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Variability in *Colletotrichum* infecting chilli plants of Andaman and Nicobar Islands, India

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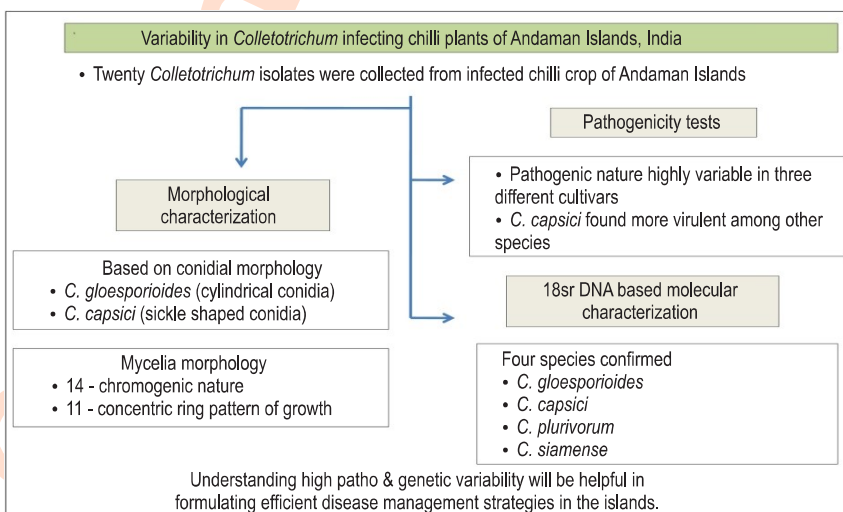
Abstract

Aim : The aim of the present study was to understand the basic pathogen dynamics of *Colletotrichum* species infecting chilli crop in the Andaman and Nicobar Islands for disease management practices.

Methodology : Twenty fungal isolates associated with chilli (*Capsicum annum*) anthracnose disease from diverse locations of tropical Andaman, India were characterized using polyphasic approaches.

Results : Upon morphological studies, out of twenty isolates, fourteen isolates were found chromogenic and eleven showed concentric ring pattern of mycelial growth. Based on conidial morphology, the isolates were grouped into two species (*C. capsici* and *C. gloesporioides*). The pathogenicity tests on three chilli varieties revealed difference in the pathogenic potential among the collected isolates. The sequence analysis using internal transcriber spacer (ITS) region revealed the presence of two other species *C. siamense* and *C. plurivorum*, in addition to *C. gloesporioides* and *C. capsici*.

Interpretation : The present study revealed the presence of high level of genetic and pathogenic variability among the *Colletotrichum* isolates infecting chilli in Andaman Islands.



Key words: Anthracnose, *Capsicum*, *Colletotrichum*, Molecular variability, Pathogenicity

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Introduction

Chilli is one of the most important cash crop and its cultivation is being practiced for several hundred years as a sustainable form of agriculture in India and many other countries. India is the largest producer and consumer of chillies in the world with a contribution of nearly 25 percent of the global output. Chilli anthracnose disease is one among the biotic factor affecting the production of chillies in major chilli-growing regions of the world which may cause yield loss upto 40% (Than *et al.*, 2008b; Diao *et al.*, 2017). Normally, the symptom of this disease includes leaf spot and fruit rot, where fruit rot on mature fruits, causing severe losses via both pre-and post-harvest fruit decay (Bosland and Votava, 2003). *Colletotrichum* is one of the major plant pathogenic fungi responsible for anthracnose (Dean *et al.*, 2012). Chilli crop grown throughout the world is susceptible to one or multiple species of *Colletotrichum* (Dean *et al.*, 2012; Weir *et al.*, 2012). Several species of *Colletotrichum* viz., *C. capsici* (Butler and Bisby), *C. gloeosporioides* (Penz.), *C. acutatum* (Simmonds), *C. atramentarium* (Berk and Broome), *C. dematium* (Pers.) and *C. coccodes* (Wallr.), *Glomerella cingulata* (Stoneman) along with *A. alternata* (Keissler) have been reported as causal agents of chilli fruit rot worldwide (Than *et al.*, 2008a). Kim *et al.* (2004) reported that different species of *Colletotrichum* can cause diseases in different parts of chilli plant.

