central Horticultural Experiment Station Chettalli, Karnataka, India (75.8°E longitude; 12.5°N latitude). The location is situated in Western Ghats of India, which receives an annual rainfall of 1500 mm which is spread over a period of more than 100 days with peak period between July and September. Scattered rain occurs during winter months. In general, the temperatures ranges from 19 ^{0}C to 32 ^{0}C. The coldest month is December-January while the maximum temperature is observed during March-April**.**

Isolation and purification of the pathogen: The selected spots from infected leaves and fruit of Carambola were washed 3 to 4 times in sterilized distilled water and then surface sterilized by dipping in 4% sodium hypochlorite (NaOCl) solution for 1 min, followed by washing with sterilized water for 4 to 5 times. Surface sterilized leaf spot pieces were then aseptically transferred into 9 cm Petri dishes containing potato dextrose agar (PDA) and incubated at 25±2°C for seven days. Thereafter, pure culture of pathogen was subcultured by selecting growing mycelia tip on PDA and aseptically transferred into another petri plate containing PDA medium, where it was grown for 15 days at 23±2°C in the BOD incubator. On the basis of their conidiophore and conidial morphology, the pathogen was identified as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc, and was purified by single spore isolation. The isolated fungal pathogen culture was maintained on PDA slants at 4°C for further use.

Fungal materials and microscopy: The anthracnose infected leaves and fruit samples of Carambola and purified pure culture of fungus conidial morphology was examined under the light microscope (Nikon Eclipse 50*i*). The 12 days old culture of the fungus grown on PDA was scraped and placed on a glass slide containing a drop of sterile water.

The specimen was observed at 400X magnification under a microscope.

Pathogenicity test: Ten ml of sterilized distilled water was added to 12 days old well sporulated pure cultures of *C. gloeosporioides* and gently swirled to mix well. Resulted conidial and mycelial suspension was filtered through two layers of sterilized muslin cloth and the concentration of conidia was adjusted to 1 x 10^6/ml using a hemocytometer**.** Matured, detached Carambola fruits were first washed with running tap water for 1 min and wiped with 70% ethanol. Then washed three times with sterilized distilled water and dried with sterile tissue papers[15]**.** Surface sterilized fruits were inoculated by wound/drop method as described by Lin et al.[10]. The inoculated fruits and controls were incubated in moisture chambers for 10 days at 27^0C. Relative humidity was maintained around 95%, placing sterilized cotton ball soaked in sterilized distilled water[18]**.** Subsequently anthracnose symptoms developed on fruit surfaces were used to re-isolated the pathogen, confirming Koch's postulates. The test was repeated twice.

Fungal genomic DNA purification: The pure culture of the fungus was grown on Potato Dextrose Broth at 25 ± 2 ºC for 7 days. The fungal mycelium was harvested by filtration through Whatman No.1 filter paper and washed with sterile distilled water and dried. Two grams of dried mycelium was used for total genomic DNA isolation by following modified protocol of CTAB method[3].The quality of the genomic DNA was checked on 0.8% agarose gel and stored at -20^0C till further use.

PCR amplification: To confirm identity of the pathogen, total genomic DNA was amplified by PCR using universal internal transcriber spacer region primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3')[21]**.**

The DNA amplification was performed with 35 cycles with cycling conditions of denaturation for 1 min at 94^0C, primer annealing for 45 seconds at 55^0C, and primer extension for 90 seconds at 72^0C, with an initial denaturation at 94^0C for 3 min and a final extension for 15 min at 72^0C. The PCR reactions were carried out in a Gene Amp PCR system 9700 (PE Applied Biosystems, Foster City, CA) thermocycler. The final volume of 25 µL PCR mix containing 2 µL DNA template, 1.5 U *Taq* polymerase, 25 mM $MgCl_2$, 2 mM dNTPs and 25 pmole of each primer were taken in a PCR tube. PCR products were electrophoresied (1 h at 80 volts) in 1.2% agarose gel in Tris-borate-EDTA buffer, pH 8.0. Gels were stained with ethidium bromide (10 mg/mL) and were visualized and documented by Alpha digidoc1000 system (Alpha Innotech Corporation, USA). The amplified PCR products (550bp) were purified by gel extraction kit (Qiagen) and sequencing was done by Eurofins Genomics India Pvt. Ltd (Karnataka, India).

