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Pathogenicity and Molecular Characterization of *Colletotrichum gloeosporioides* Causing Anthracnose Disease in Anthurium

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Anthurium is an excellent cut flower crop commercially cultivated throughout the world. Anthracnose or spadix rot disease leads to massive flower loss to anthurium growing farmers. Survey was conducted for anthurium anthracnose disease during the year 2017-2018 in anthurium growing areas of Tamil Nadu, India. *Colletotrichum gloeosporioides* cultures (10 isolates) were isolated and pathogenicity test was proved by several artificial inoculation methods. Among this, pin prick plus spraying spore suspension method has recorded the highest per cent disease index of

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64.38 on anthurium plants. Molecular analysis based on obtained sequences (MH479426) of the rDNA internal transcribed spacers (ITS1 and ITS4) resulted more than 92 per cent identical to ITS sequence of *C. gloeosporioides* published in GenBank database. The molecular study confirmed that anthracnose disease of anthurium is caused by *C. gloeosporioides.* The molecular-based clustering demonstrated the genetic relationships of the isolates and species of *Colletotrichum* and indicated that ITS rDNA sequence data were potentially useful in taxonomic species determination.

Keywords: Virulence; Colletotrichum gloeosporioides; ITS; incidence; phylogenetic; inoculation.

1. INTRODUCTION

Anthurium (*Anthurium andreanum*) is one of the beautiful cut flowers cultivated mostly in tropical humid climate. It is an excellent cut flower crop commercially cultivated throughout the world. The flowers of anthurium are popular among flower arrangers because of their attractive colours, increased vase life, bold effect and long lasting qualities [1]. The popularity of growing anthurium as cut flowers has risen tremendously in the past few years and it has now become an important export-oriented crop.

Anthracnose or spadix rot disease caused by *Colletotrichum gloeosporioides* is a major constraint to anthurium production which leads to massive losses in terms of quality and quantity. *Colletotrichum* genus was recently voted as the eighth most important group of plant pathogenic fungi in the world, based on perceived scientific and economic importance [2]. Anthracnose caused severe rotting incidence of anthurium resulting in 100 per cent death of plants in Alleppy district of Kerala [3]. Severity of anthracnose in anthurium ranged from 21.67 to 54.89 per cent in Tamil Nadu [4].

Colletotrichum is one of the most common plant pathogenic fungi in tropical or temperate regions. The symptoms can appear as small, water-soaked speckles or large necrotic and chlorotic lesions that are circular to irregular shape. Lesions are yellow, brown or black in colour, often with a bright-yellow chlorotic halo. This research paper deals about the molecular characterization of *Colletotrichum* species associated with anthracnose disease of anthurium and standardization of inoculation techniques for proving the pathogenicity.

2. MATERIALS AND METHODS

2.1 Survey and Disease Incidence

Survey was conducted in different anthurium growing areas of Tamil Nadu, India during 2017–2018 and the disease incidences were recorded. The survey includes the observation of symptoms over time and the Per cent Disease Index was calculated by McKinney [5] formula. Anthracnose disease infected leaf samples were collected from different anthurium growing areas of Tamil Nadu for isolation of the fungus, *Colletotrichum gloeosporioides.*

2.2 Isolation of *C. gloeosporioides*

The diseased leaf was first washed with tap water to remove dust and other contaminants. The infected portion was cut into small bits and surface sterilized with 10 per cent sodium hypochlorite for 5-10 minutes. In order to remove the residue of the chemical, the tissue bits were washed with three changes of sterile distilled water. The surface sterilized bits were placed on Potato Dextrose Agar (PDA) medium in sterilized Petri dishes. These plates were incubated at room temperature (28 ± 2°C) for seven days. After incubation, the cultures were purified by hyphal tip method [6] and the fungal cultures were maintained separately in agar slants/Pert plates.