Among the molecular methods, multilocus analysis is widely being used to study in depth genetic variability of *Colletotrichum*, but ITS-based identification is the basic and necessary approach prior to all recent approaches in understanding the broad genetic nature of any fungi (Schoch *et al.*, 2012). In Andaman Islands, there is 20-30% yield loss in chilli crop every year due to chilli anthracnose disease (Sakthivel *et al.*, 2019) due to prevailing climatic conditions which are congenial for a wide range of plant diseases (Sakthivel *et al.*, 2016; Sakthivel *et al.*, 2018). Analyzing that genetic diversity is necessary to understand the nature of pathogen population in particular region/locality in addition to morphological and pathological characterization. However, no such studies have been carried out on specific characterization of pathogen causing chilli anthracnose or their genetic diversity in tropical Andaman Islands. Thus, the current study aimed to characterize *Colletotrichum* isolates causing anthracnose disease of chilli in the islands through morphological, pathological and ITS based rDNA identification studies.

Materials and Methods

Pathogen isolation and morphological characterization: Chilli leaves and fruits showing symptoms of anthracnose disease were collected from various fields in different chilli-growing areas of South Andaman Islands (Table 1). The infected portions were cut into small pieces, surface sterilized in 0.1% HgCl₂ for 30 sec and rinsed three times with sterile distilled water, transferred to 9 petri dishes containing potato dextrose agar medium and incubated at room temperature (28 ± 2°C) for 2–3

days. Pure cultures of fungi were obtained by single spore isolation method. The cultures were maintained on PDA slants at 4°C. The morphological characteristics of all the isolates were studied as per the procedure described by Kumar *et al.* (2011) with slight modifications. Briefly, the cultures were grown on PDA plates and incubated at 28±2°C till the production of spores and the conidial characters were recorded. Conidial shape and size were observed under a light microscope (Olympus CX) at 10X and 40X using a micrometer, the spore photographs and spore measurements were taken with the image analyzer. For each isolate, the size of 20 conidia were measured and the mean values were calculated. Also, the mycelial nature and the presence or absence of acervuli were recorded for each isolate.

Pathogenicity tests: Pathogenicity tests were carried out following the method described of Than *et al.* (2008a) with slight modifications. *Colletotrichum* isolates were cultured on PDA at room temperature (28 ± 2°C) and the conidia from 7-day-old cultures were harvested using 5–10 ml of sterile distilled water. The conidial suspension was adjusted to a concentration of 10⁶. Three types of chilli fruits (*Capsicum annum*: medium slender type and long slender type; *Capsicum frutescens*: round type) were selected and surface sterilized with 70% ethanol.

The fruits were then thoroughly washed three-four times with sterile water and blotted dry using sterile paper tissue and wounds were made on the center of the fruits to a depth of 1 mm, using a sterile needle. A 10-µl drop of conidial suspension (10⁶ conidia ml⁻¹) was placed on the wound and the fruits were incubated in a chamber at 28±2°C with 98% relative humidity. Development of the symptom at the inoculation site was evaluated 9 days after inoculation on the basis of parent lesion size relative to the overall size of the fruit. Disease severity was scored on a 0–9 scale (0 = No infection, 1 = 1–2%, 3 = 3–5%, 5 = 6–10%, 7 = 11–25% and 9 = >25% of fruit area infected) as described by Montri *et al.* (2009). Three replications were maintained per isolate and the entire experiment was repeated twice to confirm the results. From the infection site, the pathogen was re-isolated on PDA and confirmed through conidial characters in image analyzer based on conidial morphology.

