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Phenotypic diversity, virulence and genetic characterization of *Colletotrichum gloeosporioides*, the incitant of leaf blight of small cardamom in South India

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Abstract

Small cardamom (*Elettaria cardamomum* Maton) is one of the most important spice crops in India, cultivated for its economic, culinary and medicinal values. Leaf blight incited by *Colletotrichum gloeosporioides* is a serious threat to the cultivation of cardamom in India. In the present study, an attempt was made to isolate and characterize the pathogen both morphologically and on molecular basis. Significant variations were observed in twenty isolates for conidial dimensions, the length of conidia ranged from 7.8-21.5 µm. The width of the conidia ranged from 3.3 to 7.8 µm. Isolate CD14 recorded the highest width of conidium (7.8 µm) and the lowest width was observed in CD8 isolate (3.3 µm). Variation was also observed in size and lobes, length and width of appressoria produced by the pathogen. Differential reaction of isolates to three varieties revealed that CD5, CD8 and CD7 are highly virulent based on lesion area. Molecular characterization with RAPD, SSR and ISSR primers showed that RAPD and SSR markers are not linked to any characters of pathogen but ISSR primers show linkage to the virulence of the pathogen.

Key words: Lesion area, RAPD, ISSR, SSR, *Colletotrichum gloeosporioides*, small cardamom

Introduction

Cardamom (*Elletaria cardamomum*) has the unique distinction of "Queen of spices" and is a valued gift of the evergreen forest ecosystem that stretches along the Western Ghats of South India. In India, leaf blight of cardamom is widespread in all the productive regions and is one of the greatest concerns, especially under low-shade regions and certain types, especially Malabar types. Identification of *Colletotrichum* species is nearly always difficult due to enormous variation in the morphology accepted for the same species. The establishment of disease by artificial inoculation is also essential for the studies of various aspects of plant pathology, including epidemiology, etiology, disease resistance, host-parasite interaction, and disease control and also provides a foundational understanding of ecological enrichment (Jie *et al.*, 2009). Chandramani Raj (2012) identified the virulent isolate of *Colletotrichum* from different geographic backgrounds based on the lesion diameter produced on chilli fruits.

According to Lewis Ivey *et al.* (2004), molecular tools such as PCR with species-specific primers are useful in distinguishing species of *Colletotrichum* that cannot be distinguished using morphological methods. Freeman *et al.* (2000a) reported that using specific primer of *C. acutatum* amplified specifically *C. acutatum* isolates from strawberry and anemone but did not amplify *C. gloeosporioides* and *C. fragariae* isolates. Inversely, the *C. gloeosporioides* species specific primers amplified DNA of *C. gloeosporioides* isolates alone and not that of *C. acutatum* from strawberry and anemone. Further, Maymon *et al.* (2006) used CgInt as species specific primer coupled with ITS 4 for identification of *C. gloeosporioides* from Statice (*Limonium* spp.).

Molecular tools based on DNA analysis are being used in the biotyping of variants of fungi. Many molecular markers can be used to evaluate levels of genetic diversity, phylogenetic relationships and race identification (Karatas and Agaoglu, 2010). Random Amplified Polymorphic DNA (RAPD) is a technique based on PCR that facilitates the analysis of genetic diversity and structure in natural populations and the establishment of phenotypic relationships among different species and isolates of the same species (Martin and Rygiewicz, 2005). Ratanacherdchai *et al.* (2010) conducted a study to generate the genetic diversity of 34 isolates of *Colletotrichum* spp. using six ISSR primers and showed multi-band patterns for each isolate. The primers depicted about 62-99 percent similarity and resulted in the formation of three major groups based on the geographic distribution of anthracnose fungus. Thus the present study aims to link the suitable markers with the virulence of pathogen using lesion size as a quantitative trait.

Materials and methods

Survey, isolation and identification: Surveys in Karnataka, Kerala, and Tamil Nadu collected samples with leaf blight symptoms from various altitudes and rainfall regions. Microscopic examination confirmed the presence of fungal spores. Diseased tissues were teased, examined under a binocular microscope (10x), and isolated aseptically using the standard tissue isolation method. Infected tissues were cut, disinfested with 3% sodium hypochlorite, rinsed with sterile distilled water, and transferred to potato dextrose agar (PDA) medium with streptomycin sulphate (100 ppm). After 7 days of incubation at 25˚C, growing hyphal edges were transferred to a growth medium in Petri dishes.

Cultures were identified by comparing colony and conidial characteristics with published literature and purified by single spore isolation. Pure cultures, identified as *Colletotrichum* spp., were maintained on PDA slants at 4ºC for further studies, with isolates assigned code numbers for characterization.