2.3 Pathogenicity Assay

The Pathogenicity test was conducted under protected cultivation. Anthurium plants of healthy temptation variety were maintained in pots under 75% shade. Among the ten isolates, the virulent isolate ICg1 was taken for different inoculation methods. The virulent fungal culture filtrate was prepared and the spore suspension was adjusted to 5×10^6 conidia/ml. The different inoculation methods were:

2.3.1 Leaf-clipping method

Three leaves of anthurium plants were clipped off approximately 2-3 cm from their tip by using a pair of scissors dipped in the inoculums of each isolate prepared as described earlier. Similarly control was inoculated with sterile distilled water. The plants were wrapped in moist plastic bags to

conserve moisture and placed in greenhouse at 25-27°C immediately after inoculation until optimum disease development [7].

2.3.2 Pin-pricking method

Needles were dipped into the fungal cell suspension of each isolate prepared and gently prick the leaf blades of anthurium plants maintained in pots. Similarly control was inoculated with sterile distilled water. The plants were covered with moist plastic bags to conserve moisture and placed in greenhouse chamber immediately after inoculation [8].

2.3.3 Spray inoculation method

Suspensions of different fungal isolates were sprayed directly onto anthurium plants potted separately for pathogenicity test. Then the treated pots and sterile water treated control pots were incubated for observation [9].

2.3.4 Injury by carborandom powder

The anthurium plants were artificially injured by carborandum powder randomly. Then the prepared fungal spore suspension of each isolate was sprayed onto the anthurium plant accordingly under protected cultivation. The plants sprayed with sterile water served as control and the plants were observed for disease development.

2.4 DNA Extraction and PCR Amplification

The different isolates of *Colletotrichum gloeosporioides* were grown in potato dextrose broth separately for fifteen days. The fresh mycelium from the broth was harvested by filtration separately with sterile filter paper and by using liquid nitrogen; it was grinded to a fine powder. 100-200 mg of mycelial powder of each isolate was taken separately and macerated with CTAB buffer. After maceration 700 µl solution of each isolate was transferred into centrifuge tube separately and incubated at 65°C for 25 minutes. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) were added to the incubated tubes and centrifuged at 10000 rpm for ten minutes. After centrifugation, the aqueous layer from the tubes was separated and chloroform: isoamyl alcohol (24:1) of equal volume was added to precipitate the contaminants.

The mixture was again centrifuged for ten minutes at 10000 rpm. After centrifugation 300 µl aqueous solution of each isolate was taken separately to which 5M sodium acetate: ice cold ethanol (5:2) was added. This mixture was kept at -20°C overnight. This mixture was again centrifuged at 13000 rpm at 4°C for ten minutes. After centrifugation equal volume of ethanol was added, centrifuged and kept for pellet drying. Then 50 µl of TE-buffer was added into the pellet separately, then the genomic DNA of each isolate was checked by Polymerase Chain Reaction (PCR) in order to amplify the DNA by using universal primers ITS1 forward (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 reverse (5'-TCCTCCGCTTATTGATATGC-3').

PCR amplification was done in a thermal cycler. The total volume for PCR reaction was ten µl which contained seven µl PCR master mix (Taq DNA polymerase, AMPLICON Company), one µl of free nuclease water, 0.5 µl of forward (ITS1) and reverse primer (ITS4), and one µl of DNA template. Thermo cycling procedure was carried out at initial denaturation (95°C) for two minutes, followed by 38 cycles of denaturation (95°C) for one minute, annealing at 55°C for 30 seconds, one minute extension at 72°C and final extension for ten minutes at 72°C.

2.5 Sequence Analysis

DNA sequencing of PCR product was sequenced in both directions (Eurofins Genomics India Pvt Ltd, Bangalore). The expected size of the PCR product was approximately 550bp. The obtained sequences were analysed using BLAST program with default parameters (NCBI) [10]. ITS sequences were used to construct two unrooted phylogenetic trees, using the MEGA (version 10.0.2.) program [11] with the neighbour-joining (NJ) method Saitou and Nei [12].

