Original Research Article

Morphological and molecular characterization of *Colletotrichum gloeosporioides* causing mango anthracnose

ABSTRACT

Mango (*Mangifera indica*), a fruit with high nutritional value is widely regarded as a most palatable fruit crop which is affected by number of biological constraints mainly diseases. Anthracnose caused by *Colletotrichum gloeosporioides* belongs to order *Melanconiales* is the important post harvest disease which restricts marketing of mango. In the present study, thirteen isolates obtained from various places of Tamil Nadu were collected and isolated by tissue segment approach and purified. The isolates were confirmed phenotypically using morphological characters. Molecular investigation like PCR assay using universal primers ITS1 and ITS4 produced amplicon size of 560bp. The isolates were also identified using genus specific and species specific primers which resulted in amplicon size of 280bp and 380bp. As a result, The use of morphological and molecular approaches to characterise Mango anthracnose will be useful in identifying and managing the disease.

Keywords: *Mangifera indica*; *Colletotrichum gloeosporioides*; Molecular characterization

1.INTRODUCTION

Mango (*Mangifera indica*) is considered as one of the most important tropical or subtropical fruit crops. In India, Mango is cultivated in area of 223 Lakh ha. with the production of 20336 MT with the productivity of 8.7MT/ha. The production of mango affected by a large number of biotic constraints of which, diseases caused by fungal pathogens are the major yield limiting factor. Among the diseases, Mango anthracnose caused by *Colletotrichum sp*. affects mango production both in pre and post harvest stages and reduced the yield to a extent of 5-20 percentage [11, 16]. Anthracnose disease is clearly identified by morphological symptoms like leaf spot, die back and fruit rot [22]. Sometimes, the symptoms are masked due to latent infection of pathogen due to absence of favourable environmental conditions [11]. Hence, symptom based diagnosis is not accurate and reliable. Mango anthracnose incited by several species of Colletotrichum including *Colletotrichum gloeosporioides, Colletotrichum acutatum, Colletotrichum kahawe, Colletotrichum asianum, Colletotrichum fructicola, Colletotrichum siamense* etc [13,14]. The *Colletotrichum sp*. associated with mango anthracnose in Tamil Nadu where poorly studied. Identification of pathogen associated with mango anthracnose through morphological and molecular characterization will helpful to take up proper management practices. In the present study, the pathogens associated with mango anthracnose were isolated and they were characterized morphologically and reconfirmed with molecular assays.

2. MATERIALS AND METHODS

2.1 Isolation of *Colletotrichum sp.* infecting mango

A total of thirteen mango fruits infected showing symptoms of anthracnose disease were collected from different region of Tamil Nadu during 2021-2022 and fungus associated with the disease was isolated using Tissue segment method [18, 20, and 22]. The infected mango fruits were surface sterilized with 70% ethanol and cut into thin sections. The infected sections were surface sterilized with 1% sodium hypochlorite to eliminate the saprophytes. The sections were placed onto Potato Dextrose Agar Medium and incubated at 25 °C for 7 days [1,20-22] . The fungal growth was examined from the infected portion and subcultured on PDA slants and stored at 4 °C for further studies [14].

2.2 Cultural and morphological characterization

Fungal isolates isolated from the infected mango fruits were grown on the PDA medium at a constant temperature of 25 °C for a period of 7 – 10 days[1,14]. Fungal mycelial characters, Colony character, Growth pattern and Zonations were recorded for all the isolates [15,10,20]. The conidial size of different fungal isolates was measured under 40X Magnification using Phase contrast microscope and the images were captured using the software Leica LAS version 4.11.0 (Switzerland).