Morphological characterization: Observations on macro-morphological features *viz*., colony morphology, growth rate and colour (top and reverse) were recorded by culturing the isolates on PDA medium. For morphological characterization, three 5 mm mycelial plugs were aseptically punched from actively growing areas near the edges of 7-day-old cultures of each isolate using a sterile cork borer. Each plug was subsequently transferred to the growth medium (PDA) and maintained in triplicates at a temperature range of 25 to 27 ºC for seven days. The mean radial mycelial growth (mm per day) of each isolate was recorded daily and after 7 days, colony size and colour were recorded. Further, the dimensions and shape of microscopic structures like conidia and appressoria were also recorded. For examination of conidial morphology, the isolates were cultured on PDA at 25 ºC for 7 days. The conidia harvested from the culture plates of each isolate were mounted in water, stained with lactophenol cotton blue and the size was measured at 10 X magnification. The production of conidial appressoria was induced by mixing the conidia in 2% sucrose solution. The conidial suspension was smeared on a glass slide and incubated in a moisture chamber at room temperature overnight. On a succeeding day, the slides were observed for the production of appressoria formed at the tip of germ tube from conidia and the size as well as number of lobes was recorded for each isolate (Darshana *et al.,* 2014).

Differential reaction on cardamom varieties: To study the differential reaction, clonal populations of three cardamom varieties *viz*., IISR Vijetha, IISR Avinash and Appangala 1 were inoculated with the test isolates. For preparation of the inoculum, the cultures of each isolate were grown on PDA for 7 days at 25 ºC with 12 hours of photoperiod (alternating light and dark conditions) to enhance conidial production (Hong *et al.*, 2008; Than *et al.*, 2008). The conidia were harvested by adding 10 mL of sterilized distilled water onto the Petri dish, which was subjected to gentle swirling to dislodge conidia from the mycelial mat. The concentration of conidia was adjusted to 10^6 conidia per mL using a haemocytometer and subsequently used as the standard density of inoculum. The first fully opened leaf from the spindle leaf (immature) and the second fully unfurled leaf from the spindle leaf (mature leaf) were surface sterilized with sterile distilled water and then injured gently using a sterilized needle (pin-pricking method). Each leaf had six prick points of inoculation, which was considered as single replication. Further, 5 µL of conidial suspension was placed on the wound and covered with moist cotton to ensure sufficient moisture to facilitate the infection process. The plants were observed periodically for the manifestation of symptoms. The prominence of the yellow halo surrounding the lesion area and streak as well as the lesion area were employed as criteria for recording symptomatological observations. The length (along the length of the foliage) and breadth (along the breadth of the foliage) of the lesion area were recorded on the 45th day after inoculation and the lesion area was computed using the formula π x (L X B)/ 4 (da Silva *et al.,* 2012).

Molecular characterization: In our previous study, we already characterized the pathogens based on molecular variation by using MLST techniques (Chethana *et al.,* 2016), the same isolates are used in the study. Details of which are provided in Table 1.

Molecular variability studies of *C. gloeosporioides* isolates: Knowledge of the genetic variation within and among populations is an important component of understanding the population biology of pathogenic fungi and infers the impact of driving force influencing the evolution of pathogen populations. Therefore, information on population diversity may be used for developing strategies to increase the durability of resistance. Hence, in this study, RAPD as well as ISSR were used to document variation among the isolates.

Table 1. Details of the *Colletotrichum* spp. used in the study (Chethana *et al.,* 2015)

