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Isolation, identification, molecular characterisation and proving pathogenicity of *Colletotrichum musae* causing anthracnose of banana

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Abstract
Banana (*Musa* sp.) is an important fruit crop in tropical and sub-tropical regions. The crop is well known as "Kalpataru" (Plant of Heaven), Apple of Paradise and it's known as Adams Fig due to its socioeconomic importance and variety of uses. The crop is prone to various diseases. Banana anthracnose, caused by a fungus *Colletotrichum musae* (Berk. & Curtis) is the most vital pre and post-harvest disease, which is known to reduce marketable fruit yield of 30-40%. In this context, the disease effected banana fruit was collected from KRCCH, Arabhavi, was used for fungus isolation and identified the fungus based on cultural, morphological and sequence analysis of two genomic regions [internal transcribed spacer (ITS) region and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] and further confirmed its pathogenic nature by inoculating the conidial suspension of 4×10^6 spores/ml on healthy fruits.

Keywords: Cultural, morphological, primer and conidial suspension

Introduction
Banana (*Musa* sp.) is an important fruit crop in tropical and sub-tropical regions. It originated from humid tropical regions of Indochina and Southeast Asia. It belongs to the family Musaceae (Simmonds, 1962) [27]. Presently, it represents the fifth most economically important food crop after rice, wheat, maize and potato (FAOSTAT, 2022) [9].

It is renowned for its age and has a long history, dating back to the beginning of civilization in India. The crop well known as "Kalpataru" (Plant of Heaven), Apple of Paradise and it's known as Adams Fig due to its socioeconomic importance and variety of uses. It is nutrient rich and inexpensive among fruits grown in the country and is available year-round compared to other seasonal fruits (Saurabh *et al*. 2024) [26].

Globally, the banana is cultivated in 120 countries, among which India ranks first in production and banana is the fourth largest fruit crop in the world and is cultivated in an area of 5.6 million ha, with an annual production of 135.11 million metric tons (Anon., 2024). In India, it is cultivated throughout the year and tops the list after mango and citrus in terms of production. It is cultivated in an area of 9.94 lakh ha with a production of 366.14 lakh MT and a productivity of 36.83 tons/ha. Maharashtra, Andhra Pradesh, Tamil Nadu, Uttar Pradesh, Gujarat and Karnataka are the top banana producing states of India. In Karnataka, it is grown in 994.14 thousand ha area with a production of 36614.04 thousand metric tons and a productivity of 23.8 metric tons per hectare. The major banana growing districts of Karnataka includes, Chamarajanagara, Mysuru, Shivamogga, Bellary, Chitradurga and Belagavi (Indiastat, 2023) [13]. The banana fruit is prone to a varied range of biotic and abiotic stresses that threaten the banana cultivation. Among the biotic stresses, Panama wilt (*Fusarium oxysporum* f. sp. *cubense*), Yellow sigatoka (*Mycosphaerella musicola*), Black sigatoka or black leaf streak (*Mycosphaerella fijiensis*), Anthracnose (*Colletotrichum musae*), Cigar end tip rot (*Verticillium theobromae*), Crown rot (*Colletotrichum musae*, *Fusarium* sp., *Verticillium theobromae*, *Botryodiploidia theobromae* and *Nigrospora sphaericu*), Stem-end rot (*Thielaviopsis paradoxa*), Pseudostem heart rot (*Botrydiplodia* sp., *Gloeosporium* sp., and *Fusarium* sp.,).

Banana tip over (*Erwinia carotovora*), Bacterial wilt or Moko disease (*Ralstonia solanacearum*), Banana bunchy top virus (BBTV), Banana streak virus (BSV), Banana bract mosaic (BBMV), Infectious chlorosis (CMV), Banana burrowing nematode (*Rodopholus similis*) which are serious concerned in the terms of yield reduction (Agrios, 2005) [1].

Banana anthracnose, caused by the *C. musae* (Berk. & Curtis) is the most vital pre and post-harvest disease, which results in marketable fruit loss of 30-40% (Bazie *et al*. 2014) [6]. It depreciates the nutritive value of the fruits and makes them unfit for marketing and consumption apart from reducing its fruit quality and shelf life (Mirshekari *et al*., 2012) [17]. Most of the cultivated banana are more prone to the pathogen infection. The sexual stage of the *C. musae* is *Glomerella musae*, which produces perithecia with limited number of asci, which contain typically 8 ascospores, which are one or two celled with a central oil globule (Berk. & Curtis) Arx, (1957) [5].