Molecular characterization: For molecular confirmation, DNA was extracted from the pure culture of each isolate grown on potato dextrose broth in 100 ml conical flasks for 7 days at room temperature (28 ± 2°C). The mycelia were then harvested by filtration and one gram mycelium was ground to a fine paste using CTAB extraction buffer, and DNA was extracted as per standard protocol (Murray and Thompson, 1980). The genomic DNA was checked in 0.8% agarose gel electrophoresis and stored at –20°C for further use. To identify the isolates at species level, the ITS region of the isolates were amplified with conserved primers ITS-1 (5'-GTCCTAACAAAGGTTTCCGTA-3') and ITS-4 (5'-CTCCGCTTATTGATATGC-3'). PCR was performed with a 50 µl reaction volume, 2 units of Taq polymerase, 5 µl of 10X buffer, 1.5 µl of 2.5 mM MgCl₂, 1 µl of 2.5 mM dNTP, 2 µl of 10 µM primer, 4 µl of genomic DNA and sterile

distilled water. PCR was performed with initial denaturation at 95°C for 1 min, followed by 35 cycles of 1 min at 95°C, 30 sec at 55°C and 1.20 sec at 72°C, which was followed by the final extension for 10 min at 72°C (Shenoy *et al.*, 2007) with slight modifications. Approximately, 500 base pair products were resolved by 1.5% agarose gel electrophoresis.

Results and Discussion

Morphologically, all the twenty *Colletotrichum* isolates were studied for their mycelia colour, growth pattern, conidial shape, conidial size and presence/absence of acervuli (Table 2). Based on the mycelia color, the isolates were grouped into chromogenic and non-chromogenic isolates. The chromogenic group comprised of fourteen isolates where the mycelia showed the presence of orangish conidial masses whereas in the non-chromogenic group, eight isolates showed fluffy, white or off white mycelia. Further, the isolates were classified on the presence/absence of concentric rings and regular/irregular margins. The growth pattern showed eleven isolates with concentric rings in reverse plates (Table 2). With regard to conidial shape and presence of acervuli, two isolates showed the presence of falcate shape conidia with black mass of acervuli whereas cylindrical conidial shape was observed in other eighteen isolates with no acervuli. Similarly, Than *et al.* (2008a) also observed falcate conidia in *C. capsici* and cylindrical conidia in *C. gloeosporioides*.

The maximum mean size of falcate was 25.56 cm long to 2.78 cm wide whereas in cylindrical conidia, the size varied widely from 8.38 cm to 29.30 cm long to 2.89 cm to 4.87 cm wide. The

overall results of morphological identification revealed that there might be two species, *C. gloeosporioides* and *C. capsici* responsible for chilli anthracnose disease in the Andaman Islands. Earlier many species viz., *C. gloeosporioides*, *C. fricticola*, *C. siamense*, *C. truncatum*, *C. capsici*, *C. plurivorum* have been reported to be associated with chilli an thracnose disease worldwide (Madhavan *et al.*, 2010; Kumar *et al.*, 2011; Sharma and Shenoy, 2014; Sakthivel *et al.*, 2018; Sakthivel *et al.*, 2019).

Pathogenicity tests revealed that all the isolates could induce symptoms on detached chilli fruits upon artificial infections in laboratory conditions with an exception that the infection potential varied with chilli varieties (Table 3). Among all the three different *Colletotrichum* species, *C. capsici* was found to be highly virulent with a rating of 9. Similar results were also observed by Than *et al.* (2008a) and Montri *et al.* (2009) for *C. capsici* and *C. acutatum*, respectively. This variation in pathogenic potential is also in agreement with Sharma *et al.* (2005) who reported prevalence of fifteen pathotypes of *C. capsici* existing among thirty seven isolates from different chilli growing regions of Himachal Pradesh (India) based on differential set of capsicum cultivars.

All the twenty isolates were PCR amplified with ITS 1 and ITS 4 primers for 18S rDNA. The purified products were sequenced bidirectionally, contigs were aligned in DNA Baser v4, and the sequences were subjected to BLAST analysis. The species identity was determined based on 99-100% similarity with sequences obtained from the NCBI database. The results revealed that four species viz., *C. gloeosporioides*, *C. capsici*, *C. plurivorum* and *C. siamense* were found prevalent and responsible for the anthracnose disease of chilli in the islands. All