Isolate code	Location	*Colletotrichum* sp.	Gen Bank Accession Numbers						
			ITS	GAPDH	ACT	CHS-1	GS	TUB	CYLH3
CD1	Bobbanahally, Karnataka	*C. karstii*	KJ813594	KJ813544	KJ813444	KJ813494	KJ813569	KJ813469	KJ813519
CD2	Kyanahally, Karnataka	*C. siamense*	KJ813609	KJ813559	KJ813459	KJ813509	KJ813584	KJ813484	KJ813534
CD4	Sirsi, Karnataka	*C. karstii*	KJ813595	KJ813545	KJ813445	KJ813495	KJ813570	KJ813470	KJ813520
CD5	Valparai, Tamil Nadu	*C. gloeosporioides*	KJ813602	KJ813552	KJ813452	KJ813502	KJ813577	KJ813477	KJ813527
CD6	Idukki, Kerala	*C. guajavae*	KJ813592	KJ813542	KJ813442	KJ813492	KJ813567	KJ813467	KJ813517
CD7	Yemagundi, Karnataka	*C. siamense*	KJ813610	KJ813560	KJ813460	KJ813510	KJ813585	KJ813485	KJ813535
CD8	Kodagu, Karnataka	*C. siamense*	KJ813611	KJ813561	KJ813461	KJ813511	KJ813586	KJ813486	KJ813536
CD13	Nelliahudikeri, Karnataka	*C. siamense*	KJ813612	KJ813562	KJ813462	KJ813512	KJ813587	KJ813487	KJ813537
CD14	Bhagamandala, Karnataka	*C. siamense*	KJ813613	KJ813563	KJ813463	KJ813513	KJ813588	KJ813488	KJ813538
CD15	Nagarahally, Karnataka	*C. syzygicola*	KJ813598	KJ813548	KJ813448	KJ813498	KJ813573	KJ813473	KJ813523
CD16	Ammathi, Karnataka	*Colletotrichum sp.*	KJ813604	KJ813554	KJ813454	KJ813504	KJ813579	KJ813479	KJ813529
CD18	Virajpet, Karnataka	*C. syzygicola*	KJ813597	KJ813547	KJ813447	KJ813497	KJ813572	KJ813472	KJ813522
CD19	Guddalur, Tamil Nadu	*C. siamense*	KJ813608	KJ813558	KJ813458	KJ813508	KJ813583	KJ813483	KJ813533
CD20	Wayanad, Kerala	*C. siamense*	KJ813614	KJ813564	KJ813464	KJ813514	KJ813589	KJ813489	KJ813539
CD21	Sakleshpur, Karnataka	*C. syzygicola*	KJ813600	KJ813550	KJ813450	KJ813500	KJ813575	KJ813475	KJ813525
CD23	Bettadamane, Karnataka	*C. syzygicola*	KJ813601	KJ813551	KJ813451	KJ813501	KJ813576	KJ813476	KJ813526
CD24	Bettadamane, Karnataka	*Colletotrichum sp.*	KJ813603	KJ813553	KJ813453	KJ813503	KJ813578	KJ813478	KJ813528
CD26	Sakleshpur, Karnataka	*Colletotrichum sp.*	KJ813604	KJ813554	KJ813454	KJ813504	KJ813579	KJ813479	KJ813529
CD27	Chickmagalur, Karnataka	*Colletotrichum sp.*	KJ813605	KJ813555	KJ813455	KJ813505	KJ813580	KJ813480	KJ813530
CD28	Urlikkal, Tamil Nadu	*C. guajavae*	KJ813590	KJ813540	KJ813440	KJ813490	KJ813565	KJ813465	KJ813515
CD29	Urlikkal, Tamil Nadu	*C. guajavae*	KJ813591	KJ813541	KJ813441	KJ813491	KJ813566	KJ813466	KJ813516

A set of ten RAPD (Random amplified polymorphic DNA) and seven simple sequence repeats (SSR) and five ISSR (inter simple sequence repeat) primers (Chromous Biotech Pvt. Ltd., Bangalore) as furnished in Table 2 and 3 were used to identify the molecular variability of twenty-six *C. gloeosporioides* isolates.

Table 2. List of RAPD primers

Marker systems	Nucleotide sequence 5' to 3'	PCR conditions	
OPB 07	GGTGACGCAG	Initial denaturation	94 ºC; 5 min. 41 cycles
OPC 02	GTGAGGCGTC		
OPF14	TGCTGCAGGT	Denaturation	94 ºC; 1 min.
OPF 07	CCGATATCCC		
OPL 12	GGGCGGTACT	Annealing	36 ºC; 1min.
OPL 05	ACGCAGGCAC		
OPD 07	TTGGCACGGG	Extension	72 ºC; 2 min.
OPA 09	GGGTAACGCC		
OPF 06	GGGAATTCGG	Final extension	72 ºC; 5 min.
OPC 08	TGGACCGGTG		

Table 3. List of SSR and ISSR primers

Nucleotide sequence 5' to 3'	PCR conditions		
SSR Primers			
1. $(CAG)_5$	Initial denaturation	95 ºC; 5 min.	30
2. $(GTG)_5$	Denaturation	95 ºC; 30 sec	cycles
3. $(AGG)_5$	Annealing	60 ºC; 30 sec	
	Extension	72 ºC; 1.5 min.	
	Final extension	72 ºC; 10 min.	
4. $(GACA)_4$	Initial denaturation	95 ºC; 5 min.	30
5. $(ACTG)_4$	Denaturation	95 ºC; 30 sec	cycles
6. $(GACAC)_3$	Annealing	48 ºC; 30 sec	
7. $(TGTC)_4$	Extension	72 ºC; 1.5 min.	
	Final extension	72 ºC; 10 min.	
ISSR Primers			
1. $(AG)_8$ T	Initial denaturation	94 ºC; 2 min.	35
2. $(AG)_8$ C	Denaturation	94 ºC; 1 min.	cycles
3. $(GA)_8$ T	Annealing	52 ºC; 1min.	
4. $(TG)_8$ A	Extension	72 ºC; 1.5 min.	
5. $(GA)_8$ YG	Final extension	72 ºC; 6 min.	