The statistical reliability of the branches was evaluated by performing bootstrap analysis (1000 replicas). Sequence data matrix was aligned using the ClustalW option and the alignment was corrected where necessary. ITS sequences of the species *C. gloeosporioides* (GenBank accession numbers MH479426) were compared with eleven ITS sequences belonging to *Colletotrichum* sp. showed more than ninety per cent identity.

3. RESULTS AND DISCUSSION

3.1 Survey and Isolation

Survey was conducted during 2017-2018 in various places of Tamil Nadu. The occurrence of

anthracnose disease on anthurium cultivars was recorded (Table 1). Symptoms typical of anthracnose caused by *Colletotrichum gloeosporioides* were frequently observed on anthurium plants in various districts of Tamil Nadu, India. In general, the common symptoms consist of necrotic spots on leaves were more or less circular, light brown with a prominent yellow margin that in a later phase of infection formed the fruiting bodies of the fungus. Anthracnose appeared as small, irregular and sunken lesions, with a diameter of 0.5 cm or little more, which could turn to black, increase in size and form acervuli. Infection starts as a tiny dark spots that expands later on. In advanced stages under wet conditions, a general rot of the entire spadix may occur [13].

The present survey revealed that the disease incidence of anthracnose on leaves caused by *C. gloeosporioides* was the most serious problem in the cultivation of anthurium in Tamil Nadu, India. Severe symptoms were frequently observed on leaves as well as flowers. This paper explained about the pathogenicity and identification of different pathogenic isolates of *Colletotrichum gloeosporioides* based on molecular characterization.

A total of ten *C. gloeosporioides* isolates were isolated of which all the ten were identified as *C. gloeosporioides* through morphological and molecular analysis. Colonies grown on PDA were initially white-orange and then turned to greenish-grey (Fig. 1a). In the reverse side, colonies were brownish-orange to grey (Fig. 1b). Bright orange to pink spore masses were produced. Conidia were hyaline, unicellular and cylindrical. The length and width of conidia produced *by C. gloeosporioides* sized from 10 to 16 µm in length and 3 to 5 µm in width. The maximum sporulation was observed at 28°C in all five *C. gloeosporioides* isolates [14].

3.2 Pathogenicity of *C. gloeosporioides*

The virulence of different isolates of *C. gloeosporioides* was tested in pot culture under protected cultivation. The results showed that the isolates induced the anthracnose symptoms on the inoculated anthurium leaves as like the natural symptoms. Among the ten isolates, ICg1 was found to be the most virulent isolate.

The different inoculation techniques were experimented with the virulent isolate ICg1. The result showed that pinprick+spraying spore suspension method has recorded the highest disease incidence of 64.38 per cent disease index (PDI) followed by carborandom. Injuries by pin prick (47.77 PDI) and pin prick+mycelial disc (42.21 PDI). The least percentage was observed in leaf clipping method (29.97 PDI) (Table 2).

Several studies have been conducted for comparing different inoculation methods against various pathogens for screening different varieties. Co et al. [15] evaluated three different smut inoculation techniques: soaking, wounding along with paste and pastes without wounding in sugarcane seedlings and found wounding along with paste method was the best method. Bayern and Sharma [16] compared five stem inoculation methods with respect to phytoalexin accumulation and *Fusarium* wilt development in carnation and found injection method to be the more effective method. Thangamani et al. [17] confirmed pathogenicity of (*Colletotrichum musae*) anthracnose disease of banana by pin prick method. Patel [18] proved pathogenicity of *Colletotrichum gloeosporioides* from leaf spot of ornamental orchid in which pin pricking injury method found best on the leaves.

3.3 PCR Amplification and Sequence Analysis

A PCR product of approximately 550 bp was obtained using primers ITS1 and ITS4 (Fig. 2). The amplicons were purified from agarose gel and sequenced.

