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Morphological and molecular characterization of two devastating chilli pathogens *Colletotrichum acutatum* (Chilli die-back/ Anthracnose) and *Fusarium brachygibbosum* (Chilli wilt)

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Abstract
Among all of the spices and condiments, the chilli (*Capsicum annum* L.) is the fourth most important around the world. Within Asia, it has been established as most important spice. Out of more than forty diseases incited by fungi, the chilli die-back/anthracnose and wilt disease caused by *Colletotrichum* spp. and *Fusarium* spp. respectively have been established most serious constraint in the chilli production especially in India. The cultural and morphological characteristics studies revealed that the colony *C. acutatum* had smooth margin with white to greyish-black zonation. The topography of mycelia was raised fluffy mycelial growth with dense crowding. The microscopy work revelaed that *C. acutatum* produced fusiform conidia with a size ranged from 8.3 µm to 9.6 µm length and 3.6 µm to 4.7 µm width. On the other hand, the colony colour of *Fusarium* isolate was pinkish white to white with browned tinch smooth to irregular margin. The mycelial topography was flat to fluffy. Following the microscopy studies, koch postulates for pathogenicity were also followed by both pathogens positively. Furthermore, universal ITS primers-based molecular characterization were carried out for both chilli pathogens. The results revealed that PCR amplification of ITS gene region for both pathogens resulted in an amplicon fragment of 550 bp and 560 bp while in the *F. brachygibbosum* analogous to the region of the 18S-28S rRNA intervening sequence. Later, the respective amplicons were sequenced followed by sequence-submission in the NCBI database with accession numbers of MF063316 and MF150000 respectively. Therefore, in this present study, the *C. acutatum* and *F. brachygibbosum* pathogens were identified based on its cultural, morphological and molecular characteristics.

Keywords: Chilli, anthracnose**,** *Colletotrichum acutatum*, *Fusarium brachygibbosum*, morphological characterization, molecular characterization, ITS primers.

Introduction
Chilli (*Capsicum annum* L.) is the most important essential vegetable cum spice crop in the world. Among all countries, India is the biggest producer, consumer as well as exporter worldwide. Within India, the chilli cultivation covers an 116,578-ha area with yield upto 1.4 million tons of dry fruits and 2 million tonnes of fresh fruits (AGRISTAT, 2019) [1]. Among Indian states, Andhra Pradesh is the largest chilli producing which comprises about 26% to the total cultivating area followed by Maharashtra (15%), Karnataka (11%), Odisha (11%) and Madhya Pradesh (7%) and other states contributing 22% to the total area under chilli cultivation (Jagtap *et al.*, 2012) [13]. Nutritically, it contains many organic ingredients including steam-volatile oils, fatty oils, mineral elements, vitamins C, carotenoids, pigments proteins and an alkaloid-nature capsaicinoids (Bosland and Votava, 2000; Kumar and Tata, 2009; Zhuang *et al*., 2012) [38]. Additionally, This alkaloid compound is actually responsible for the hot taste and used effectively in many industries including neurology and pharmaceutical (Hayman and Kam, 2008) [12]. Furthermore, it has many biochemical, pharmacological and medicinal properties including antioxidant, anti-inflammatory, antiallergeic, antitumor and anti-carcinogenic activities and antimicrobial properties (Lee *et al*., 2005; Nishino *et al*., 2009; Wahba *et al*., 2010) [37].