2.3 Molecular confirmation of *Colletotrichum sp.*

2.3.1 Genomic DNA extraction

Using a modified CTAB method. Total DNA was isolated from the *Colletotrichum sp*. Mycelial mats [5]. A mortar and pestle were used to macerate about 100mg of dried mycelial mats using CTAB buffer (10 percent CTAB, 1M Tris base, 5M Nacl, 0.5 M EDTA). The mixture was transferred to microfuge tubes, vortexed for 2 minutes, and then incubated at 65 °C for 20 minutes. An equal amount of phenol, chloroform and isoamyl alcohol (25:24:1) was added to the mixture and centrifuged at 13,000 rpm for 10 min. After incubation, the supernant was transferred to a new Eppendorf tube and to which double the volume of ice-cold isopropanol was added and incubated at -20°C overnight. After an overnight incubation period, the tube was centrifuged for 10 min at 13,000 rpm. The DNA pellet was treated with 70% ethanol, and the tube was air dried and resuspended in 30 µL of double distilled water. The amount of genomic DNA in the sample was quantified using NanoDrop spectrophotometer (Thermo Scientific. Wilmington, DE) to measure and quantify the samples.

2.3.2 PCR amplification using universal primers and genus specific primers

A PCR assay was performed for molecular confirmation of *Colletotrichum sp.* Using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 primers (5'-TCCTCCGCTTATTGATATGC-3')[4,11]. Temperature profile followed for the amplification of ITS region is initial denaturation of 94 °C for 3 minutes followed by 35 cycles of denaturation of 94°C for 1 minute, annealing at 55 °C for 1 minute, extension of 72 °C for 1 minute and final extension at 72 °C[9,11]. A highly conserved Actin genomic region was targeted and detected using primers such as ACT 512F

(ATGTGCAAGGCCGGTTTCGC) ACT 783R (TACGAGTCCTTCTGGCCCAT) [3,14]. The PCR program for amplification of ACT genomic region including initial denaturation of 94°C for 2 min, followed by 35 cycles of denaturation of 94°C for 1 minute, annealing at 60°C for 45 seconds, extension of 72 °C for 1 minute and final extension at 72°C for 10 minutes[14]. All PCR amplification reaction mixture consisted of 5 µL of 2X master mix, 1 µL each of forward and reverse primers, 1 µL of genomic DNA (50 ng/ µl), and 2 µL of nuclease free water. The amplified PCR product was confirmed using 1 percent agarose gel dissolved in 1X TAE buffer amended with 2 µL of EDTA at 70V for 1 hr. The results were visualized using gel documentation unit (MultImage TM light cabinet, USA). PCR amplified product of ITS region was partially sequenced and the sequences were submitted to Genbank database and accession numbers were obtained.

2.3.3 Molecular Confirmation of *Colletotrichum gloeosporioides* using species specific primer

Molecular confirmation of *Colletotrichum gloeosporioides* isolates were performed using PCR reaction using species specific primers developed by Kamle et al (2013). The primer used in this study were CgF (TTGCTTCGGCGGGTAGGGTC) and CgR (ACGCAAAGGAGGCTCCGGGA) [11]. The PCR reaction mixture consists of 5 µL of 2X master mix, 1 µL each of Forward primer and Reverse primer, 1 µL of template DNA and 2 µL of nuclease- free water. The PCR program including initial denaturation of 94°C for 2 minutes followed by 35 cycles of denaturation of 94°C for 1 minute , annealing at 60°C for 45 seconds, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes[11,12]. Finally, amplified products were confirmed by 1% agarose gel dissolved in 1X TAE buffer amended with 2 µL of EDTA at 70V for 1hr and documented in gel documentation unit.

