

## First Report of *Collectotrichum acutatum* J.H. Simmonds Associated with Anthracnose Disease in Malayan Apple from India

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### ABSTRACT

The genus *Collectotrichum* contains an extremely diverse number of fungi including both plant pathogens and saprophytes. Plant pathogenic species are important worldwide, causing diseases commonly known as anthracnose of vegetables, fruits, and perennial tree crops. Anthracnose diseases appear in developing fruit in the field (pre-harvest) and those damaging mature fruit during storage (post-harvest). The anthracnose disease infected samples of Malayan apple fruit and leaves were collected from Central Horticultural Experiment Station, Chettalli, Karnataka state of India. The fungus was isolated from infected leaves and fruits by standard fungal isolation method. The Koch's postulates of the fungus were confirmed by re-inoculated on the fruit and leaves of the same host. Further the fungus was identified based on pathogenic, morphological and amplification of internal transcribed spacer (ITS) region by PCR using universal ITS specific primers. The amplified PCR product were cloned and sequenced. The sequence analysis showed that Malayan apple isolate was showed maximum nucleotide identity of 100% with *Collectotrichum acutatum* infecting *Lupinus albus* flowering plant.

**Key words:** Fungi, PCR, Malayan apple, Species

### INTRODUCTION

Malayan apple (*Syzygium malaccense* Merr. & Perry.) is a non-climacteric tropical fruit species. It is also known as red guava, Malay rose apple, jambu bol or ball guava. The fruits are pear-shaped usually pink, light-red, red, green, sometimes greenish white, or cream-colored and are generally crisp, with a subtly sweet taste or aromatic flavor<sup>15</sup>. The species

presumably originated in Indo-Malaysia region or Southeast Asia. The tree species is being cultivated for long time in tropics especially in Indo-Malaysia, Southeast Asia, Melanesia, Polynesia and Macronesia<sup>26</sup>. There are three species of *Syzygium*, namely the Malay apple (*Syzygium malaccense*), water apple (*Syzygium aquem*) and wax apple (*Syzygium samaragense*) bear edible fruits.

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The delicious fruit can be eaten directly and is known to be beneficial for human health, due to its significant levels of vitamins and minerals, organic compounds, flavonols, phenolic compounds, and sesquiterpenes, as well as limited amounts of carotenoids, all of which act as antioxidants<sup>11</sup>. The tree is also widely used in traditional medicines. Basically, it was grown as an ornamental tree in south east Asia, but due to increase in acceptance of exotic fruits in international fruit market the fruits crop has been grown in parts of West Bengal, Kerala, Karnataka and Tamil Nadu states of India.

A few selected superior germplasm of Malayan apple is maintained at experimental Plot in Central Horticultural Experiment Station Chettalli, Karnataka, India are showing symptoms of anthracnose on leaves, flowers and fruit. But, in the past two years, the disease incidence was increasing sharply in severe farm on new flash of leaves, flowers and fruits. The level of incidence differed significantly about 50 to 60 % on different accession of Malayan apple. Therefore the present study aim was to identify the causal agent of anthracnose observed on Malayan apple in Karnataka.

## MATERIAL AND METHODS

**Collection Isolation and maintenance of *Collectotrichum acutatum*:** The Malayan apple infected leaves, flowers and fruit samples with typical anthracnose symptoms were collected from Malayan apple germplasm maintained at experimental Plot in Central Horticultural Experiment Station Chettalli, Karnataka, India (75.8°E longitude; 12.5°N latitude with an annual rainfall of 1500 mm which is spread over a period of more than 100 days with peak period between July and September-2016). Pieces of the diseased tissues 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 several rinses with sterile distilled 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, the pure culture *Collectotrichum acutatum* was isolated 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 as described by Simmons<sup>20</sup> the pathogen was identified as *Collectotrichum acutatum* J.H. Simmonds, and purified by single spore isolation method. Monoconidial culture fungus was maintained on potato dextrose agar (PDA) slants at 4°C

**Fungal materials and microscopy:** The anthracnose infected leaves and fruit samples of Malayan apple and purified pure culture of fungus conidial morphology was examined under the light microscope (Nikon Eclipse 50i). The 15 days old of culture of the fungus was grown PDA were 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:**

Pathogenicity test of *Collectotrichum acutatum* was conducted on symptomless, detached Malayan apple fruits and leaves. The leaves and fruits were surface-sterilized with ethanol (70%) and then washed three times with sterilized distilled water and dried with sterile tissue papers. The surface sterilized fruits were inoculated with 15 µl of the conidial suspension (10<sup>6</sup> conidia /ml, adjusted with Haemocytometer) by creating the wound with sterile needle as described by Lin et al.<sup>14</sup>. Control fruit and leaves were inoculated with 15µl of sterile distilled water. The fruits and leaves were then incubated in a plastic container at 25°C and >95% relative humidity, and examined for lesion development 5 days after inoculation. After 7 days, anthracnose symptoms development on fruit surfaces was re-isolated from the disease fruit and leaves

and subcultured onto PDA plates, which were incubated at 25°C in darkness. The resultant cultures were checked for colony and spore morphology to confirm Koch's postulates. The test was repeated twice.