The *C. musae*, which infects young fruits in the field but remains quiescent until fruit ripens, rapid germination of fungal spores and produces appressoria under suitable conditions (low temperature and high humidity) leads to the development of anthracnose symptoms. The typical symptoms of disease become evident at the ripening stage. The major symptoms of the anthracnose appear on fruit peel as brown to black spots, which becomes sunken lesions with salmon or orange-colored acervuli. Additionally, wound anthracnose can severely damage the fruit if it develops during transport and reducing the quality of the bananas before they reach the ripening stage (Thangamani *et al*., 2011) [29].

The present investigation on banana anthracnose includes isolation and identification and proving pathogenicity of the pathogen causing anthracnose of banana. Information obtained from cultural and morphological studies are insufficient to exactly identify any fungal isolate, it may causes overlapping and misidentification of the isolate. Thus, it is necessary to use molecular techniques to compensate for the limitations of cultural and morphological characterization.

Materials and Methods

Fungal isolation and identification

The present investigations were carried out during 2023-2024 in the Department of Plant Pathology, K.R.C.C.H, Arabhavi, Karnataka state.

Colletotrichum musae isolate was isolated from banana fruits showing typical symptoms of anthracnose disease. Pathogen was isolated using standard tissue isolation technique on PDA media as described by Prashanth (2007) [22] with minor modifications. Infected tissues of banana fruits along with healthy tissue portions were cut into pieces of 1-2 mm in size. The tissue pieces were then surface sterilized with 0.1 per cent sodium hypochlorite or 70 per cent ethanol for about 15 seconds. Subsequently, the bits were transferred into petri dishes containing double distilled water and then placed on sterile tissue paper to eliminate excess moisture. The bits were then transferred to petri plates filled with 15 ml of potato dextrose agar with sterilized needles and forceps and monitored regularly for the fungal development. The petri dishes were incubated at 27±1 ºC for 8 days. Pure culture of the fungus was obtained by the single spore isolation on two per cent water agar. The fungus was identified and maintained on PDA medium, with active growing colonies being sub cultured at intervals of 20-25 days. Pathogen was identified based on spore morphology, colony characters and molecular characterization.

Molecular characterization of *Colletotrichum* sp

Fungal isolate was cultured in 150 ml Erlenmeyer flasks containing potato dextrose broth and was incubated at 27±1 °C for 4 days. After incubation, well developed mycelial mat was collected by filtration. Genomic DNA extraction was conducted by Cetyl trimethyl ammonium bromide (CTAB) method as the protocol outlined by Murray and Thomson (1980) [19] and Raeder and Broda (1985) [23] with certain modification. DNA was qualified by means of 0.8 per cent agarose gel electrophoresis. The quantity of DNA was analyzed using Nano drop (Nanodrop ThermoFisher). The quality of the DNA sample was judged based on whether DNA shows clear, sharp band (good quality) or over smear (Poor quality/degraded) under the gel documentation unit. The ITS region of the rDNA was amplified using primers mentioned in table 1. The PCR reactions were carried out in a PCR mixture mentioned in table 2. The DNA amplifications were performed in a thermocycler (*Eppendorf mastercycle* Vapo. Protect 96 wells) using the cycle parameters as mentioned in table 3 and table 4. The PCR product was sent to Juniper Life Sciences Pvt. Ltd, for sequencing. Based on the chromatogram, noisy portions were removed using the BioEdit sequence alignment editor. Edited sequences were submitted as a nucleotide query at the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). Sequences of the isolate was deposited in GenBank of NCBI. By using the MEGA 11 software with the Kimura 2-parameter model a phylogenetic tree was constructed using the maximum likelihood method. The tester isolate of *C. musae* which was isolated and the reference isolate of different *Colletotrichum* species were also used on the tree for comparison with the isolate generated in this study.