3. RESULT

3.1 Isolation of pathogen associated with Mango anthracnose

The location of mango anthracnose samples collection and their GPS Coordinates were presented in Table 1. A total of 13 fungal isolates were consistently isolated from the mango fruits showing typical symptom of Mango anthracnose. The colony characters of the fungi varies from white fluffy to dark grey mycelial growth. The mycelia are hyaline, septate and branched. Among the thirteen fungal isolates, four isolates produced zonations in the culture plate were as others did not record any concentric zonations. All the fungal isolates produced single celled, cylindrical or dumbbell shaped conidia with the size varies from 9.9 – 17.19 in length, 3.2 – 6.1 in breadth (Table 2). Based on the cultural and morphological characters of fungal mycelium and conidia, The fungal isolates were identified as *Colletotrichum gloeosporioides.*

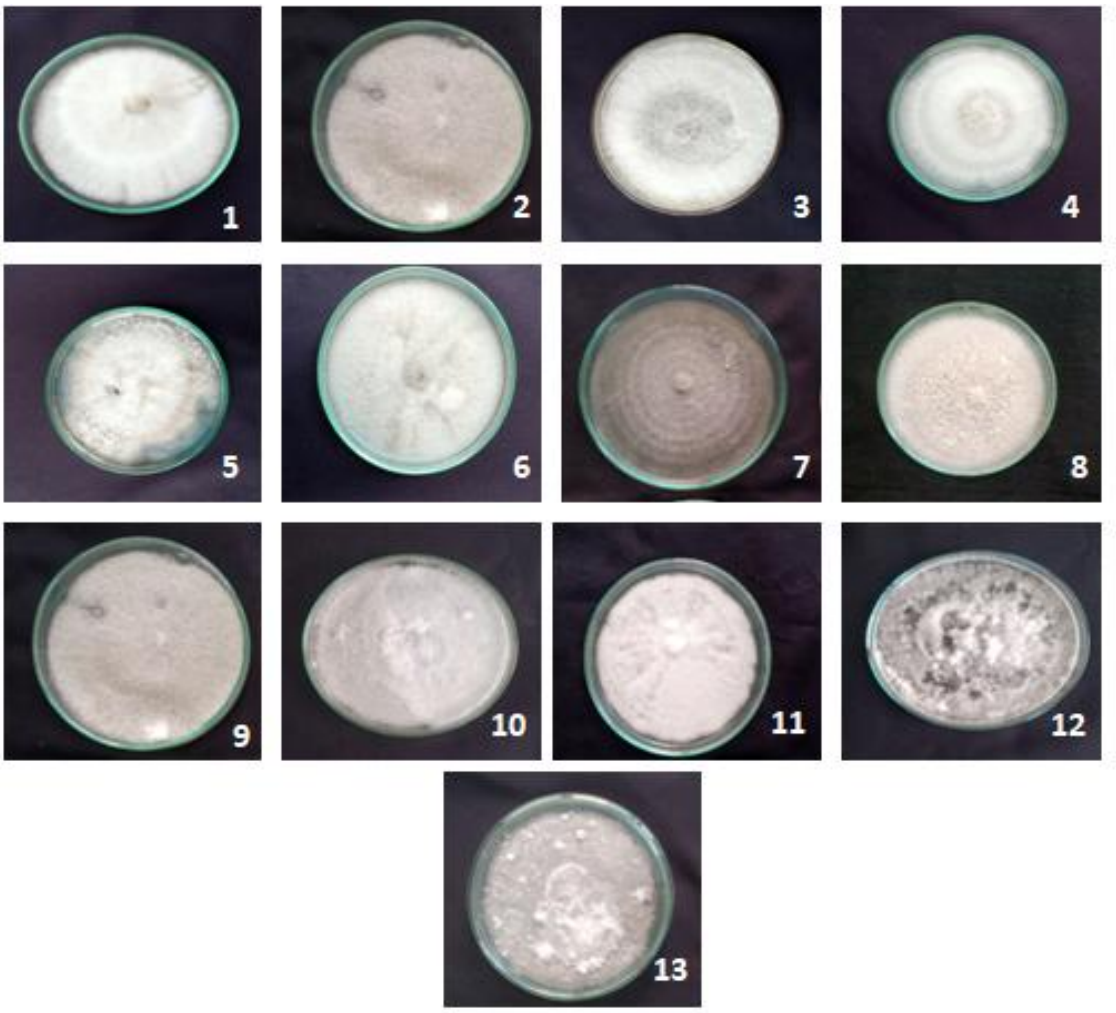

Figure 1. Morphological characters of *Colletotrichum gloeosporioides* isolates 1- CBE 1, 2- SVG 1, 3- CBE 2, 4- CBE 3, 5- VG 1, 6- TH 1, 7- CBE 4, 8- VG 1, 9- KR 1, 10- PL1, 11- SA 1, 12- SA 2, 13- MDU 1.

