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Morphological and molecular characterization of *Colletotrichum gloeosporioides* (penz) sac. isolates causing inflorescence die-back and leaf spot disease in arecanut

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Abstract
Inflorescence die-back and leaf spot disease is an economically important disease of areca nut across the world. The causal agent of the disease i.e., *Colletotrichum gloeosporioides* has often been confused with other *Colletotrichum* species, especially *C. acutatum*. To confirm the disease roving survey was conducted in major areca nut growing districts *viz*. Chikkamagaluru, Shivamogga and Davanagere of Karnataka state. Among the different pathogen isolates isolated, four isolates were characterized through morphological and molecular means. The data revealed that, all the isolates were found to be *C. gloeosporioides* and exhibited greyish white colony colour, whereas the reverse side appeared white to dark grey as well as orange colour. The conidia were cylindrical with both apices rounded or with one apex rounded and the other end partly pointed. The polymerase chain reaction (PCR) assay with the *C. gloeosporioides* using species-specific primer (Cg INT) with ITS4 yielded a single band of 450bp. Nucleotide sequences of the Cg INT region of the ribosomal DNA of all isolates had 99% homology with *C. gloeosporioides*. Thus, the present study proved the association of *C. gloeosporioides* in causing inflorescence die-back and leaf spot disease through detached leaf technique under *In-vitro*.

Keywords: Inflorescence die-back, leaf spot, areca nut, *Colletotrichum gloeosporioides*

Introduction
Areca nut (*Areca catechu* L.) is a palm belongs to the family Arecaceae, which is grown in most parts of the tropical and subtropical regions of Asia and East Africa. It is thought to be originated from Malaysia or Philippines. Areca nut is extensively cultivated in the plains and foothills of Western Ghats and North Eastern regions of India. Area and production in different states indicate that Karnataka, Kerala and Assam account for more than 90 per cent of the total output in India. (Kulkarni and Mulani, 2004) [4].

Profitable cultivation of arecanut is becoming very difficult in recent years. As areca nut palms are vulnerable to various abiotic and biotic stresses resulting drastic reduction of the yield. Since areca nut is a perennial crop, it is exposed to various pests and diseases around the year at all crop growth and development stages. Among the diseases that infects areca nut, inflorescence die back and leaf spot disease caused by *C. gloeosporioides* is an emerging and serious disease of areca nut crop. Wide variations in cultural and morphological characters, pathogenicity and host range have been reported among isolates of *C. gloeosporioides*. The objective of the present study was to understand the pathogen involved in Inflorescence die-back and leaf blight disease in areca nut through morphology and molecular characterization using species-specific primers and ITS sequencing.

Material and Methods
Symptomatology
The typical die-back symptom was observed on the inflorescence of arecanut palms. The disease appeared first on the rachillae of male flowers as dark brown patches which soon spread and caused drying and shrivelling of male rachis starting from tip towards base. Eventually shedding of female flowers and young buttons was observed. Light brown patches were observed on the primary, secondary and tertiary rachis and on primary rachis production of concentric rings of pink coloured conidial mass of the pathogen were seen. As the disease progressed, the whole inflorescence dried and was found drooping on the palm without getting detached.

The initial symptoms of the leaf blight disease appeared as small, circular or oblong to irregular brownish spots surrounded by larger yellow halo. As the disease progresses the centre of the spots turns grey or straw colour and in the advanced stages these spots coalesced to give a blighted appearance to the leaves.

Collection and isolation of the pathogen

The areca nut inflorescence showing typical symptoms of inflorescence die back and leaves showing spots and blight symptoms suspected to be infected by *Colletotrichum gloeosporioides* were collected with the help of plant climbers from the farmers field located at three different districts *viz*. Shivamogga, Davanagere and Chikkamagaluru. The specimen was preserved in polytene bags while transmit. Upon bringing to the laboratory specimens were preserved in the refrigerator at 4 ± 1 °C and were utilized for further studies

The standard tissue isolation procedure was followed to isolate the pathogen from the diseased specimens. The infected bits of rachis along with some healthy portions measuring 2 mm to 5 mm were incised from the affected inflorescence and surface sterilized with 0.1 per cent sodium hypochlorite solution for 30 seconds and washed thrice using sterilized distilled water for 2 minutes to remove the traces of sodium hypochlorite if any and then transferred aseptically to sterilized Petri plates containing potato dextrose agar medium. Such Petri plates were incubated at room temperature (27±1 °C) and observed periodically for the growth.