Table 1: Sequences of primers used in the study

Primers	Sequence 5’ – 3’	Reference
ITS 1 F	5’ TCC GTA GGT GAA CCT GCG G 3’	White *et al*. (1990) [31]
ITS 4 R	5’ TCC TCC GCT TAT TGA TAT GC 3’	
GD F	5’ GCC GTC AAC GAC CCC TTC ATT GA 3’	Huang *et al*. (2021) [12]
GD R	5’ GGG TGG AGT CGT ACT TGA GCA TGT 3’	

Table 2: Composition of PCR reaction for amplification of different regions of *Colletotrichum*

Sl. No.	Reaction mixture	Quantity (µl)
1	10X Taq Enzyme assay buffer (NEB, M0273L)	2.0
2	dNTPs mixture (2 mM)	2.0
3	Taq DNA polymerase (5U/ µl)	0.23
4	Primer (5 pM) F and R	2.0
5	Template DNA (100 ng/ µl)	1.0
6	Distilled water (nuclease free)	12.77
Total volume (µl)		20

Table 3: PCR programme followed for amplification of ITS region of *Colletotrichum*

PCR stage		Time	Temperature (ºC)	Cycles
Pre PCR	Hot start	3 min.	94	1
PCR	Denaturation	45 sec.	94	35
	Annealing	45 sec.	54	
	Extension	1 min.	72	
Post PCR	Final extension	7 min.	72	1
Hold at 4 ºC				

Table 4: PCR programme followed for amplification of GAPDH primers of *Colletotrichum*

PCR stage		Time	Temperature (ºC)	Cycles
Pre PCR	Hot start	3 min.	95	1
PCR	Denaturation	30 sec.	95	35
	Annealing	30 sec.	55	
	Extension	45 sec.	72	
Post PCR	Final extension	5 min.	72	1
Hold at 4 ºC				

Pathogenicity assay of *Colletotrichum musae*

The fungal inoculum of conidial suspension was prepared by adjusting the conidial concentration to 4×10^{-6} cfu/ml by adding sterile double distilled water to the inoculum. Fully mature banana fruits were brought from the research field, KRCCH, Arabhavi, Belgaum district. Later, the fruits were inoculated with the help of an automizer by spraying with a spore suspension of 4×10^{-6} cfu/ml. The fruits were covered with polythene bags for 48 hours to maintain sufficient humidity and to ensure successful penetration of the pathogen into the host tissue. Control fruits were maintained by spraying with sterile distilled water in a similar manner. Observations were recorded for the appearance and development, re-isolation was done from the artificially infected fruits. The isolate obtained was compared with the original culture to confirm its pathogenic nature.

Results and Discussion

Symptomatology

The symptoms initially appear on green fruits as brown sunken spots, as the fruit starts ripening, characterized dark brown to black lenticular sunken lesions with a pale margin of variable sizes are formed; these lesions coalesce to become sizable sunken patches on fruit peel after ripening. Under favourable moist conditions, on the infected fruit tissue they may bear salmon coloured acervuli, severely affected fruits become blackened and rot. (Plate 1). Comparatively similar observations were observed by Gaikwad *et al*., (2000) [10] in apple, Lim *et al*. (2002) [15] in banana, Anthony *et al*. (2004) [4] in banana, Ranasinghe *et al*. (2005) [24] in banana, Chillet *et al*. (2007) [8] in young bananas, Thangamani *et al*. (2011) [29] in banana, Zhimo *et al*. (2017) [32] in immature banana, Saurabh *et al*. (2023) [26] reported similar symptoms in banana.

Isolation and identification of pathogen

The fungus was isolated from banana fruit exhibiting typical symptoms of anthracnose using the standard tissue isolation technique on PDA medium. The pure culture was obtained through the single spore isolation method. On potato dextrose agar, the fungus appeared whitish to salmon pink and developed septate mycelium. The conidia were cylindrical and straight with rounded ends, every so often featuring one to two oil globules at the tips. On the pure culture plate, the conidia formed small pink clusters, acervuli was observed at the back of petri plate. (Plate 2). Based of the above findings the pathogen was preliminarily identified as *Colletotrichum musae*. Similar exertion has been done by Naik (1985) [20] and Bhat (1987) [7] isolated *Colletotrichum* sp. by single spore isolation technique and identified as *C. musae* (Berk. and Curt.) Arx based on the morphological and cultural characteristics (Thangamani *et al*., 2011) [29], Lim *et al*. (2002) [15] isolated *Colletotrichum* using tissue segment method on PDA, Peres *et al*. (2002) [21] isolated by tissue segment method, Prashanth and Sataraddi (2011) [22] isolated *C. gloeosporioides* from pomegranate, Sakinah *et al*. (2014) [25] isolated thirty-eight *Colletotrichum* isolates from banana, Unnithan and Thammaiah (2017) [30] isolated *C. musae* from dark-brown anthracnose lesions on banana (*Musa* sp.).