Table 1. Location and GPS Coordinates of the Mango anthracnose samples collection

Isolate	Location	District	GPS Coordinates
CBE 1	Kondayampalayam	Coimbatore	11° 04' 19.4" N 76° 93' 51.4" E
SVG 1	Karaikudi	Sivaganga	10° 05' 48.86" N 78° 80' 89.29" E
CBE 2	TNAU Farm	Coimbatore	09° 42' 14.8" N 77° 49' 46.8" E
CBE 3	Saravanampatti	Coimbatore	11° 07' 40.75" N 76° 99' 61.16" E
VG 1	Velanganni	Thanjavur	10° 40' 83.8" N 79° 69' 25.4" E

TH 1	Thenkasi	Thirunelveli	09° 06' 86.8" N 77° 32' 94.1" E
PL 1	Pollachi	Coimbatore	10° 66' 09.1" N 77° 00' 48.12" E
CBE 4	Annur	Coimbatore	11° 22' 18.12" N 77° 10' 57.82" E
MDU 1	Kallupatti	Madurai	09° 42' 08.7" N 77° 49' 42.5" E
SA 1	pappampadi	Salem	11.64'44'' N 77.57'5.4° E
SA 2	Tharamangalam	Salem	11.67'40.6'' N 77.58'4.8'' E
KR 1	Hosur	Krishnagiri	12° 74' 09.1" N 77° 82' 53.8" E
ER 1	Sathyamangalam	Erode	11.50'34.5'' N 77.2444° E

Table 2: Cultural and morphological characters of Mango anthracnose pathogen *Colletotrichum gloeosporioides*

Isolate	Colony character	Zonation	Conidial size µm
CBE 1	White fluffy mycelial growth	Present	9.95 x 4.78 µm
SVG 1	Pale grey mycelial growth	Absent	13.10 x 5.83 µm
CBE 2	White fluffy at margin and grey at centre	Absent	12.89 x 4.45 µm
CBE 3	White, smooth mycelial growth	Present	12.44 x 4.32 µm
TH 1	White to grey mycelium	Present	9.95 x 4.253 µm
CBE 4	Greyish white fluffy mycelial growth	Absent	15.60 x3.751 µm
VG 1	Dark grey mycelial growth	Present	17.19 x 5.99 µm
KR 1	Pale grey mycelial growth with White margin	Absent	14.73 x 5.73 µm
PL 1	Pale grey fluffy mycelial growth	Absent	12.67 x 6.10 µm
SA 1	White flat mycelial growth	Absent	11.54 x 4.67 µm
SA 2	Whitish grey fluffy mycelial	Absent	10.76 x 5.23 µm

	growth		
ER 1	Thick black mycelial growth	Absent	9.87 x 4.65 µm
MDU 1	Blackish flat mycelial gowth	Absent	10.87 x 5.43 µm