The fungus was isolated from the infected leaves of areca nut following the standard procedures. The infected diseased samples along with healthy tissues were cut into small pieces around 2 mm to 5 mm and surface sterilized by dipping in 0.1 per cent sodium hypochlorite solution for 30 seconds. The treated plant tissues were then washed three times with sterilized distilled water. Excess water was drained down by soaking with sterilized blotting paper. The cut pieces were then placed in Petri dishes containing sterilized PDA (three pieces / dish). These plates were incubated at a temperature of 27±1 °C and the growth was examined four to five days after incubation. The fungus thus obtained was identified using microscopes and upon confirmation of pathogen was purified using single spore isolation method.

Ten ml of clear filtered two per cent water agar solution was poured into sterile Petri plates and allowed to solidify. The dilute spore suspensions were prepared in sterile distilled water from ten days old culture. Two ml of spore suspension was spread uniformly using glass spreaders on water agar medium. After four hours of incubation at 27±1 °C, the plates were examined to locate the presence of germinated conidia. Then a single isolated and germinated conidium was marked with permanent marker on the glass surface of the agar and transferred to PDA slants in such a way that the conidium bearing surface was in contact with PDA surface and incubated at 27±1 °C. The pure culture was sub cultured once in a month and preserved in refrigerator at 4 °C and used for further studies. All the four pathogen isolates were properly labelled and used for further studies.

Proving the pathogenicity

Pathogenicity test was carried out to know whether the fungus is capable of producing typical symptoms of inflorescence die back under artificial inoculations. To prove Koch's postulates healthy unopened areca nut inflorescence was collected from the areca nut plant, washed thoroughly with tap water, swabbed with 0.1 per cent sodium hypochlorite and washed with sterile distilled water. Fungal mycelial bits from seven days old culture was harvested and placed on the inflorescence, cotton moistened with sterile water was placed above the mycelial bit. The inflorescence placed with moistened cotton without fungal cultures above the inflorescence served as the control. Symptom development was observed closely and keenly.

When the typical symptoms of the disease appeared on the inoculated inflorescence of areca nut, re-isolation of the pathogen from artificially induced diseased material was again made and the fungus thus obtained was compared with the original culture. After confirming its identity as *Colletotrichum gloeosporioides*, it was used in all the further studies.

Morphological characterization of *Colletotrichum gloeosporioides* on different solid media

The pathogen isolate used for proving Koch's postulate was utilized to study morphological characteristics

Twenty ml of PDA medium was poured aseptically into 90 mm diameter Petri plates. After solidification, five mm disc of the pathogen was selected from actively growing hyphae of twelve day old culture using a sterile cork borer and a single disc was placed at the centre of Petri dish and incubated at room temperature. Later morphological characters of conidia such as, length, shape, and septation was also recorded using phase contrast microscope.

Molecular characterization of the pathogen isolates

Total genomic DNA from fungal isolates was extracted using the CTAB DNA extraction protocol.

DNA extraction and purification buffers

- 1 M Tris- Cl, pH 8
- 0.5 M EDTA, pH 8
- 4 M NaCl
- 10 per cent CTAB
- 2 per cent β-mercaptaethanol
- Phenol-chloroform-isoamylalcohol (25:24:1)
- Ice-cold isopropanol
- Wash buffer: 70 per cent ethanol

Isolation of DNA from *Colletotrichum gloeosporioides* was done by following the standard CTAB method with certain modifications (Patil, 2009) [5].

1. 2-3 g of fungal mat grown on potato dextrose agar was taken and homogenized using pestle and mortar in liquid nitrogen.
2. To the above solution, 1 ml of DNA extraction Buffer was added and were kept in water bath at 65 °C for 1 hour.
3. The suspension was taken out, allowed for cooling. 1ml of phenol: Chloroform: isoamyl alcohol (25:24:1 ml) was added in centrifugation tube, centrifuged at 10,000 rpm for 15 minutes at 4 °C.
4. The supernatant was taken in a fresh centrifuge tube and 4 µl RNase was added and incubated at 37 °C for 30 minutes.
5. Cooled isopropanol of about 1/3rd volume (300-400 µl) was added and stored at -20 °C overnight.
6. Next day, tubes were centrifuged @ 13,000 rpm for 10 minutes at 4 °C then supernatant were discarded.
7. About 500 µl of 70 per cent ethanol was added for pellet

and again centrifuged at 13,000 rpm for 10 minutes.