Molecular characterization of *C. musae*

The total genomic DNA of the *Colletotrichum* sp. was analysed using ethidium bromide (10 mg/ml) stained 0.8% agarose gel in Tris-acetate-EDTA buffer (pH 8). The clear DNA bands were pictured with an agarose gel documentation unit and utilized for subsequent analysis.

The total genomic DNA of *Colletotrichum* isolate underwent PCR amplification using ITS and GAPDH primers. The resulted PCR amplicon was approximately 616 base pairs for the ITS and 257 base pairs for the GAPDH of *Colletotrichum* isolate was amplified. The amplified PCR products were sequenced in both forward and reverse directions using the same primers and depicted in plate 3 and 4, respectively on gel electrophoresis.

The analysis revealed that isolate KRCCHACM is indeed *C. musae*, assigned accession number PQ345387 for ITS primer and PQ664489 for GAPDH primer. It exhibited a high similarity of 99.43 and 100.00 per cent to a previously deposited sequence of *C. musae* ZEHCM (OP010082.1) and *C. musae* isolate ICMP:18701 (JX010047.1), respectively. To illustrate the genetic relationships built on the ITS rDNA and GAPDH gene sequences, the phylogenetic tree was constructed using the maximum likelihood method.

Isolate name	Accession number	Per cent similarity
KRCCHACM	PQ345387	99.43
KRCCHACM	PQ664489	100.00

The phylogenetic tree illustrates the evolutionary relationships among several species of *Colletotrichum*. The combined ITS+GADPH dataset for the *C. musae* species complex comprised 22 strains, including an out-group taxon from the *Monilochaetes infuscans* CBS 869.96. The aligned data matrixes comprised 873 characters (ITS: 616 and GAPDH: 257). The *Colletotrichum musae* KRCCHACM formed clade with reference strains of *Colletotrichum musae* CBS 125356 and *Colletotrichum musae* CBS 192.31 (plate 5)

The results were aligned with the findings of Peres *et al*. (2002) [21] identified based on molecular characterisation using ITS4 and ITS5 primers, Sakinah *et al*. (2014) [25] employed ITS and β-tubulin primers, obtained per cent similarity of 98 to 100% for ITS regions and 97 to 100% for β-tubulin, Mosca *et al*. (2014) [18] identified different species of *Colletotrichum* using species specific primers (Coll1F-Coll3R), Sudarma *et al*. (2021) [28] obtained 650 bp using ITS1/ITS4 primers and obtained homology of 98-100%, Ma

et al. (2022) studied molecular characteristics using species specific primer genes (ACT and GAPDH) and obtained 300 bp and 250 bp respectively, Kurera *et al*. (2023) [14] identified *C. musae* using primer pairs of ITS1 and ITS4, GD92F1 and GDR1, GSF1 and GSR1 and, BT2a and BT2b.

Pathogenicity test

The inoculated fruits developed characteristic symptoms of banana anthracnose, closely resembling those observed in natural conditions. Typical symptoms appeared within 4 to 5 days post-inoculation. The fungus was productively reisolated from the inoculated fruits, fulfilling Koch's postulates and exhibited morphological characteristics consistent with the original isolate. (Plate 7). Similar observations were observed by Griffee and Burden (1976) [11], Peres *et al*. (2002) [21] proved pathogenicity of *C. musae*, Prashanth and Sataraddi (2011) [22] proved pathogenicity of *C. gloeosporioides* in pomegranate, Unnithan and Thammaiah (2017) [30] reisolated *C. musae* from artificially inoculated banana fruits and proved its pathogenicity.