3.2 Molecular characterization of *Colletotrichum gloeosporioides*

In the present study, All the thirteen fungal isolates were subjected to molecular characterization using Polymerase Chain Reaction by using universal ITS 1 and ITS 4 primers. The PCR products of fungal isolates are subjected 1 percentage gel electrophoresis assay, Which yielded an amplicon size of approximately 560 bp (figure 2). The amplicon of PCR assay was further partially sequenced and the sequences were submitted to NCBI Genbank database and accession numbers were obtained (Table 3). From the accession numbers, all the 13 isolates were confirmed as *Colletotrichum gloeosporioides*. Though, PCR amplification of ITS region is one of the most widely used molecular technique. For species level identification of the fungus, it is not highly specific. Hence, we have targeted ACTIN gene present in *Colletotrichum sp.* and also *Colletotrichum gloeosporioides* and amplified through the PCR reaction using ACT gene primers such as ACT512F, ACT783R and species specific primers CgF and CgR. The PCR reaction of above primers yielded an amplicon size *of* approximately 280bp and 380bp (Figure 3&4) respectively.

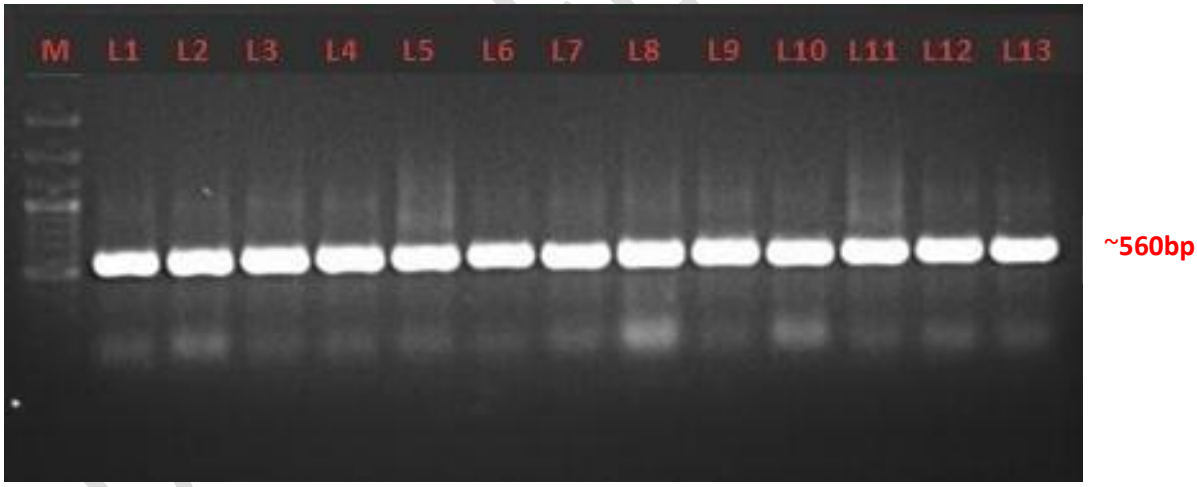

Figure 2. PCR product of ~560bp amplified from Genomic DNA of *Colletotrichum gloeosporioides* isolates using ITS 1 & ITS 4 primers. L1-L11 *Colletotrichum gloeosporioides* isolates M- 100bp DNA Ladder (Genei Pvt. L. Bangalore).