8. Then supernatant was discarded, tubes were kept to air dry and pellet was re suspended in 500 µl of TE buffer.

The quality of the DNA isolated was checked and quantified by spectrophotometry.

Polymerase chain reaction (PCR)

The ribosomal DNA (r DNA) unit contains genetic and non-genetic or spacer region. Each repeat unit consists of copy of 18S, 5.83S and 28S like r DNA and its spacer like internal transcribed spacer (ITS). The r DNA has been employed to analyse evolutionary events because it is highly conserved, whereas ITS rDNA is more variable; hence, it was used for investigation.

Details of the primers used in the experiment

Organism	Primer code	Sequence
Specific primer	CgINT -f	5'-GGCCTCCCGCCTCCGGGCGG-3'
	ITS -4- r	5'-TCCTCCGCTTATTGATATGC-3'

PCR condition for ITS region, *Colletotrichum gloeosporioides*

Steps	Specific primer	
	Temp (°C)	Duration (min)
Initial denaturation	94	5
Denaturation	94	1
Annealing	54	1
Extension	72	2
Final extension	72	10
Hold	4	20
No. of cycles Denaturation Annealing Extension	35	

Separation of amplified products by agarose gel electrophoresis

Agarose gel electrophoresis was performed to resolve the amplified product using 1.4 per cent agarose in 1X TBE (Tris Borate EDTA) buffer, 0.5 µ1 ml-1 of ethidium bromide and loading buffer (0.25% Bromophenol Blue in 40% sucrose). Four µl of the loading dye was added to 20 µ1 of PCR product and loaded to the agarose gel. Electrophoresis was carried at 75 V for 1.5 h. The gel was observed under UV light and documented using a gel documentation unit

Sequencing of ITS region

The amplified DNA was sequenced for the four isolates of *Colletotrichum gloeosporioides* to confirm the organism. The sequence homology search was performed using the BLAST program through the internet server at the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov/blast/). Sequences and accession numbers for compared isolates were retrieved from the Gen Bank database. Sequence pair distances among related and different fungal isolates were scored with the Clustal W program and phylogenetic tree analysis was performed with the Mega software.

Results

Symptomatology

Inflorescence die-back disease initially appeared on the tip of the rachillae of male flowers as yellowish lesion followed by dark brown necrotic patches which soon spreads and leads to drying and shrivelling of male rachis from tip to the base. As disease progresses it results in shedding of female flowers and young buttons. Apart from that on primary rachis production of concentric rings of grey coloured conidial mass of the pathogen were also seen. Such infected inflorescence turns black dries, droop and hangs on the stem.

The initial symptoms of the leaf spot disease appeared as small, circular or oblong to irregular brownish spots. The centre of the spots were grey or straw colour surrounded by yellow halo. In the advanced stages spots coalesced to give a blighted appearance to the leaves.

The pathogen was identified based on the colony and conidial characters as well as morphology of the fungus. On PDA the fungus produced dense, cottony, dirty white to greyish mycelium with even margin. Microscopic observations revealed the presence of one celled spores which were hyaline, cylindrical with rounded ends with oil globules. The conidia were oblong or cylindrical or slightly dumbel, hyaline, aseptate with rounded ends and with one or two oil globules. Conidia measured 11.48 - 20.01 µm in length x 4.25- 6.62 µm in length and the average being 15.74 x 5.43 µm. Conidiophores were simple, Filiform measuring 10 to 12.5 µm. Conidia measured 12.78 µm in length x 4.52 µm in width, one celled, hyaline and cylindrical with rounded ends.

Proving the pathogenicity

Artificial inoculations were conducted as described under 'Material and Methods'. Under laboratory conditions, the inflorescences started browning 3- 4 days after inoculation and greyish conidial mass appeared on the tertiary, secondary and primary rachis after twelve days of inoculation. The un-inoculated rachis neither showed the presence of conidial mass or brownish discolouration. Re-isolation of the pathogen was made from the infected rachis and the fungus which was found to be identical to the original ones.

Morphological characterization of *Colletotrichum gloeosporioides* on different solid media

The conidia were oblong or cylindrical or slightly dumbel, hyaline, aseptate with rounded ends and one to two oil globules. Conidia on the culture media were found to be in orange mass. The conidia collected from potato dextrose agar measured 12.02 to 12.70 µm × 3.5 to 4 µm.