Amplification reactions were performed in volumes of 25 µL containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001 percent gelatin, 100 µM each of dATP, dCTP, dGTP and dTTP (Pharmacia), 0.2 µM primer, 25 ng of genomic DNA and 0.5 unit of Taq DNA polymerase (Perkin Elmer Cetus). Amplification was performed in an Eppendorf master cycle gradient thermal cycler programmed with appropriate PCR conditions using the fastest available transitions between each temperature. Amplification products were analyzed by electrophoresis in 1.2 percent agarose gel detected by staining with ethidium bromide.

Band scoring and data analysis: The banding patterns were scored for RAPD, SSR and ISSR primers in each *C. gloeosporioides* isolate starting from the small size fragment to large sized one. Presence and absence of each band in each isolate were coded as 1 and 0, respectively. The scores were used to create a data matrix to analyze genetic relationships using the NTSYS-pc program version 2.02 (Exeter Software, New York, USA) described by Rohlf (1990). A dendrogram was constructed based on Jaccard's similarity coefficient (Jaccard, 1908) using the marker data from the *C. gloeosporioides* isolates with the unweighted pair group method (UPGMA).

Results and discussion

Survey, isolation identification and characterization: Cardamom belongs to the family *Zingiberaceae*, called as "queen of spices," grown throughout the Western Ghats of India and is regarded as one of the world's most important spice crops. Generally, spices play a vital role in culinary purposes and medicinal properties. One of the limiting factors that influence spices' economic value is caused by plant pathogens. Saju *et al.* (2011) reported a yield loss of up to 48 percent in large cardamom due the *C. gloeosporioides*.

The surveys revealed that cardamom is cultivated under diverse agro-ecosystems, which also comprise other component crops. Twenty isolates of *Colletotrichum gloeosporioides* were collected from different cardamom growing tracts, especially, the Western Ghats region, identified at species level based on morphological characteristics and were assigned appropriate code numbers to facilitate the characterization procedures. These isolates were used to study the colony, conidial and appressorial characteristics. Morphological examination of these *C. gloeosporioides* isolates revealed a great level of variation, as reported by Sutton (1980). The colony, conidial and appressorial characteristics of isolates were found to be diverse, establishing variability existing in the field populations of *Colletotrichum.* The results revealed that all the isolates produced hyaline cylindrical conidia. However, significant variations were noticed for conidial dimensions among the isolates. The length of conidia ranged from 7.8-21.5 µm. The highest length of conidia was observed in CD16 isolate (21.5 µm) followed by, CD27 isolate (20.2 µm) and the shortest conidial dimension was recorded in CD8 (7.8 µm). The width of the conidia ranged from 3.3 to 7.8 µm. Isolate CD14 recorded the highest width of conidium (7.8 µm) and the lowest width was observed in CD8 isolate (3.3 µm). Variations were also observed in the size and lobes of appressoria produced by the pathogen. The length of the appressorium ranged from 6.5 to 15.6 µm. The highest length of appressorium was observed in CD1 isolate (15.6 µm) followed by CD5 isolate (15.3 µm) and the shortest was recorded in CD18 and CD20 (6.5 µm). The width of the appressoria ranged from 4.5 to 9.7 µm. Isolate CD4 recorded the highest width (9.7 µm) and the lowest width was observed in CD2, CD6 and CD20 isolate (4.5 µm) (Table 4). This variation was observed for all the isolates and is within the limits indicated for *C. gloeosporioides* according to Freeman *et al.* (2000b) and a majority of the isolates had straight cylindrical conidia that overall fitted into the commonly accepted description of *C. gloeosporioides* (Sutton, 1980).

Differential reaction on cardamom varieties: The differential reactions of twenty isolates were evaluated in three different varieties of cardamom (IISR Avinash, IISR Vijetha and Appangala I) both in immature and mature leaves. The maximum lesion area of 40.82 mm^2 was observed in the isolate CD20 against the variety IISR Avinash and the least was observed in CD28 (4.91 mm^2) in immature leaf, similar results were also observed in mature leaf. Whereas in the variety IISR Vijetha, the maximum leaf lesion area was noticed in CD5 (60.45 mm^2) followed by CD27 (35.72 mm^2) in immature leaf. Maximum lesion area in the variety Appangala I was noticed in CD5 (38.47 mm^2) followed by CD 20 (21.98mm^2) in immature leaves, whereas in mature leaves the reaction was controversial and maximum lesion area was observed in CD8 (54.95 mm^2) followed by CD7 (52.99 mm^2). Thus, from the results it can be concluded that CD5, CD8 and

Table 4. Colony, conidial and appressorial characteristics of *C. gloeosporioides* infecting cardamom