Sequence analysis: Sequence similarity searches were performed by comparing sequences available in the database using BlastN[1]. The sequences showing highest scores with the present isolates were obtained from database[2] and aligned using SEAVIEW program[6]. The sequence identity matrixes for the ITS-region of the fungus were generated using Bioedit Sequence Alignment Editor (version 5.0.9)[9] and phylogenetic tree was generated by MEGA 6.01 software[17] using the neighbour joining method with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously.

RESULT AND DISCUSSION

Symptoms: Initially anthracnose disease symptoms were noticed on leaves, producing small brown colour circular spots (Fig 1a). These spots turn into lesions with ash colour centre demarcated with black margin. Later the spots coalesce to form large number of irregular lesions. On fruits tiny, slightly depressed light to dark brown spots were observed which expand and make fruit soft. On soft fruit acervuli was found in lesions which were orange colour. Speckle also appears on fruit as tiny brown or black spots that are visible soon after the fruit set and until harvest (Fig 1b).

Morphological characterization

Arial mycelium of the *Collectotrichum gloeosporioides* was whitish and occurred as tufts in some places. Huge amount of orange,

sessile conidial masses were observed mainly at the centre of the culture (Fig 2a). Reverse side of the culture was pale orange in the centre and off-white towards outer region. Conidiogenous cells were hyaline, cylindrical to clavate. Conidia were hyaline, single celled, (8-20 × 2.5-5 µm) either cylindrical with obtuse ends or ellipsoidal with rounded apex and a narrow, truncate base (Fig 2b). Grey to light brown, dark walled appressoria were clavate, oval or irregular shaped and variable in size. Acervuli develop in lesions on leaves, branches and fruits and conidia in acervuli remain viable for long period. On the basis of colony morphology and conidial characters isolate was initially identified as *Collectotrichum gloeosporioides.*

Pathogenic characters

Fruits inoculated with *Collectotrichum gloeosporioides* started showing disease symptoms two days after inoculation and at the 5th day typical anthracnose lesions were observed with fruit discoloration.

Molecular characterization

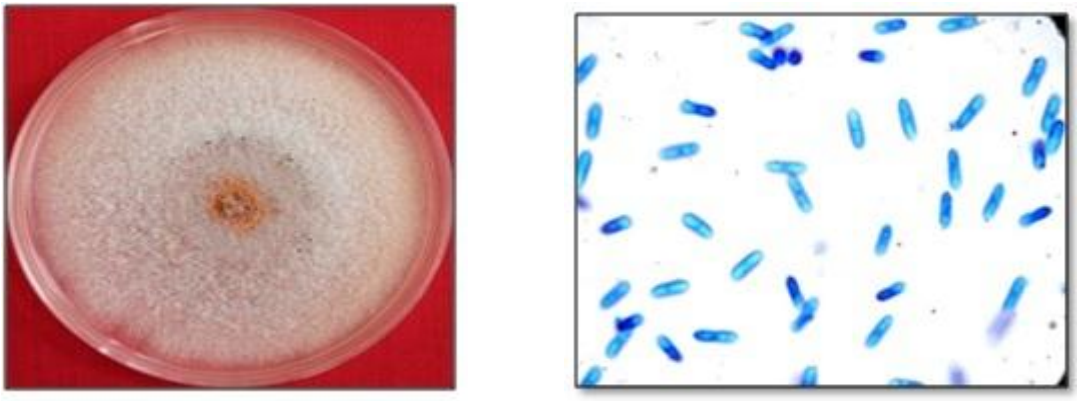
The total genomic DNA of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc was amplified by using universal Internal transcriber spacer region (ITS) specific primers. The expected PCR amplicon size of 550bp was amplified (data not shown). The amplified PCR product was cloned and sequenced. The sequence is available in database under following accession number, KU933356. The ITS sequence of *C gloeosporioides* isolated from carambola was compared with *Colletotrichum* species available in the database[2]. The sequence analysis showed that the carambola isolate had 100% similarity with *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.infecting tobacco crop.

The phylogenetic analysis was carried out by comparing the Internal transcribed spacer of *C. gloeosporioides* with other corresponding region of different *Colletotrichum* species available in database[2]. The result revealed that, the Carambola isolate formed close cluster with *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc (Fig 3). Therefore, the fungus that caused anthracnose on Carambola was identified as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc based on morphological, pathogenicity test and amplification of ITS region.