Table 1: Details of *Colletotrichum* isolates of chilli analyzed in the study

Name	Host	Place of collection	Molecular Identification	GenBank ID
Cg_Mg1	Chilli Leaf	Manglutan	<i>Colletotrichum gloeosporioides</i>	KX449532
Cg_Mg2	Chilli Leaf	Manglutan Junction	<i>Colletotrichum plurivorum</i>	KX449533
Cg_Gp1	Chilli Leaf	Guptapara	<i>Colletotrichum gloeosporioides</i>	KX449534
Cg_Gp2	Chilli Leaf	Guptapara	<i>Colletotrichum gloeosporioides</i>	KX449535
Cg_Mg3	Chilli Leaf	Manglutan	<i>Colletotrichum siamense</i>	KX449536
Cg_Bt1	Chilli Leaf	Bimbliton	<i>Colletotrichum gloeosporioides</i>	KX449537
Cg_Mg4	Chilli Fruit	Manglutan	<i>Colletotrichum gloeosporioides</i>	KX449538
Cc_Gp3	Chilli Leaf	Guptapara	<i>Colletotrichum capsici</i>	KX449539
Cg_Tb1	Chilli Leaf	Tushnabad	<i>Colletotrichum gloeosporioides</i>	KX449540
Cg_Hp1	Chilli Leaf	Habdipur	<i>Colletotrichum gloeosporioides</i>	KX449541
Cg_Hp2	Chilli Leaf	Habdipur	<i>Colletotrichum gloeosporioides</i>	KX449542
Cg_Hp3	Chilli Fruit	Habdipur	<i>Colletotrichum gloeosporioides</i>	KX449543
Cg_Tb2	Chilli Leaf	Tushnabad	<i>Colletotrichum gloeosporioides</i>	KX449544
Cg_Cp1	Chilli Leaf	Collinpur	<i>Colletotrichum gloeosporioides</i>	KX449545
Cg_Cp2	Chilli Leaf	Collinpur	<i>Colletotrichum gloeosporioides</i>	KX449546
Cg_Cp3	Chilli Leaf	Collinpur	<i>Colletotrichum gloeosporioides</i>	KX449547
Cg_Tr1	Chilli Leaf	Tirur	<i>Colletotrichum gloeosporioides</i>	KX449548
Cg_Tr2	Chilli Leaf	Tirur	<i>Colletotrichum gloeosporioides</i>	KX449549
Cg_Tr3	Chilli Leaf	Tirur	<i>Colletotrichum gloeosporioides</i>	KX449550
Cc_Mg5	Chilli Leaf	Manglutan	<i>Colletotrichum capsici</i>	KX449551

Table 2: Morphological characteristics of *Colletotrichum* isolates isolated from chilli anthracnose of Andaman Islands