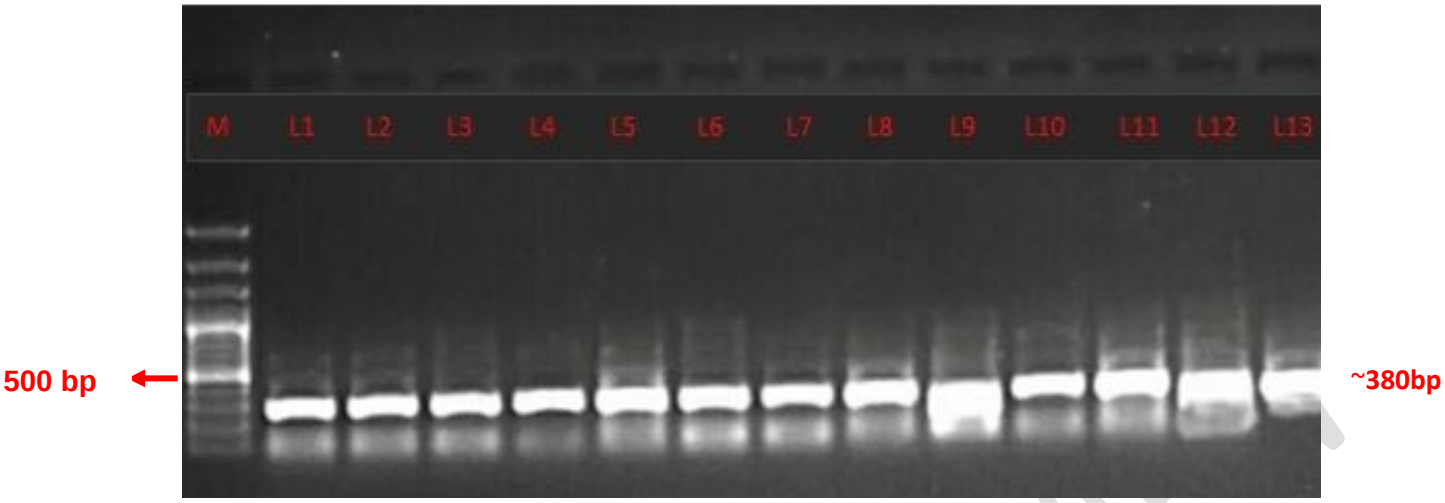

Figure 3. PCR amplification of C. gloeosporioides species specific primers amplified a band of ~380bp. L1-L13: Colletotrichum gloeosporioides isolates. M- 100 bp DNA Ladder.

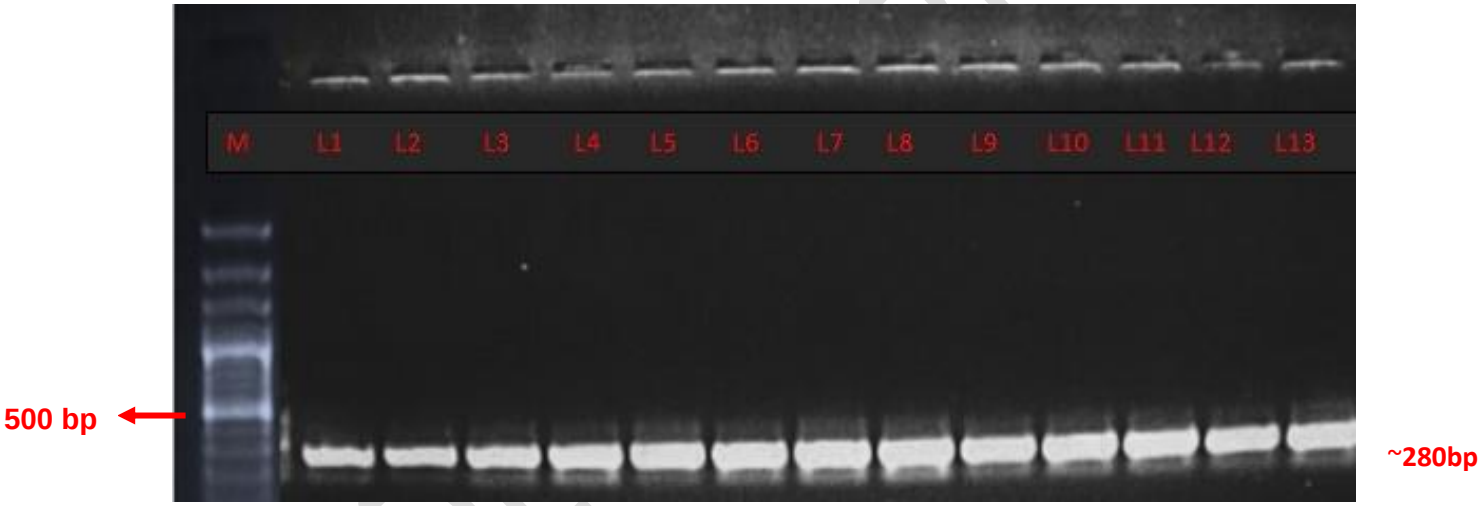

Figure 4. PCR amplification of Colletotrichum gloeosporioides ACTIN genomic region showing amplicon size of ~280bp L1-L13 : *Colletotrichum gloeosporioides* isolates amplified ~280bp. M- 100bp DNA Ladder.