On PDA the pathogen produced dense, cottony, dirty white to greyish mycelium with even margin. It produced abundant aerial mycelium in the centre of the colony without any zonation. Later, it produced conidiophores either arising singly or closely packed together in rows (Plate). Conidiophores were single celled, hyaline and aseptate with one or several conidial scars. The conidia were oblong or cylindrical or slightly dumbel, hyaline, aseptate with rounded ends and with one or two oil globules. Conidia measured 11.48 - 20.01 µm in length x 4.25- 6.62 µm in length and the average being 15.74 x 5.43 µm (Plate 1). Conidiophores were simple, filiform measuring 10 to 12.5 µm. Conidia measured 12.78 µm in length x 4.52 µm in width, one celled, hyaline and cylindrical with rounded ends.

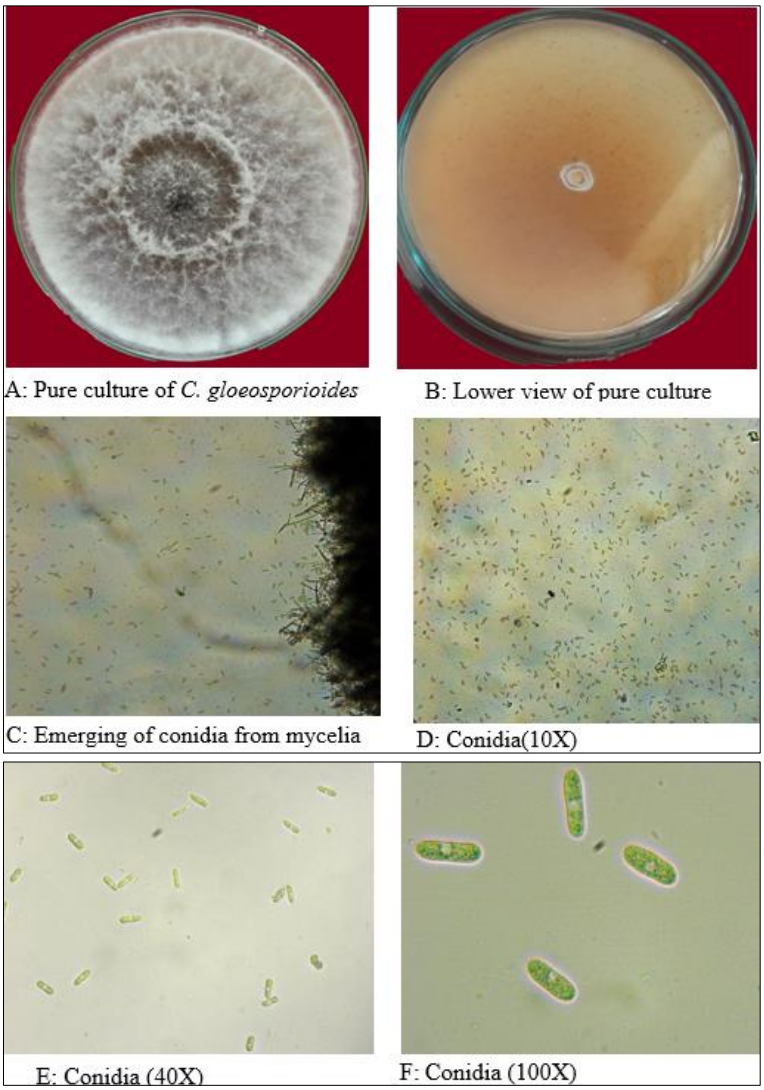


Plate 1: Morphological identification of *C. gloeosporioides*

Molecular characterization of *C. gloeosporioides*

Isolation of Genomic DNA

Genomic DNA of the fungus was isolated by CTAB method. The DNA obtained was observed by running on 1.0 per cent Agarose gel electrophoresis.

Amplification of CgInt and ITS4 region

Specific amplification of *C. gloeosporioides* was done with specific primer CgInt. DNA amplicon was observed at 450 bp for all four isolates (Plate 1).

DNA sequencing

The PCR product was sequenced by using CgInt as forward and ITS4 as reverse primers at Biokart India Pvt Ltd., Bengaluru. The obtained DNA sequences of four different isolates were given below.