However, the chilli production suffers hugely from an array of diseases caused by multiple fungi, bacteria and virus diseases. Out of more than 83 different diseases reported, more than 40 diseases are incited by fungi (Rangaswami, 1979) [27]. Among them, die-back/anthracnose caused by cosmopolitan *Colletotrichum* spp. is the most destructive disease in India as it causes total of 50-60% yield losses (Bagri *et al.*, 2004: Sharma *et al.*, 2005) [30]. In other words, estimated loss due to this disease is about US$ 1.33 million (Mahasuk *et al.*,

2009b) [21]. This pathogen distribution is cosmopolitan by nature and show very broad host range. Etiologically, most *Colletotrichum* species viz., *C. acutatum*, *C. coccodes*, *C. dematium*, and *C. gloeosporioides* were intimately associated with single host plant and cause anthracnose in chilli (Park and Kim, 1992) [25]. Among all of them, *C. acutatum* is highly genetically diverse and has been considered a species complex which contains 31 examples of phylogenetic organisms (Damm *et al*., 2012) [10]. It causes various symptoms dieback of shoots, leaf spots and fruit rots in field as well as in storage conditions (Bansal and Grover, 1969) [5]. Typically, anthracnose symptoms include sunken necrotic tissues with concentric acervuli rings, fused lesions on chilli fruits with appearance of conidial masses with high severity.

Next important disease of chilli is *Fusarium* wilt disease reported to affect more than 90% of the chilli cultivation in country (Singh and Singh, 2004; Madhavi *et al.*, 2006) [20]. The most common species of *Fusarium* are *F. oxysporum* and *F. solani*, whereas, *F. moniliforme* and *F. pallidoroseum* are found in some parts only. The pathogen is typically soil-borne necrotroph whose infection is enhanced by dry weather condition and excessive soil moisture (Booth, 1971; Khan *et al.*, 2018) [15]. The typical disease symptoms include brown discoloration of vascular tissue followed by upward and inward rolling top leaves followed by wilting and plant death (MacHardy and Beckman, 1981; Khan *et al.*, 2018) [15].

Nonetheless, numerous reports are submitted by many researchers in the literature related to detection and characterization of *Colletotrichum* and *Fusarium* isolates from different crops around the globe (Guerber *et al*., 2003; McKay *et al*., 2009) [22]. However, there is no such literature with the present context paper *i.e.* the identification and molecular characterization of *Colletotrichum acutatum* and *Fusarium brachygibbosum* isolates. As a result, the present study was conducted as an attempt for proper identification and characterization of these causal agents for timely management of the diseases and to suggest strategies to control these two pathogens.

Materials and Methods

Isolation and maintenance of fungal culture

Typical anthracnose infected chilli (Fig.1a) and wilt affected chilli (Fig.1b) samples were collected from the Coimbatore region of Tamil Nadu State, India. A small piece of infected chilli fruits and root along with bit healthy tissue were subjected to superficial sterilization with 0.1% $HgCl_2$ for 30 seconds and subsequently rinsed three times with sterile distilled water. Following this step, the rinsed samples were then placed aseptically on the surface of Potato Dextrose Agar (PDA) medium by half plate technique and incubated at the room temperature (28 ± 2^0C) for 7 days in incubator (Innova42-Incubator shaker series). After 7-days of incubation, pure cultures of the fungi were collected by hyphal tip method and maintained on PDA slants (Riker and Riker, 1936) at 4 ºC for further usage. A bit of fungal culture was taken on a glass slide and observed with lyca image analyzer under 40X magnifications for the presence of microconidia, macro conidia and chlamydospore.

A **B**

Fig 1: Diseased specimen showing typical Anthracnose and wilt symptoms **a.** Typical sunken lesion on fruits on infected fruit caused by *Colletotrichum* spp (left). **b.** Drooping and wilting symptom due to *Fusarium* spp. **(**right)

Pathogenicity study

To confirm the pathogenicity under glass house condition, rapid root-dip transplanting technique was followed for *Fusarium* sp. referring the protocol described by Naik *et al.* (1996) [23]. Similarly, for *Colletotrichum* the freshly plucked ripe red chilli fruits of highly susceptible local variety K1 were gathered from the field and thoroughly washed under running tap water. Then, the fruits were blot-dried and surface sterilized with 70% ethanol and followed by 1% sodium hypochloride solution for two minutes and subsequently washed thrice with sterile distilled water. Nine-millimeter of mycelial disc of pathogen was placed on pin pricked fruits and incubated at 28±2 ^{0}C for 6 to 8 days. Three replications were maintained and monitored on the regular basis till re-isolation step. The fungi were re-isolated to establish pathogenicity from the artificially-inoculated fruits showing typical anthracnose and wilt symptoms. The established cultures were compared with the original culture for the morphology and colony characters (Ratanacherdchai *et al.*, 2010) [28].