Isolate Code	Colony Diameter (cm)	Characteristics	Conidium						
			Shape	Size(µm)		No. of lobes	Size(µm)		
				Range	Mean		Range	Mean	
CD1	6.8	Grayish white mycelium concentric zonations outer circle white	C	13.69-16.3x3.2-6.52	15.25x4.8	U	12.3-15.6x9.1-10.4	13.4x9.7	
CD2	9.0	Grayish white mycelium, puffy growth , black colour	ECC	11.7-15.6x5.8-6.52	14.08x6.1	U	9.7-11x4.5-5.8	10.2x5.0	
CD4	8.5	Grayish puffy growth ,black colour with radial bottom	E	12.3-13.6x5.2-6.5	12.7x6.1	1-2	13.0-14.3x6.5-9.7	13.4x7.3	
CD5	7.0	Puffy growth, centre yellow mycelium, outer whitish growth	E	14.9-16.3x5.8-7.1	15.7x6.7	U	13.3-15.3x7.8-9.1	14.3x8.3	
CD6	6.8	Grayish white mycelium puffy growth	C	13.6-15.6x5.8-7.71	14.7x6.52	U	7.1-9.7x4.5-5.8	8.6x5.4	
CD7	9.0	Irregular growth white scanty mycelium, Slight pink colouration at the center	E	12.3-14.9x5.8-8.4	13.4x6.9	U	9.1-13.6x3.2-6.5	10.1x4.8	
CD8	9.0	Very fast growing grey colour mycelium(initially white) with puffy growth, black bottom	C	7.8-11.7x3.3-6.5	9.0x4.1	1-3	9.78-13.6x5.8-7.1	12.2x6.5	
CD13	9.0	Dull white mycelium scanty growth	E	12.3-13.6x5.8-6.5	13.1x6.1	U	11.7-13.0x5.8-7.1	12.3x6.4	
CD14	8.5	Whitish colony growth, White Bottom	E	16.9-18.2x7.1-7.8	17.7x7.6	U	8.4-13.0x5.8-7.1	9.6x6.3	
CD15	8.75	Grayish puffy growth towards the center portion , Black colour with zonations bottom	CCC	13.13-19.5x5.8-7.1	16.4x6.3	SL	9.1-10.4x5.8-6.5	9.6x6.0	
CD16	8.5	Grayish colony with well marked sectoring, Black bottom	ED	18.9-21.5x5.8-7.1	20.0x6.7	U	13.0-14.3x5.8-6.5	13.5x6.1	
CD18	8.75	Dull grayish puffy colony , black with zonations bottom	D	16.3-18.2x7.1-7.8	16.9x7.4	SL	6.5-7.8x5.8-6.5	7.1x6.1	
CD19	8.0	White cottony growth with slight sporulation towards center, White bottom	E	11.7-13.0x5.8-6.5	12.5x6.3	SL	13.0-14.3x5.8-6.5	13.5x6.1	
CD20	4.75	Grayish puffy growth, Black bottom	C	17.6-18.9x5.8-7.1	18.3x6.6	U	6.5-7.1x4.5-6.5	6.7x5.5	
CD21	6.5	White Mycelium with sectoring , irregular margin, slight creamish bottom	C	13.0-14.3x5.2-6.5	13.4x6.1	U	13.6-14.3x6.5-7.1	14.0x6.9	
CD23	8.75	Grayish white colony with sectoring and puffiness, black bottom	E	13.6-14.9x5.8-7.1	14.4x6.6	1-2	8.4-9.7x7.1-9.1	9.4x8.3	
CD24	8.75	White mycelium with grayish colony, Slight orangish bottom	ET	12.3-16.3x5.8-6.52	14.3x6.1	1-3	13.0-14.3x6.52-7.8	13.7x6.9	
CD26	8.75	Dull black cottony growth with clear zonations , Black bottom	DT	13.0-14.9x5.8-6.52	13.6x6.1	1-2	7.8-8.4x5.2-5.8	8.2x5.6	
CD27	9.0	Dark grayish cottony growth, Black bottom	CT	18.2-20.2x5.8-7.1	19.1x6.3	U	7.8-9.7x5.8-6.5	8.6x6.3	
CD28	9.0	Grayish cottony with clear radial growth, Black colour with radial growth and zonations bottom	D	13.0-17.6x6.52-7.1	15.3x6.9	U	7.1-9.7x3.9-5.2	8.4x4.6	
CD29	8.75	Dull blackish cottony growth, Black colour bottom	D	13.0-15.6x5.8-7.1	14.6x6.5	1-2	7.1-9.7x5.8-7.1	8.3x6.52	

Table 5. Differential reaction of *C. gloeosporioides* isolates on cardamom variety, IISR Avinash