Table 3 : NCBI Genbank accession numbers of *Colletotrichum gloeosporioides* isolates:

S.No	Isolates	Accession number
1	KR 1	ON025567
2	CBE 1	ON025546
3	PL 1	ON025539
4	TH 1	ON025542
5	CBE 2	ON176165
6	CBE 3	ON176197
7	VG 1	ON176571
8	CBE 4	ON176680
9	SVG 1	ON176198
10	ER 1	ON606233
11	MDU 1	ON614154
12	SA 1	ON714922
13	SA 2	ON714930

4.DISCUSSION

Colletotrichum is the most important and common fungal pathogen causing anthracnose in fruit, vegetables and ornamental crops [7]. Several species of *Colletotrichum* were found associated with mango anthracnose like *Colletotrichum gloeosporioides, C. acutatum* [2]*, C. asianum, C. fructicola, C. siamense*[14].

In the present study, thirteen fungal isolates of *C. gloeosporioides* were isolated from the mango fruits showing typical symptom of anthracnose disease. The colour of the colonies showed variations like white, pale grey, dark grey and black with fluffy or smooth mycelial growth with or without zonations. The conidial size of different isolates of *Colletotrichum sp.* also showed variations. Morphological characters such as colony colour, conidial shape, conidial length and width, mycelial growth rate for the identification of *C. gloeosporioides [15]*. However, the existence of variation in the cultural characteristics and conidial size of *C. gloeosporioides* was reported from Bangladesh [1]. The cultural characteristics of *Colletotrichum* species associated with mango anthracnose and found that existence of variation in an conidial size of different *Colletotrichum* species [13]. Disadvantages of morphological characters in identified *C. gloeosporioides* isolates and their morphological features express differentially on the media and host plant which lead to confusion on the identification on species level [6]. Identification of fungal pathogens based on Polymerase Chain Reaction using Internal Transcription Spacer region and species specific primers is now widely used especially for identifying the symptomless infection [8,17].

In the present study, thirteen isolates of *Colletotrichum* species were confirmed as *Colletotrichum gloeosporioides* through PCR assay using ITS1 and ITS4 primers, *Colletotrichum* genus specific primers *and C. gloeosporioides* specific primers.PCR assay yielded 560bp for ITS 1 and ITS 4 universal primers, 280bp for *Colletotrichum* gene specific primers and 380 bp for species specific primers. From the PCR assay with species specific primer, it was confirmed that all the thirteen fungal isolates belongs to *Colletotrichum gloeosporioides*. Thirty fungal isolates associated with mango anthracnose as *C. gloeosporioides* through PCR assay using species specific primer from Lucknow, India [11]. They also validated the species specific primers with other *Colletotrichum species* like *C. truncatum, C. acutatum, C.musae* and *C. capsici* was found that, these primers specifically amplified the *Colletotrichum gloeosporioides* DNA with the amplicon size of 380 bp. The use of species specific primer in PCR assay for the identification of Colletotrichum gloeosporioides was also reported [7,11,16,19,].

5. CONCLUSION

The fungal associated with mango anthracnose in Tamil Nadu was identified by *Colletotrichum gloeosporioides* by morphological characters such as colony morphology and conidial morphology. The fungus was confirmed by PCR assay using universal primer, genus specific and species specific primers. The PCR products of all thirteen isolates were partially sequenced and accession numbers were obtained from NCBI Genbank.

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