**Fungal genomic DNA purification:** The pure culture of the fungus grown on PDB at 25 ± 2 °C 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 by following modified protocol of CTAB method<sup>3</sup>. The isolated DNA was dried under vacuum, resuspended in 50 ml of TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0). The quality of the genomic DNA was checked on 0.8% agarose gel and stored at -20°C for further use.

#### PCR amplification

Furthermore to confirm the exact identity of the pathogen, total genomic DNA was amplified by PCR using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTA TTGATATGC-3')<sup>27</sup>. The DNA amplification was performed with 35 cycles with cycling conditions of denaturation for 1 min at 94°C, primer annealing for 45 seconds at 55°C, and primer extension for 90 seconds at 72°C, with an initial denaturation at 94°C for 3 min and a final extension for 15 min at 72°C. 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<sub>2</sub>, 2 mM dNTPs and 25 pmole of each primer were taken in a PCR tube. PCR products were electrophoresed (1 h at 80 volts) in 0.8% 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 sequence to sequences available in the database using BlastN<sup>1</sup>. The sequences showing highest scores with the present isolates were obtained from database and aligned using SEAVIEW program<sup>8</sup>. The sequence identity matrixes for the big bud phytoplasmas were generated using Bioedit Sequence Alignment Editor (version 5.0.9)<sup>10</sup> and phylogenetic tree was generated by MEGA 6.01 software<sup>24</sup> using the neighbour joining method with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously.

## RESULT AND DISCUSSION

**Symptoms:** The infected leaves showed a small black lesion that coalesces and gives scorched appearance to the canopy (Fig 1a). On fruit sunken concentric rings, which are pinkish in color around 5mm in diameter were observed<sup>4</sup>. These lesions may coalesce entire surface of the fruit, which leads to inside pulp become giving bad odour (Fig 1c). The area of lesions is firm at first, but eventually become soft and putrid. The infection of anthracnose was also observed on emerging buds of flowers, as small black lesions later coalesce to form big lesion may leads to premature dropping flower at early stage, thereby reducing fruit set (Fig 1b).

#### Morphological characterization

Colonies of *Collectotrichum acutatum* were dense aerial, initially white or cream white, becoming gray and then turning dark gray, as the cultures aged on PDA (Fig 2 a). Colony reverse was white to pinkish in colour. Bright orange spore masses were produced outward from the center of the colony. The cultures developed black acervuli around the centre of the colony. Fungal colonies on PDA medium grew to 70-80 mm diameter in one week at room temperature (25 ± 2°C). The isolated

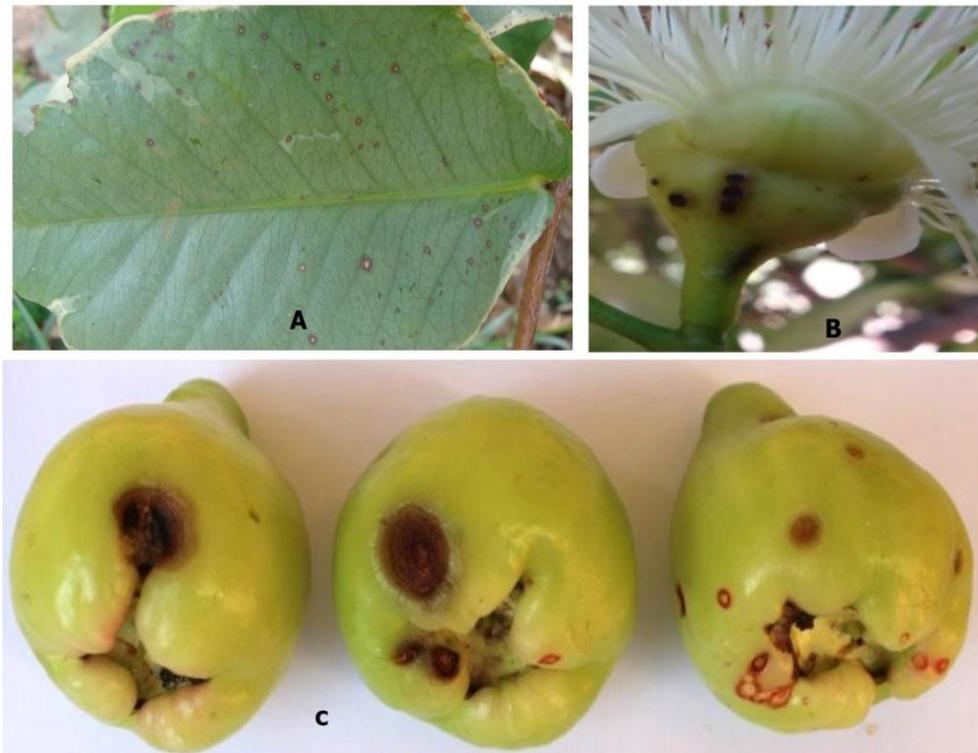
pathogen produced orange spore mass on plates. Mycelial was branched, septate and hyaline. Conidia were hyaline, one-celled, straight, smooth, fusiform, (8-15 × 2.5-5 µm) with pointed ends (Fig 2 b). Conidiophores are branched at base, hyaline in early stage later turning into brown colour. Acervuli are superficial without setae. The fungus was initially identified as *Collectotrichum acutatum* J.H. Simmonds, based on characters like culture morphology and conidium shape and size<sup>21,22,6</sup>. The cultures did not form sectors after 10 days of incubation.