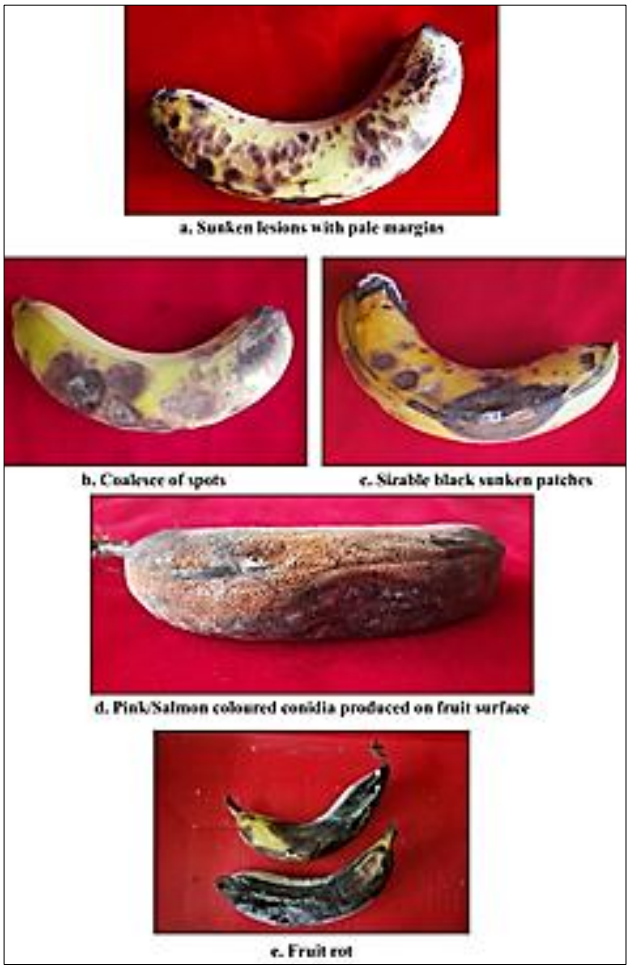

Plate 1: Symptomatology of anthracnose disease caused by *C. musae*

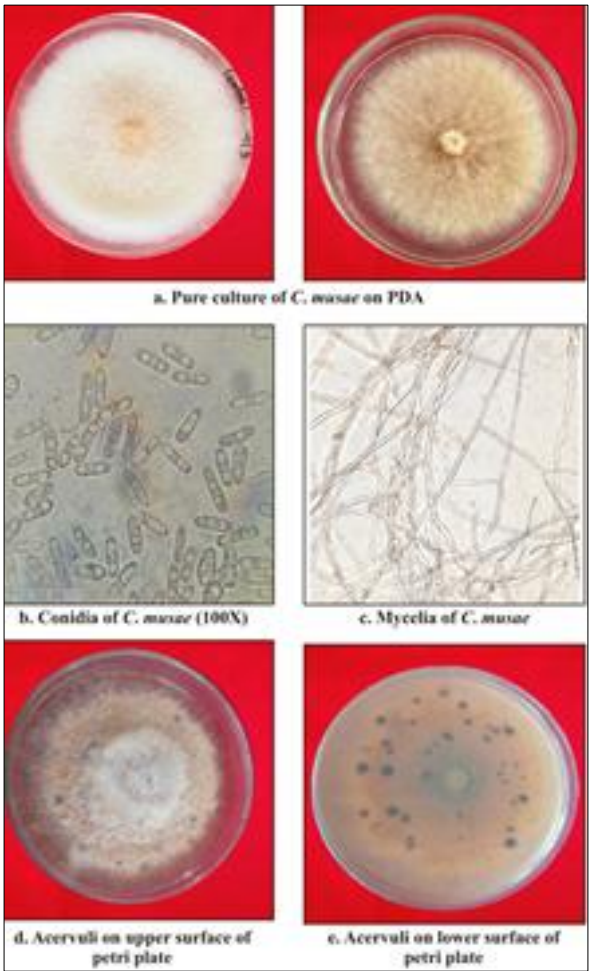

Plate 2: Morphological characteristics of *C. musae* on potato dextrose agar

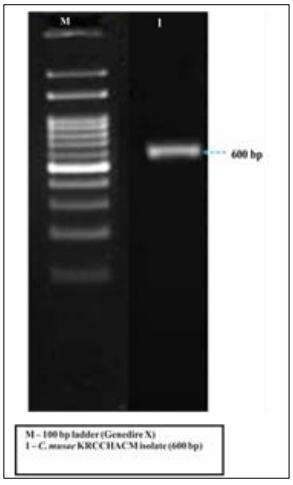

Plate 3: Gel picture of PCR amplified ITS region of *C. musae* using the primer pairs of ITS 1 and ITS 4