Isolate code	Immature leaf						Mature leaf					
	Symptomatology			Lesion Area			Symptomatology			LesionArea		
	Halo (Yellow)	Margin	Streak`	Length (Range)	Breadth (Range)	Area (mm^2)	Halo (Yellow)	Margin	Streak`	Length (Range)	Breadth (Range)	Area(mm^2)
CD1	Prominent	REB	NPROM	4-7(5.5)	4-7(5.5)	23.75±1.31	Prominent	REB	NPROM	2-4(3)	3-5(4)	9.42±0.54
CD2	Prominent	REB	NPROM	4-8(6)	4-6(5)	23.55±1.36	Prominent	NoREB	NPROM	4-6(5)	3-5(4)	15.70±0.90
CD4	NPROM	REB	NPROM	1-4(2.5)	3-4(3.5)	6.87±0.39	NPROM	NoREB	Nostreak	2-3(2.5)	1-3(2)	3.93±0.22
CD5	Prominent	REB	NPROM	2-5(3.5)	3-5(4)	10.99±0.634	Prominent	NoREB	NPROM	3-6(4.5)	3-5(4)	14.13±0.81
CD6	Prominent	REB	NPROM	3-4(3.5)	3-5(4)	10.99±0.634	NPROM	NoREB	Nostreak	2-3(2.5)	2-4(3)	5.89±0.34
CD7	Prominent	REB	NPROM	2-5(3.5)	2-4(3)	8.24±0.47	NPROM	REB	Nostreak	2-3(2.5)	3-4(3.5)	6.87±0.39
CD8	NPROM	REB	NPROM	3-10(6.5)	3-5(4)	20.41±1.17	NPROM	REB	Nostreak	2-3(2.5)	3-5(4)	7.85±0.45
CD13	NPROM	REB	NPROM	3-4(3.5)	3-4(3.5)	9.62±0.55	NPROM	REB	Nostreak	2-3(2.5)	3-4(3.5)	6.87±0.39
CD14	NPROM	REB	NPROM	3-5(4)	3-5(4)	12.56±0.72	NPROM	NoREB	Nostreak	2-4(3)	3-5(4)	9.42±0.54
CD15	NPROM	REB	Nostreak	3-6(4.5)	3-4`(3.5)	12.36±0.71	NPROM	REB	Nostreak	3-4(3.5)	3-5(4)	10.99±0.634
CD16	NPROM	REB	NPROM	2-3(2.5)	3-4(3.5)	6.87±0.39	NPROM	REB	Nostreak	2-4(3)	3-5(4)	9.42±0.54
CD18	Prominent	REB	NPROM	5-7(6)	4-5(4.5)	21.20±1.22	NPROM	REB	Nostreak	3-5(4)	3-6(4.5)	14.13±0.81
CD20	Prominent	REB	Prominent	6-10(8)	4-9(6.5)	40.82±2.35	Prominent	REB	NPROM	4-6(5)	4-6(5)	19.63±1.13
CD21	NPROM	REB	Nostreak	3-7(5)	3-4(3.5)	13.74±0.79	NPROM	REB	Nostreak	3-4(3.5)	3-5(4)	10.99±0.63
CD23	NPROM	REB	NPROM	2-6(4)	3-4(3.5)	10.99±0.63	NPROM	REB	Nostreak	3-4(3.5)	4-6(5)	13.74±0.79
CD24	NPROM	REB	NPROM	2-4(3)	3-5(4)	9.42±0.54	NPROM	NoREB	Nostreak	1-3(2)	3-4(3.5)	5.50±0.31
CD26	Prominent	REB	NPROM	2-6(4)	3-5(4)	12.56±0.72	NPROM	REB	Nostreak	2-3(2.5)	2-4(3)	5.89±0.34
CD27	NPROM	REB	NPROM	3-4(3.5)	3-5(4)	10.99±0.64	NPROM	REB	Nostreak	2-3(2.5)	2-4(3)	5.89±0.34
CD28	NPROM	REB	NPROM	2-3(2.5)	2-3(2.5)	4.91±0.28	NPROM	REB	Nostreak	2-3(2.5)	2-3(2.5)	4.91±0.283
CD29	NPROM	REB	NPROM	3-7(5)	4-6(5)	19.63±1.13	NPROM	REB	Prominent	1-3(2)	1-4(2.5)	3.93±0.22
C.D. *P*=0.05						2.75						1.68

Not Prominent: NPROM. Redish Brown: REB

Table 6. Differential reaction of *C. gloeosporioides* isolates on cardamom variety, IISR Vijetha