Genomic DNA extraction

The genomic DNA was extracted from mycelial mat of both the pathogens by using CTAB method (Cetyl trimethyl ammonium bromide) with slight modifications of Knapp and Chandlee (1969) [16]. For fungal colonies, young mycelium was grown at room temperature (28±2 °C) for 15 days. The mycelial powder was obtained after finely grounding the fungal mat with liquid nitrogen. After this, 20 ml of DNA extraction buffer consists of 0.1 mol/L of Tris, 0.01 mol/l of EDTA and 1.5 mol/l of NaCl was addition into the frozen powder. With the periodic stirring, the suspensions were kept at 65 °C for 1 hour. Then, the obtained homogenates were vortexed and centrifuged with chloroform-isoamyl alcohol (24:1). The upper phase was collected and blended with of sodium acetate (3 mol/ l) and of ice-cold isopropanol in the ratio of 0.1:0.6 v/v respectively. It was centrifuged after this step and remaining isopropanol was decanted. The obtained transparent pellets were washed twice with 70% ethanol. The isolated nucleic acid was subsequently dissolved in 50 µl TE buffer (pH 8) followed by checking of the concentration and purity of the extracted DNA with Nanodrop (ND1000) spectrophotometer (Thermo Fisher Scientific, USA). The DNA concentration was set to 100ng/µl for the PCR amplification and stored for future use at -20 ºC.

PCR amplification and partial sequencing of ITS region

The universal primers ITS1 (Forward) and ITS4 (Reverse) were used to amplify the ITS regions of *Colletotrichum* spp. ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-

TCCTCCGCTTATTGATATGC-3'. PCR reaction mixture comprises of 10µl *viz*., 5 µl of PCR master mix, 1 µl of forward primer and 1 µl reverse primer, template DNA 2 µl and 1 µl of sterile water. The PCR for ITS regions (ITS1-5.8S-ITS2) was performed by using thermocycler (Thermo Fisher Scientific, USA) and programmed with initial denaturation temp 94 ºC for 1 min; followed by 30 cycles of denaturation temp 94 ºC for 60s, for annealing temp was adjusted to 58^{0}C for 2 min totally; extension temp 72 ºC for 1 min and final extension temp 72 ºC for 5 min. The PCR products were analyzed by electrophoresis by resolving on 1.2% agarose gel and visualized under UV light imager (Alpha Innotech, California, USA). The unpurified amplified PCR product (30 µl) was sent to for the partial sequencing (Agrigenome Labs Pvt. Ltd, Cochin, Kerala). The DNA sequencing was done using universal primers ITS1 and ITS4 (10 µl). Sequence identities of study isolates were compared by retrieving the available sequence from the GenBank database (Altschul *et al*., 1990) [2]. Newly obtained sequences for the study related isolates were deposited to National Center for Biotechnological Information (NCBI) database, GenBank, New York, USA and the accession number were obtained.

Construction of phylogenetic tree

The obtained sequence for both chilli fungal pathogen representative isolates were compared with the previous available sequence in GenBank (https://blast.ncbi.nlm.nih.gov/). Multiple sequence alignments have been carried out using the Clustal Omega multiple sequence alignment program of MEGA Version 6.0 with selected parameters (Tamura *et al*., 2013) [35]. Further, by using Neighbor-joining (NJ) and maximum-parsimony (MP) methods the required trees were constructed by using MEGA, version 6.0 (Tamura *et al.*, 2013) [35]. The relative stability of the branches and confidence values were assessed and evaluated by performing bootstrapping with 1,000 replications.