The sequences obtained from the mycelia isolated from leaves resulted more than 92% identical to ITS sequences of *C. gloeosporioides* published in GenBank database. The obtained ITS sequence (GenBank accession number MH479426) and ten ITS sequences of the genus *Colletotrichum* retrieved from GenBank, were used to construct phylogenetic trees. The molecular identification of the ICg1 isolates as *C. gloeosporioides* was confirmed by phylogenetic analysis (Fig. 3).

The inaccuracies of identifying *C. gloeosporioides* solely by morphological criteria have been largely overcome by the use of molecular methods for differentiating *Colletotrichum* species. Ribosomal DNA sequences have been used extensively for species delineation within the genus *Colletotrichum* [19,20] and analysis of the

Table 1. Survey and isolation of anthurium anthracnose disease on different anthurium growing areas of Tamil Nadu, India

S. no.	Village name	District	Isolate code	Per cent Disease Index (PDI)*	Geographical location		
					Latitude	Longitude	Altitude (Ft.)
1.	Thandikudi	Dindugal	ICg1	56.66[a]	10° 17' 36.8658"N	77° 42' 33.3324"E	3832.021
2.	Pechipparai	Kanyakumari	ICg2	48.88[d]	8° 26' 26.145"N	77° 18' 15.7536"E	393.701
3.	Thuckkalay	Kanyakumari	ICg3	38.88[f]	8° 14' 33.0246"N	77° 18' 53.9382"E	32.8084
4.	Parasalai	Kanyakumari	ICg4	52.21[c]	8° 19' 37.8834"N	77° 9' 29.7354"E	104.987
5.	Moolachanvilai	Kanyakumari	ICg5	31.10[h]	8° 12' 14.0106"N	77° 9' 29.7354"E	131.234
6.	Yercaud	Salem	ICg6	31.10[i]	11° 47' 44.8398"N	78° 12' 42.8148"E	4921.26
7.	Nagloor	Salem	ICg7	17.77[j]	11° 50' 7.0218"N	78° 12' 22.809"E	5324.803
8.	TNAU	Coimbatore	ICg8	36.66[g]	11° 0' 56.0298"N	76° 55' 51.312"E	1430.45
9.	Pandrimalai	Dindugal	ICg9	54.43[b]	10° 20' 38.6478"N	77° 45' 20.7828"E	3973.097
10.	Wellington	Nilgiris	ICg10	41.10[e]	11° 21' 44.4204"N	76° 47' 21.228"E	6085.958

**Mean of three replications*

Means in a column followed by same superscript are not significantly different by Duncan's Multiple Range Test at P =0.05

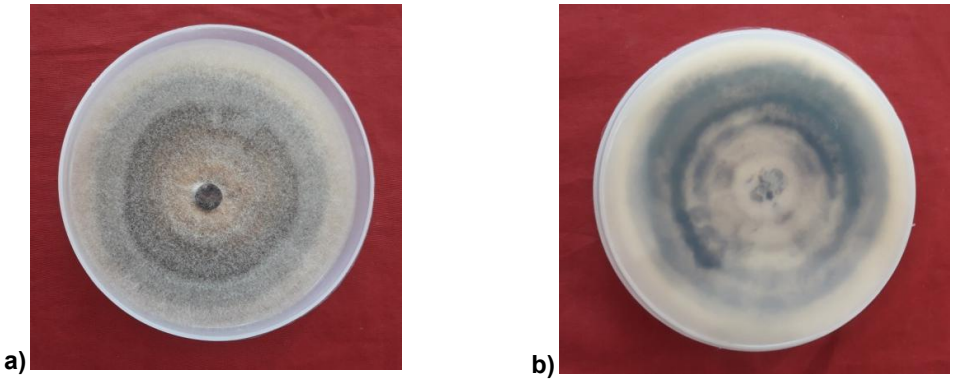

Fig. 1. Appearance of *C. gloeosporioides* culture on PDA medium. a) Front side, colonies grown were initially white-orange and then turned to greenish-grey. b) reverse side, colonies were brownish-orange to grey

Table 2. Artificial inoculation method for pathogenicity test for anthurium anthracnose caused by *Colletotrichum gloeosporioides*