Sequences of *Colletotrichum gloeosporioides* isolates

>ON979675.1 *Colletotrichum gloeosporioides* isolate Thirthahalli

CTTCGAAATTTACAATATAAAAACAGAGTTTGGGGG
TCCTTCGGCGGGCCGTCCCCGTTTTTACCCGGGAGGG
GGGTTGTTCCCGCCGAGGCCAACCAATGGTATGTTC
CCCAGGGGTTTGGGAGTGTAAACTCGGAAAGAATTC
TGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTT
ACAACTCCCAAACCCCTGTGAACATACCAATTGTTG
CCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGAC
GGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATAT
GTAACTTCTGAGTAAAACCATAAATAAATCAAAACT
TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA
GAACGCAGCAAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATT
GCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGA
GCGTCATTTCAACCCTCAAGCCTGCAGCTTCCATTGC
GTAGTAGTAAAACCCTCGCAACTGGTAGCGCGGCGC
GGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGA
CCTCGG

>ON974405.1 *Colletotrichum gloeosporioides* isolate Shikaripura

ACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGA
GGACCCCTAACTCTGTTTCTATAATGTTTCTTCTGAG
TAAAACAAGCAAATAAATTAAAACTTTCAACAACGG
ATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGA
AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACATTGCGCCCGCCAG
TATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAA
CCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCG
GAGGCCCTCCGTGGGCACACGCCGTCCCCCAAATAC
AGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAG

CTAACACCTCGCAACTGGAGAGCGGCGCGGCCACGC
CGTAAAACCCCCAACTTCTGAAAGTTGACCTCGAAT
CAGGTAGGAATACCCGCTGAACTTAAGCATATCAAT
AAGCGGAGGAAAAGAAACCAACAGGGATTGCCCTA
GTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGA
AATCTGGCCC

>ON974402.1 *Colletotrichum gloeosporioides* isolate Chikkamagaluru

AACTCGGAAAGAATTCTGTAGGTGAACCTGCGGAGG
GATCATTACCGAGTTTACAACTCCCAAACCCCTGTG
AACATACCAATTGTTGCCTCGGCGGATCAGCCCGCT
CCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTA
AACTCTGTTTCTATATGTAACTTCTGAGTAAAACCAT
AAATAAATCAAAACTTTCAACAACGGATCTCTTGGT
TCTGGCATCGATGAAGAACGCAGCAAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT
CTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCG
GGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCC
CCCGGGTTTGGTGTTGGGGATCGGCGAGCCCTTGCG
GCAAGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTG
CAGCTTCCATTGCGTAGTAGTAAAACCCTCGCAACT
GGTAGCGCGGCGCGGCCAAGCCGTTAAACCCCCAAC
TTCTGAATGTTGACCTCGGATCAGGTAGGAATACCC

>ON974313.1 *Colletotrichum gloeosporioides* isolate Davanagere

CTTCGAAATTTACAATATAAAAACAGAGTTTGGGGG
TCCTTCGGCGGGCCGTCCCCGTTTTTACCCGGGAGGG
GGGTTGTTCCCGCCGAGGCCAACCAATGGTATGTTC
CCCAGGGGTTTGGGAGTGTAAACTCGGAAAGAATTC
TGTAGGTGAACCTGCGGAGGGATCATTACCGAGTTT
ACAACTCCCAAACCCCTGTGAACATACCAATTGTTG
CCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGAC
GGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATAT
GTAACTTCTGAGTAAAACCATAAATAAATCAAAACT
TTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA
GAACGCAGCAAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATT
GCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGA
GCGTCATTTCAACCCTCAAGCCCCCGGGTTTGGTGTT
GGGGATCGGCGAGCCCTTGCGGCAAGCCGGCCCCGA
AATCTAGTGGCGGTCTCGCTGCAGCTTCCATTGCGTA
GTAGTAAAACCCTCGCAACTGGTAGCGCGGCGCGGC
CAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTC
GG

DNA sequences of four isolates were compared using bioinformatics tool like NCBI (National Centre for Bioinformatics) blast programme. Based on sequence comparison, nucleotide sequences of the CgInt region of the ribosomal DNA of all four isolates had 99% homology with *C. gloeosporioides* isolates available in the NCBI. Thus, all the four isolates were confirmed as *Colletotrichum gloeosporioides.*

Phylogenetic analysis

The Cladogram (Fig.1) obtained from MEGA X software showed that sequences from Thirtahalli, Davangere and Chikkamagalur group together. The sequence of Shikaripura isolate was the outlier and most distant from the other organisms.