Carambola or Star fruit is a quintessential exotic fruit tree originally from tropical Asia, is now grown in many tropical and subtropical regions of the world due to it multipurpose therapeutic potentials. The main carambola producing areas are in Taiwan, Malaysia, Indonesia, Hawaii, Florida, and India[5]. Although India is one of the largest producers of carambola, it's still, the crop considered as minor fruit in this country due to its production in restricted area, popularity and awareness among the people, although it is a very important crop and contributes a good share of economy in many localities. The fruit tree is largely cultivated in hilly regions up to 1,200 meters in warm, moist climates, primarily in the southern states and along the west coast, extending from Kerala up to West Bengal. The main constrains for production of carambola is anthracnose. The disease is caused by *Colletotrichum gloeosporioides* produces different kind of symptoms such as speckle, black spot and scab disease on fruits. The pathogen is found to be infecting all the above ground parts of the tree *i.e.* flowers, leaves, twigs and fruits. In India the disease was first reported by Rana and Upadhyaya[14]. Though, the crop has been grown in India from ancient time but still it has not caught the required pace in cultivation. In many areas the fruit are produced on very small plots or as a backyard trees. As Carambola cultivation increases around the world and new cultivars are introduced, it is logical to assume that diseases will increase importance. In countries like Malaysia were the fruit is grown predominantly, anthracnose disease is reported as one of the important fruit disease. The disease is severe especially during rainy season when temperature and humidity is congenial for dispersal of conidia[16]. The primary source of inoculums for *C. gloeosporioides* is infected fruits that produce conidia[4]. Therefore further studies are required on etiology and epidemiology of anthracnose caused by *C. gloeosporioides* to contain the disease effectively.

A B C

Fig. 1: Carambola tree showing the symptoms of anthracnose on (a) Leaf, (b) fruits under natural conditions



A B

Fig. 2: (a) Pure culture of *Colletotrichum gloeosporioides* on Potato dextrose agar, (b) Conidia having pointed ends of *Colletotrichum gloeosporioides*

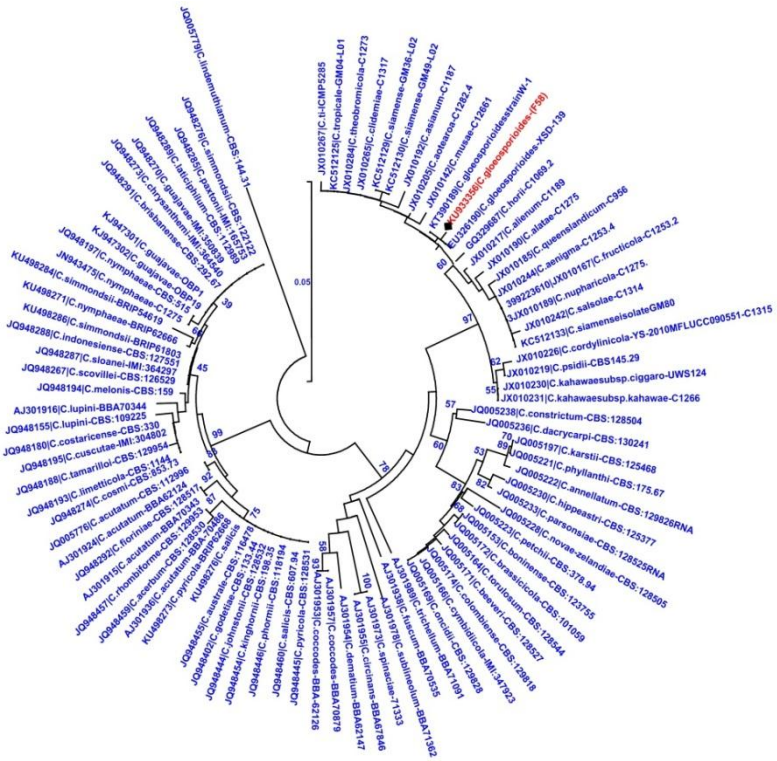


Fig. 3. Phylogenetic trees based on sequences of ITS region of *Colletotrichum gloeosporioides* with corresponding ITS regions of other *Colletotrichum species* using Neighbor-joining algorithm. Horizontal distances are proportional to sequence distances, vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1000 replicates was performed and the bootstrap percent values more than 50 are numbered along branches. The GenBank accessions of different *Colletotrichum* strains used for analyses in this study as given by Alvarez et al. (2014).

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Conflict of Interest statement

The authors declare that they have no conflict of interest.

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