### Molecular characterization

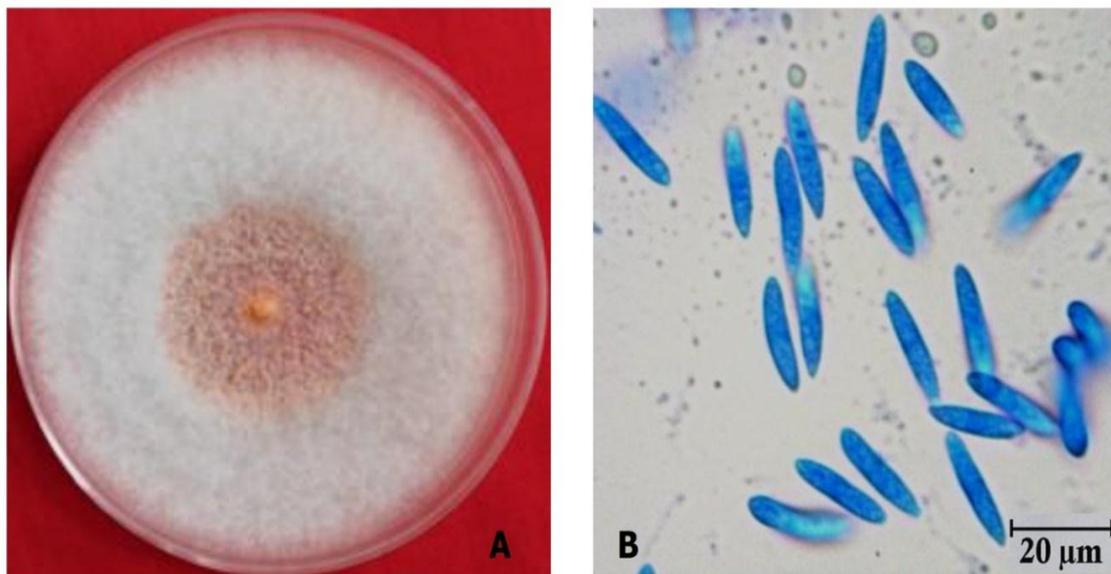
The total genomic DNA of *C. acutatum* was amplified by using universal intergenic transcriber spacer region (ITS) specific primers. The expected PCR amplicon size of 550bp was amplified (data not shown). The amplified PCR product was cloned and sequenced. The sequence is available in GenBank (NCBI) with the accession number, KU933355. The ITS sequence of *C. acutatum* isolated from Malayan apple was compared with other *Collectotrichum* species available in the database<sup>2</sup>. The sequence analysis showed that the isolate showed 100% similarity with *Collectotrichum acutatum* infecting *Lupines albus* and *Fragaria* sp. The phylogenetic analysis was carried out by comparing the Internal transcribed spacer of *C. acutatum* with other corresponding region of different *Collectotrichum* species available in database<sup>2</sup>. The result revealed that, the Malayan apple isolate formed closely cluster with *C. acutatum* infecting *Lupines albus* and *Fragaria* sp. (Fig 3). Therefore, the fungus that caused anthracnose on Malayan apple was identified as *Collectotrichum acutatum* based on morphological, pathogenicity test and amplification of ITS region.