Isolate name	Mycelial characters			Acervulus– Present (+) / Absent (-)	Conidia shape	Conidial size (µm)	
	Margin	Colour	Nature			Length	Width
Cg_Mg1	Circular	Chromogenic	Flat, white mycelia, orangish concentric rings	-	Cylindrical	15.72 ± 0.23	4.41 ± 0.16
Cg_Mg2	Circular	Non chromogenic	Fluffy, off –white mycelia without concentric rings	-	Cylindrical	10.39 ± 0.65	2.89 ± 0.15
Cg_Gp1	Circular	Chromogenic	Flat, white mycelia, orangish concentric rings	-	Cylindrical	10.58 ± 0.66	3.42 ± 0.13
Cg_Gp2	Circular	Chromogenic	Flat, white mycelia, orangish concentric rings	-	Cylindrical	14.87 ± 0.32	4.74 ± 0.17
Cg_Mg3	Circular	Chromogenic	Flat, white mycelia, orangish concentric rings	-	Cylindrical	12.32 ± 0.45	3.21 ± 0.14
Cg_Bt1	Irregular	Chromogenic	Flat, white mycelia, orangish concentric rings	-	Cylindrical	5.95 ± 0.22	2.22 ± 0.09
Cg_Mg4	Irregular	Chromogenic	Flat, white mycelia, orangish concentric rings	+	Cylindrical	29.30 ± 0.67	2.70 ± 0.17
Cc_Gp3	Circular	Non chromogenic	Fluffy, off –white mycelia without concentric rings	+	Falcate	25.56 ± 0.45	2.78 ± 0.21
Cg_Tb1	Circular	Chromogenic	Flat, white mycelia, orangish concentric rings	-	Cylindrical	13.49 ± 0.27	5.06 ± 0.18
Cg_Hp1	Circular	Chromogenic	Flat, white mycelia, orangish concentric rings	-	Cylindrical	14.97 ± 0.26	4.24 ± 0.16
Cg_Hp2	Circular	Chromogenic	Fluffy, off –white mycelia without concentric rings	-	Cylindrical	12.05 ± 0.28	3.30 ± 0.16
Cg_Hp3	Circular	Chromogenic	Flat, white mycelia, orangish concentric rings	-	Cylindrical	13.62 ± 0.18	4.87 ± 0.17
Cg_Tb2	Irregular	Non chromogenic	Fluffy, off –white mycelia without concentric rings	-	Cylindrical	12.40 ± 0.26	4.75 ± 0.18
Cg_Cp1	Circular	Non chromogenic	Fluffy, off –white mycelia without concentric rings	-	Cylindrical	14.07 ± 0.34	4.39 ± 0.18
Cg_Cp2	Circular	Chromogenic	Flat, white mycelia, orangish concentric rings	-	Cylindrical	14.03 ± 0.33	4.03 ± 0.16
Cg_Cp3	Circular	Non chromogenic	Fluffy, off –white mycelia without concentric rings	-	Cylindrical	13.23 ± 0.22	3.77 ± 0.11
Cg_Tr1	Circular	Non chromogenic	Fluffy, off –white mycelia without concentric rings	-	Cylindrical	13.91 ± 0.15	4.06 ± 0.08
Cg_Tr2	Irregular	Chromogenic	Flat, white mycelia, orangish concentric rings	-	Cylindrical	8.38 ± 0.63	2.92 ± 0.30
Cg_Tr3	Circular	Non chromogenic	Fluffy, off –white mycelia without concentric rings	-	Cylindrical	14.49 ± 0.45	4.84 ± 0.22
Cc_Mg5	Circular	Non chromogenic	Fluffy, off –white mycelia without concentric rings	+	Falcate	24.61 ± 1.10	2.39 ± 0.16

the gene sequences were submitted and the GenBank Accessions numbers (KX449532- KX449551) were derived. From the results, it is also observed that prevalence of *C. gloeosporioides* found to be the major species group (16 isolates) in infecting chilli crop of Andaman islands when compared with other species viz., *C. capsici* (2 isolates), *C. siamense* (2 isolates) and *C. plurivorum* (1). Earlier, the association of *C. capsici* were found more prevalent in India (Sharma et al., 2005; Madhavan et al., 2010) compared to *C. gloeosporioides* complex. However *C. siamense* inciting chilli anthracnose in India was reported only by

Sharma and Shenoy, (2014). In conclusion, the results of this study revealed the existence of four *Colletotrichum* species causing anthracnose disease of chilli in the Andaman Islands. In addition, this study also revealed the presence of high level of genetic and pathogenic variability among the *Colletotrichum* isolates which needs special attention while formulating successful eco-friendly management practices. Moreover, this would be the first systematic investigation of chilli anthracnose pathogen variability in the Andaman Islands which may be further confirmed using recent molecular approaches.

Table 3: Anthracnose severity scores on different sized chilli fruits

Isolates	Small Max score	Medium Max score	Large Max score
Cg_Mg1	9	9	9
Cg_Mg2	9	7	9
Cg_Gp1	7	7	3
Cg_Gp2	9	9	9
Cg_Mg3	9	5	7
Cg_Bt1	7	7	5
Cg_Mg4	9	7	9
Cc_Gp3	9	9	9
Cg_Tb1	9	9	9
Cg_Hp1	7	5	9
Cg_Hp2	7	7	9
Cg_Hp3	7	3	9
Cg_Tb2	9	7	5
Cg_Cp1	9	9	7
Cg_Cp2	9	9	9
Cg_Cp3	9	9	9
Cg_Tr1	9	5	9
Cg_Tr2	9	9	9
Cg_Tr3	7	7	7
Cc_Mg5	9	9	9

*Severity of scoring was recorded as per Montri et al., 2009

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