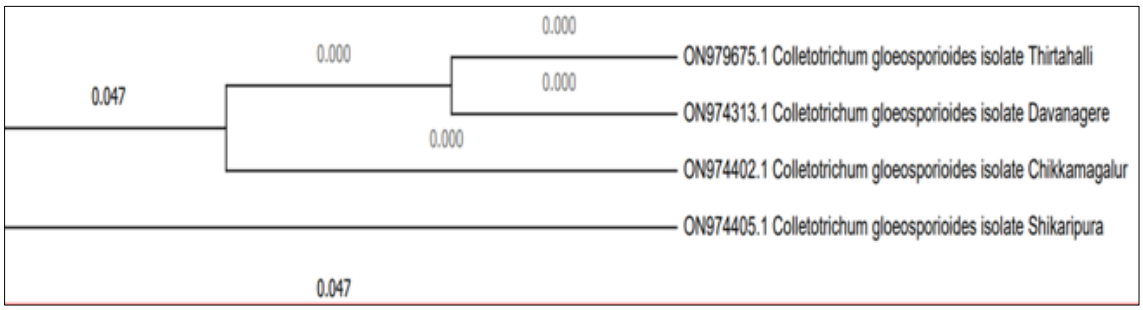
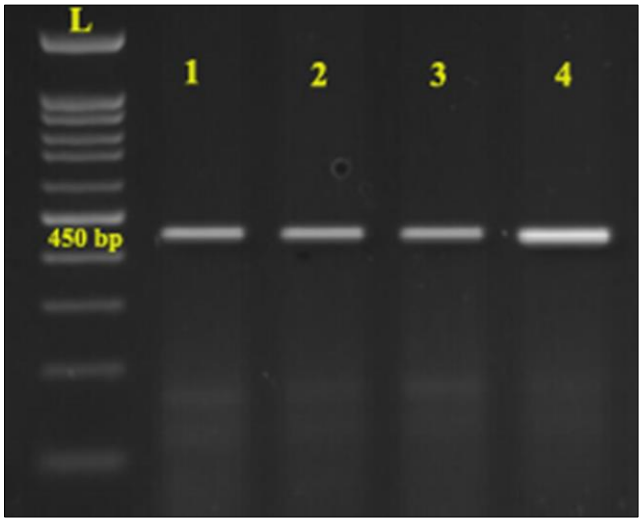


Fig 1: Phylogenetic analysis of different isolates of *C. gloeosporioides*



Thirthahalli isolate L- 100 bp Ladder Shikaripura isolate Chikkamagaluru isolate Davanagere isolate

Plate 2: PCR amplification of *C. gloeosporioides* isolates using species-specific primer (CgINT)

Discussion

The study on morphological characters showed conidia of *Colletotrichum gloeosporioides* were oblong or cylindrical or slightly dumbel, hyaline, aseptate with rounded ends and with one or two oil globules and it measured 15.74 µm × 5.43 µm. These characters were similar with the earlier descriptions by Hegde *et al*. (1986) [3], Hegde *et al*. (1988) [2] and Pradeepkumar (2000) [7].

C. gloeosporioides on PDA medium exhibited greyish white colony colour, whereas the reverse side of colony appeared to be white to dark grey as well as orange colour. The conidia were found to be cylindrical with both apices rounded or with one apex rounded and the other end pointed. The results are in similarity to earlier works of Ansari *et al*. (2018) [1] and Pedraja *et al*. (2019) [6] who reported that *C. gloeosporioides* from different crops showed dense, cottony, puffy and white colour aerial mycelium and orange coloured conidial masses on the front side of the plate with regular margin and moderate growth (4.8 mm/day), on the reverse side of the plate, the colonies were of white to pale grey mycelium and dark grey at the point of inoculation.

The four different isolates of pathogen isolated were used for amplification by PCR (Polymerase chain reaction). The isolates which were characterized as *Colletotrichum gloeosporioides* based on cultural and morphological characters were subjected for molecular confirmation in PCR by using species specific primers (CgINT). The PCR amplification using Forward primer CgINT (5'-GGCCTCCCGCCTCCGGGCGG-3′) and reverse primer ITS-4-r (5'-TCCTCCGCTTATTGATATGC-3') yielded a single band at 450 bp. Their identity as *Colletotrichum gloeosporioides* were molecularly confirmed in NCBI database and the sequences of four isolates were deposited in the gene bank and the accession numbers were obtained. The results obtained are in agreement with the results obtained by earlier workers Serra *et al.* (2011) [8] and Zivkovic (2017) [9]. The results from phylogenetic tree showed that Shikaripura isolate had distant sequence from other isolates. Thirthahalli, Davangere and Chikkamagalur isolates had higher similarity compared to Shikaripura isolate.

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