Malayan apple has become widely cultivated in recent years as a Indian traditional herb in northeast and south India. The main drawback of this production is

anthracnose and fruit fly under natural condition. Therefore to effective management of the disease, one should know the morphogenic symptom of the disease on the plant as well as pathogen. In the present study Malayan apple showing the typical anthracnose symptoms was confirmed by infecting of *Collectotrichum acutatum* based on phenotypic traits (colony appearance, and character of vegetative and reproductive structures) and sequences of rDNA ITS region. The colour of cultures may vary considerably within and between species of *C. acutatum* and *C. gloeosporioides*. Colonies of *C. gloeosporioides* were usually gray in appearance, while *C. acutatum* colonies had a chromogenic (pink) or nonchromogenic (white to gray) phenotype<sup>7,12,6,28</sup>. *Collectotrichum acutatum* causes anthracnose diseases on numerous host plants in worldwide<sup>5</sup>. Originally the disease was described on *Papaya*, *Capsicum* and *Delphinium ajacis* in Australia by Simmonds<sup>19</sup>. Today *C. acutatum* species complex is known infect many fruits like strawberry<sup>9</sup>, Citrus<sup>16</sup>, Apple<sup>13</sup>, Olive<sup>23</sup>, Cranberry<sup>18</sup> and Blueberry<sup>25</sup>. The pathogen also found to be infecting avocado, bread fruit, Carambola, citrus, fig, guava, kiwifruit, lychee, mango and papaya<sup>17</sup>. The information would be helpful for growers to manage the disease successfully in Malayan apple. Anthracnose caused by *C. acutatum* is an emerging disease that may threaten the profitability of Malayan apple as well other fruits crops considering its broad host range. More likely that the inoculum can disseminate from one crop to another and survive for long duration in the under fields. Additional studies on etiology and epidemiology of anthracnose caused by *C. acutatum* are needed to define further the disease management strategies. To our knowledge, this is the first report of *Collectotrichum acutatum* J.H. Simmonds infected Malayan apple in India.



**Fig. 1: Malayan apple showing the symptoms of anthracnose on (a) Leaf, (b) fruits, (c) flower under natural conditions**



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

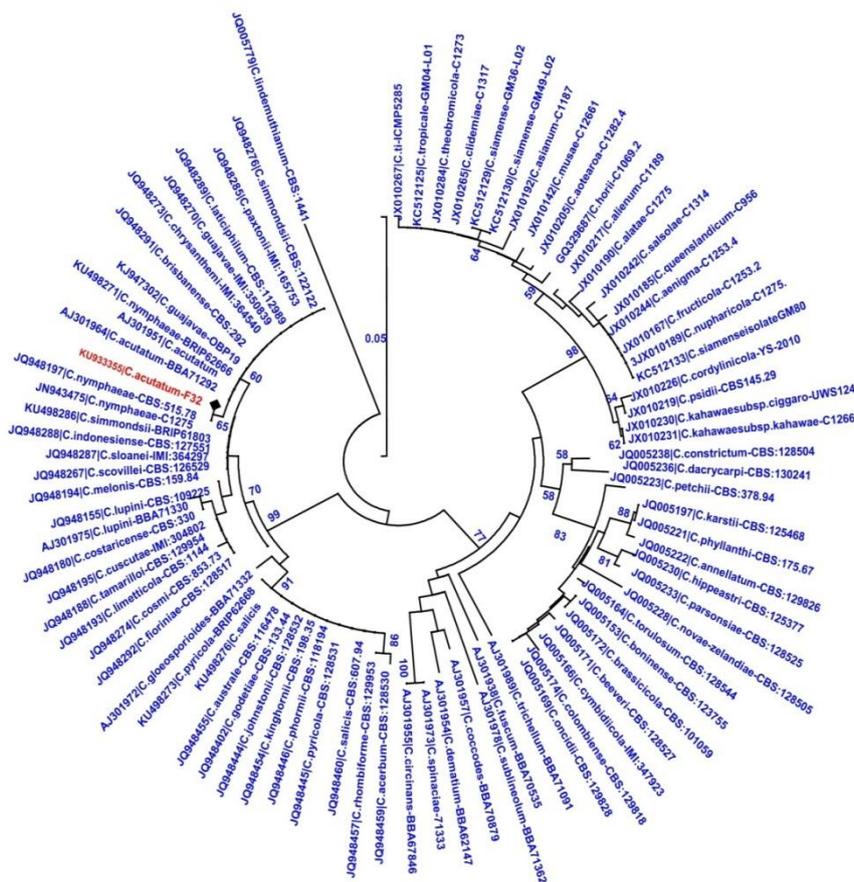


Fig. 3: Phylogenetic trees based on sequences of ITS region of *Colletotrichum acutatum* with corresponding ITS regions of other *Colletotrichum* species using Neighbor-joining algorithm. Horizontal distances are proportional to sequence distances, vertical distances are arbitrary. The trees are unrooted. 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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