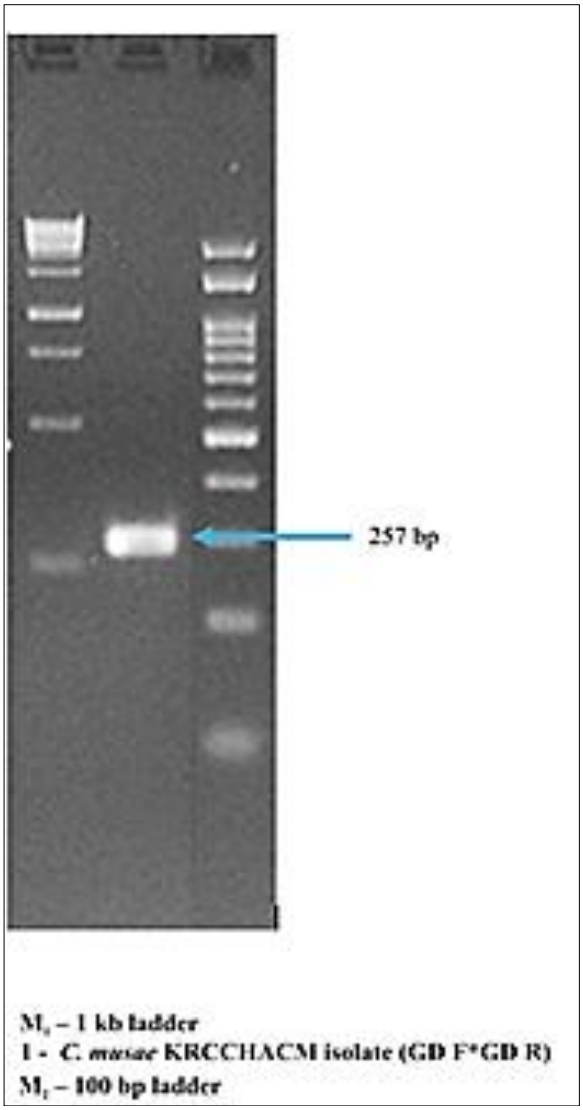

Plate 4: Gel picture of PCR amplified *C. musae* using the primer pairs of GD F and GD R

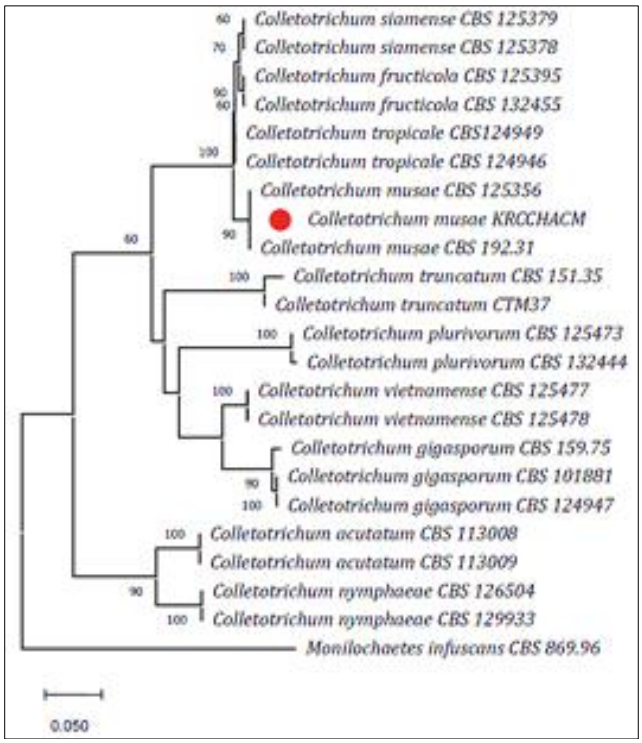

Plate 5: Phylogenetic relationships in the *C. musae* isolate KRCCHACM based on combined ITS+GAPDH loci using maximum likelihood method. The isolate derived from the present study is marked in red.

Plate 6: Proving pathogenicity of *C. musae* on banana fruits

Conclusion

The study identified *Colletotrichum* sp. associated with anthracnose of banana in Belagavi region of Karnataka. Based on molecular characterisation, pathogen was identified as *Colletotrichum musae* using two different primers *viz*., ITS and GAPDH primer pairs and yielded 600 bp and 257 bp, respectively, amplicon for *C. musae*. According to phylogenetic analysis, *C. musae* KRCCHACM isolate showed 99.43 and 100.00 per cent similarity to the earlier deposited sequence of *C. musae* ZEHCM (OP010082.1) and *C. musae* isolate ICMP:18701 (JX010047.1), respectively and further confirmed its pathogenic nature by inoculating the conidial suspension of 4×10^6 spores/ml on healthy fruits.

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