Results and Discussion

Cultural and morphological characterization

In this study, *Colletotrichum* spp. were isolated from infected chilli fruit samples using Potato Dextrose Agar (PDA) medium by half plate technique. The cultural and morphological characteristics studies revealed that the colony colour of *C. acutatum* had smooth margin and white to greyish black with zonation. The topography of mycelia was raised fluffy mycelial growth and densely crowded (Fig. 2a &2b). It produced fusiform conidia with a size ranged from 8.3 µm to 9.6 µm length and 3.6 µm to 4.7 µm width (Fig. 2d). Based on the cultural and morphological characters viz., size and shape of conidia, appressoria and cultural characters of these isolates were identified as *C. acutatum* as per the description by Sutton (1992) [34] and Than *et al.* (2008). Similarly, in case of *Fusarium* isolate F1 exhibited white coloured colony in front side and pinkish coloured in the reverse of the petri plate. The colony margin was smooth to irregular with the mycelial topography being flat to fluffy (Fig. 2e &f). *Fusarium* produced micro, macro conidia and chalmydospores. Small, oval shaped, hyaline, single or bicelled microconidia was observed and the sizes varies from 7.89 to 13.65 µm in length and 4.67 to 5.75 µm in width. However, macroconidia were hyaline, sickle shaped and multi-celled with 2-3 septate. The size of macro conidia varies from 16.90 to 31.60 µm in length and 3.89 to 5.53 µm in width. The microconidia were more in number where macro conidial numbers were scattered (Fig. 2g). Terminally and intercalary chlamydospores were abundant and which are globose to oval-shaped (Fig. 2h). These findings were accepted by several authors Booth (1971), Singh and Singh (2004) and Joshi *et al.* (2013).

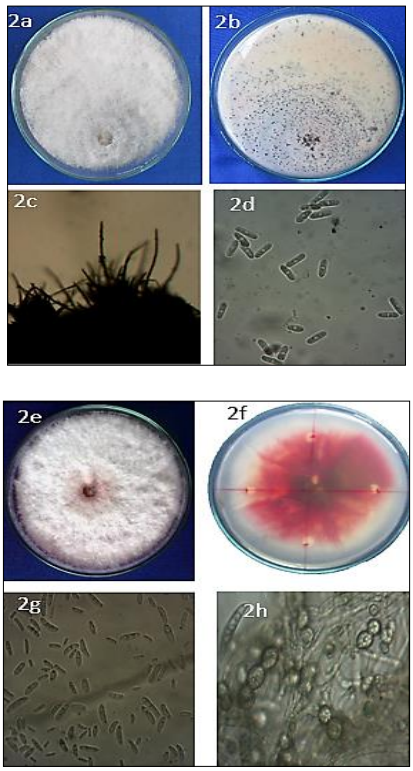

Fig 2: Cultural and morphological characteristics of *C. acutatum and F. brachygibbosum.*

Mycelial front and back side view of *C. acutatum* (2a &2b) and *F. brachygibbosum* (2e &2f) mycelia. Acervuli with black setae (2c) and Fusiform conidia (2d) of *C. acutatum.* Micro with macro conidia (2g) and Chlamydospore (2h).

Molecular characterization of the pathogen

In the present study, ITS 1 and ITS 4 universal primers were used for molecular characterization. The amplification of 550 bp fragment corresponding to the ITS 1, 5.8 S and ITS 4 regions of the rDNA gene for the *C. acutatum* was examined. The pathogen *C. acutatum and F. brachygibbosum* were amplified for ITS region with a fragment size of 550-560bp (Fig. 3). The amplicon (560 bp) obtained from the PCR amplification of virulent strain of *C. acutatum* and *F. brachygibbosum* obtained with the primer pair ITS1 and ITS 4 were partially sequenced. Molecular identification by using ITS region sequences was done through NCBI-BLAST (http://blast.ncbi.nlm.nih.gov) for the species identification. The result revealed that 96-100% nucleotide homology with *C. acutatum* was observed with other isolates available in NCBI database. Similarly, for *F. brachygibbosum* was upto 99% homology with *F. brachygibbosum* available in NCBI data.