Isolate code	Immature leaf						Mature leaf					
	Symptomatology			Lesion Area			Symptomatology			LesionArea		
	Halo (Yellow)	Margin	Streak`	Length (Range)	Breadth (Range)	Area (mm^2)	Halo (Yellow)	Margin	Streak`	Length (Range)	Breadth (Range)	Area (mm^2)
CD1	Prominent	REB	NPROM	5-7(6)	6-8(7)	32.97±2.85	Prominent	REB	NPROM	3-7(5)	4-10(7)	27.48±2.38
CD2	Prominent	REB	Prominent	4-6(5)	4-6(5)	19.63±1.70	Prominent	REB	NPROM	3-5(4)	4-6(5)	15.70±1.36
CD4	Prominent	REB	NPROM	3-7(5)	3-6(4.5)	17.66±1.52	Prominent	REB	NPROM	5-7(6)	4-9(6.5)	30.62±2.65
CD5	Prominent	REB	Prominent	4-10(7)	5-17(11)	60.45±5.23	Prominent	REB	NPROMt	4-10(7)	10-16(13)	71.44±6.18
CD6	NPROM	REB	Nostreak	3-4(3.5)	4-7(5.5)	15.11±1.30	NPROM	REB	Nostreak	3-4(3.5)	4-6(5)	13.74±1.20
CD7	Prominent	REB	Nostreak	2-4(3)	3-5(4)	9.42±0.81	NPROM	REB	Nostreak	2-5(3.5)	3-7(5)	13.74±1.19
CD8	Prominent	REB	Nostreak	3-6(4.5)	3-6(4.5)	15.90±1.37	NPROM	REB	Nostreak	2-5(3.5)	3-6(4.5)	12.36±1.07
CD13	NPROM	REB	Nostreak	3-6(4.5)	5-8(6.5)	22.96±1.98	NPROM	REB	Nostreak	3-6(4.5)	6-8(7)	24.73±2.90
CD14	NPROM	REB	NPROM	1-4(2.5)	3-5(4)	7.85±0.68	NPROM	REB	Nostreak	2-4(3)	3-7(5)	11.78±1.02
CD15	Prominent	REB	NPROM	3-7(5)	7-10(8.5)	33.36±2.89	Prominent	REB	NPROMt	3-6(4.5)	8-11(9.5)	33.56±2.90
CD16	NPROM	REB	Prominent	3-6(4.5)	4-6(5)	17.66±1.52	NPROM	NoREB	Nostreak	2-4(3)	3-7(5)	11.78±1.02
CD18	Prominent	REB	NPROM	3-9(6)	3-11(7)	32.97±2.85	Prominent	REB	Nostreak	4-6(5)	7-10(8.5)	33.36±2.88
CD20	NPROM	REB	NPROM	3-6(4.5)	4-6(5)	17.66±1.52	NPROM	REB	Nostreak	2-4(3)	3-6(4.5)	10.60±0.91
CD21	NPROM	REB	Prominent	2-5(3.5)	5-8(6.5)	17.86±1.54	NPROM	REB	NPROM	3-6(4.5)	6-10(8)	28.26±2.44
CD23	Prominent	REB	Prominent	4-8(6)	4-10(7)	32.97±2.85	NPROM	REB	Nostreak	2-5(3.5)	2-6(4)	10.99±0.95
CD24	NPROM	REB	NPROM	3-6(4.5)	5-8(6.5)	22.96±1.98	NPROM	REB	Nostreak	3-6(4.5)	5-10(7.5)	26.49±2.29
CD26	Prominent	REB	Prominent	4-7(5.5)	3-6(4.5)	19.43±1.68	NPROM	REB	Prominent	2-5(3.5)	3-7(5)	13.74±1.19
CD27	NPROM	REB	NPROM	2-11(6.5)	3-11(7)	35.72±3.09	NPROM	REB	Nostreak	3-5(4)	4-6(5)	15.70±1.36
CD28	NPROM	REB	Nostreak	2-4(3)	2-5(3.5)	8.24±0.71	NPROM	REB	Nostreak	2-5(3.5)	3-6(4.5)	12.36±1.07
CD29	Prominent	REB`	NPROM	3-6(4.5)	3-7(5)	17.66±1.52	NPROM	REB	Nostreak	3-4(3.5)	5-10(7.5)	20.61±1.78
C.D.*P*=0.05						6.42						2.24

Table 7. Differential reaction of *C. gloeosporioides* isolates on cardamom variety, Appangala I