S. no.	Inoculation method	Per cent Disease Index (PDI)*
1.	Spraying spore suspension	36.54[e]
2.	Injury by pinprick	47.77[c]
3.	Pin prick + spore suspension spray	64.38[a]
4.	Leaf clipping method	29.97[f]
5.	Injury by carborandom powder	53.32[b]
6.	Pin prick + mycelia disc	42.21[d]
7.	Control (water spray)	-
CD (P=0.05)		**1.170**

**Mean of three replications*

Means in a column followed by same superscript are not significantly different by Duncan's Multiple Range Test at P =0.05

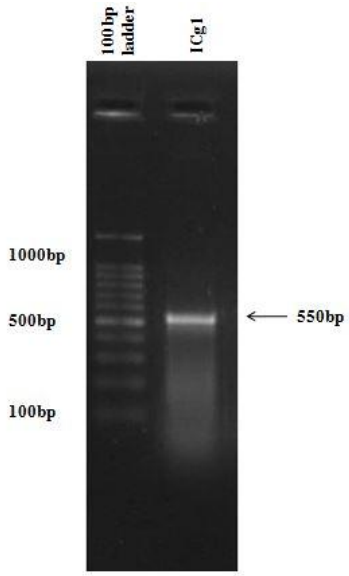

Fig. 2. PCR amplification of *C. gloeosporioides* ITS primer amplified a band of 550 bp ICg1 virulent isolate. Lane 1 is 100 bp DNA Ladder and Lane 2 is ICg1

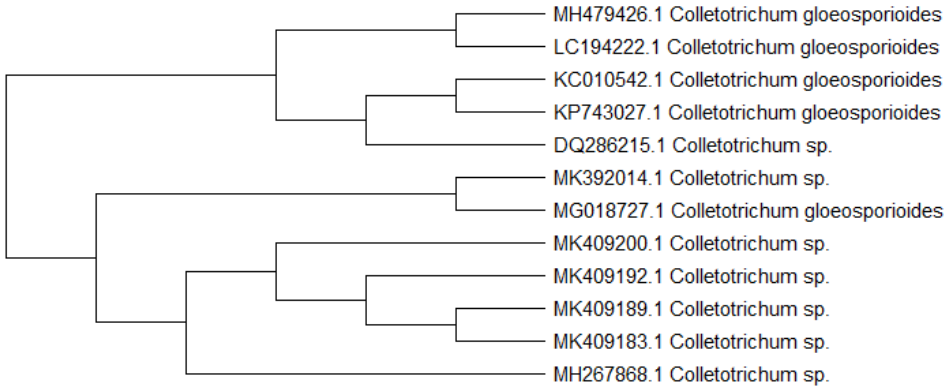

Fig. 3. Phylogenetic tree based on maximum parsimony analysis of the ITS sequences of *Colletotrichum gloeosporioides* (GenBank accession number MH479426) and the closest relatives from GenBank

variable ITS regions – especially the ITS1 portion – provides sufficient information to infer phylogenetic relationships among *Colletotrichum* species [21]. Kamle et al. [22] reported that DNA fragment of approximately 580 bp were amplified for *C. gloeosporioides* using ITS (Internal Transcribed spacer) primer. The *Colletotrichum* isolates were identified using PCR with species specific primers, complemented by phylogenetic analysis of nucleotide sequences of the internal transcribed spacer region and partial glyceraldehyde-3-phosphate dehydrogenase gene [23]. For this reason, ITS sequence analysis was used to verify the identity of the pathogen causing anthurium anthracnose.

4. CONCLUSION

Among the artificial inoculation methods, pin prick + spraying spore suspension has caused the highest percentage of disease incidence in anthurium plants. For diagnostic purposes, the sequencing method developed here showed its objective value by confirming that the strain MH479426, recently isolated from a subcutaneous infection in Brazil and identified according to its morphological features as *C. gloeosporioides* [24].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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