Based on the higher homology, the pathogens were identified as *C. acutatum* and *F. brachygibbosum* and obtained accession number MF150000 and MF063316 respectively. Similarly, sequence analysis by using internal transcribed spacer (ITS) regions has been widely used and proved very useful in studying phylogenetic relationships among

Colletotrichum species (Sreenivasaprasad *et al*., 1996; Moriwaki *et al*., 2002 and Photita *et al*., 2005).

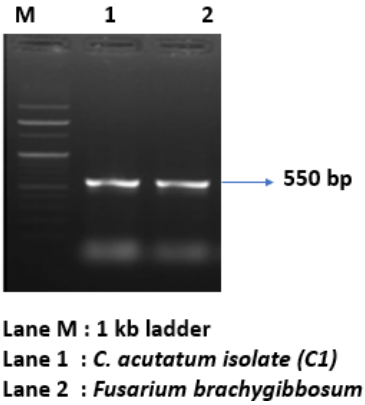

Fig 3: PCR amplification of ITS region of *C. acutatum and F. brachygibbosum.*

Phylogenetic analysis

A phylogenetic analysis was constructed in order to compare the ITS nucleotide sequence collected in this research and the reference strains of the *Colletotrichum* species complex available in the GenBank database using the NJ and MP methods (MEGA, version 6.0). The study isolates of *Colletotrichum* spp. under study were showing 100% similarity with *Colletotrichum scovillei* (MT036562.1; MN121801.1; MN121800.1; MN121799.1; MN121798.1; MN121796.1) and *Colletotrichum fioriniae* (MT068552.1) (Fig. 4), offering a variety of evolutionary closely related lineages with different phenotypic characteristics to all reference sequences and classified into well-supported clades with a bootstrap support of 100% and Bayesian posterior probability value of 1.00. *Colletotrichum* spp. consist of different cryptic species due to this it's very difficult to characterize because of unreliable morphological features and more genetic diversity (Sreenivasaprasad and Talhinhas, 2005; Cai *et al*., 2009; Damm *et al.*, 2012; Baroncelli *et al*., 2017). Similar study was done several workers to study the species complex in *Colletotrichum.* Many *Colletotrichum* species, *including C. acutatum, C. gloeosporioides,* and *C. capsici*, infect pepper (Bailey and Jeger, 1992; Than *et al*., 2008) [36]. similarly, the same analysis was done for *Fusarium* sp. and it was evident from the dendrogram (Fig.5) that the different species of fusarium grouped into two major clusters and shared maximum similarity with the *Fusarium brachygibbosum* (MF063317.1; MK752430.1; MK752429.1; MN626364.1; MN128229.1).

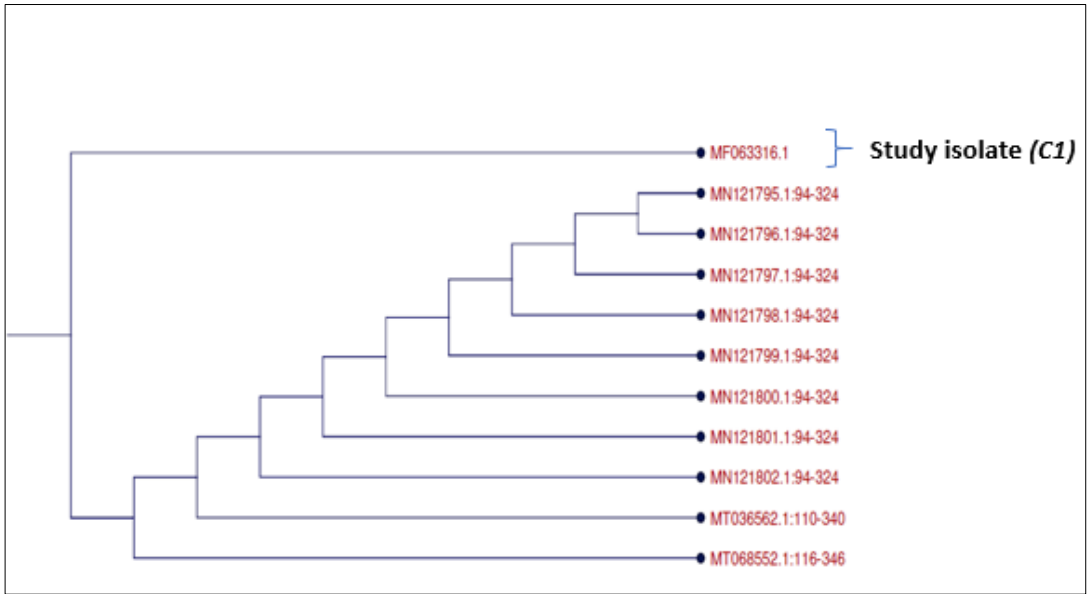

Fig 4: Phylogenetic analysis using the maximum-parsimony method to compare the ITS sequences of study isolate with *Colletotrichum* species references sequences retrieved from Gen Bank

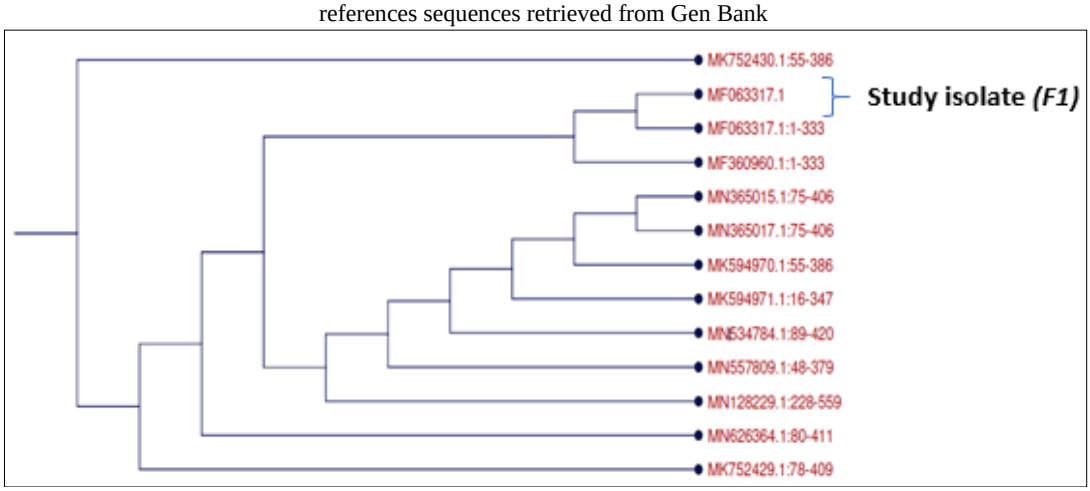

Fig 5: Phylogenetic analysis using the maximum-parsimony method to compare study isolate *Fusarium* sp. with references ITS sequence retrieved from GenBank

Conclusion

Chilli, *Capsicum annum* L. cultivation has existed since several hundred years in India as well as many other countries as a sustainable form of agriculture. It suffers from many biotic agents such as fungi, bacteria, viruses, rodents, viroids and nematodes. Among them, the fungal diseases anthracnose (or) fruit rot and wilt are most destructive and prevalent ones in India specially. For instance, *C. acutatum* is one of the most important fungal pathogen species complexes comprising several important fungi that cause economically significant losses of various crops. Therefore, in this study, the *C. acutatum* and *F. brachygibbosum* pathogens were identified based on its cultural, morphological and molecular characteristics. The advent of molecular biology tools for identification and diagnostics, based mostly on the rDNA-ITS region enabled such further discrimination. The information generated in the current study will be highly advantageous for developing future epidemiological models and development of suitable disease management practices of anthracnose in chilli is crucial in sense. Therefore, precautionary action is needed to prevent any future outbreaks of this disease.

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