Isolate code	Immature leaf						Mature leaf					
	Symptomatology			Lesion Area			Symptomatology			LesionArea		
	Halo (Yellow)	Margin	Streak`	Length (Range)	Breadth (Range)	Area (mm^2)	Halo (Yellow)	Margin	Streak`	Length (Range)	Breadth (Range)	Area (mm^2)
CD1	Prominent	REB	NPROM	3-5(4)	7-10(8.5)	26.69±2.31	Prominent	REB	NPROM	3-5(4)	5-10(7.5)	23.55±1.36
CD2	NPROM	REB	NPROM	3-5(4)	3-6(4.5)	14.13±1.22	NPROM	REB	NPROM	3-5(4)	3-10(6.5)	20.41±1.78
CD4	NPROM	REB	NPROM	4-8(6)	5-10(7.5)	35.33±3.06	NPROM	REB	NPROM	4-7(5.5)	7-10(8.5)	36.70±3.17
CD5	Prominent	REB	Prominent	4-10(7)	4-10(7)	38.47±3.33	NPROM	REB	Prominent	3-5(4)	8-13(10.5)	32.97±2.85
CD6	NPROMt	REB	NPROM	4-6(5)	4-9(6.5)	25.51±2.20	NPROM	REB	NPROM	3-5(4)	5-9(7)	21.98±1.90
CD7	NPROM	REB	NPROM	3-5(4)	5-10(7.5)	23.55±2.04	NPROM	REB	Prominent	4-11(7.5)	7-11(9)	52.99±4.58
CD8	NPROM	REB	NPROM	2-6(4)	6-10(8)	25.12±2.17	NPROM	REB	Nostreak	4-10(7)	8-12(10)	54.95±4.75
CD13	NPROM	REB	Prominent	3-4(3.5)	9-13(11)	30.22±2.61	NPROM	REB	Prominent	2-5(3.5)	5-11(8)	21.98±1.90
CD14	NPROM	REB	NPROM	3-5(4)	5-9(6.5)	20.41±1.76	NPROM	REB	Nostreak	3-5(4)	6-8(7)	21.98±1.92
CD15	NPROM	REB	NPROM	2-4(3)	4-6(5)	11.78±1.02	NPROM	REB	Nostreak	3-4(3.5)	4-8(6)	16.49±1.48
CD16	NPROM	REB	NPROM	4-5(4.5)	6-13(9.5)	33.56±2.90	NPROM	REB	NPROM	3-5(4)	11-15(13)	40.82±3.52
CD18	NPROM	REB	NPROM	3-5(4)	3-5(4)	12.56±1.08	NPROM	REB	No-streak	4-6(5)	4-5(4.5)	17.66±1.52
CD20	NPROM	REB	NPROM	3-5(4)	5-9(7)	21.98±1.90	NPROM	REB	Nostreak	3-6(4.5)	6-8(7)	24.73±2.14
CD21	NPROM	REB	Nostreak	4-9(6.5)	4-7(5.5)	28.06±2.43	NPROM	REB	Nostreak	4-10(7)	8-10(9)	49.46±4.28
CD23	NPROM	REB	NPROM	5-6(5.5)	5-9(7)	30.22±2.61	NPROM	REB	Nostreak	4-7(5.5)	4-10(7)	30.22±2.61
CD24	NPROM	REB	NPROM	4-7(5.5)	4-9(6.5)	28.06±2.43	NPROM	REB	Nostreak	3-7(5)	5-8(6.5)	25.51±2.20
CD26	Prominent	REB	Prominent	4-13(8.5)	4-7(5.5)	36.70±3.17	Prominent	REB	NPROM	3-5(4)	6-11(9.5)	29.83±2.58
CD27	NPROM	REB	NPROM	3-6(4.5)	5-7(6)	21.20±1.83	NPROM	REB	NPROM	4-6(5)	6-10(8)	31.40±2.71
CD28	NPROM	REB	NPROM	4-6(5)	7-11(9)	35.33±306	NPROM	REB	NPROM	3-6(4.5)	9-13(11)	38.86±3.36
CD29	NPROM	REB`	Prominent	3-6(4.5)	7-10(8.5)	30.03±2.60	NPROM	REB	Nostreak	3-5(4)	5-7(6)	18.84±1.62
C.D. *P*=0.05						6.87						3.00

CD7 are the most virulent isolates (Table 5, 6, 7; Fig. 1). Garg *et al.* (2013) have also explained the differential reaction of *C. capsici* upon artificial inoculation to 41 genotypes of chilli, eleven highly resistant lines have also been identified. It can be argued that variation in the isolates may be inherent since, isolates were collected from different locations, and the physiological characters are influenced by environmental conditions through natural chance mutations which may be responsible for such variability. There was no clear relationship between the seven morphological groups and pathological groups. The isolates which were under the same morphological group, differed in virulence groups. Combination of these two characteristics has been successfully used to categorize *Colletotrichum* species. Thind and Jhooty (1990) successfully used morphological and pathological characteristics to categorize 150 isolates of *C. capsici* and *C. gloeosporioides* causing chilli anthracnose.

Assessing genetic diversity through RAPD, SSR and ISSR markers: In RAPD, SSR and ISSR analysis, polymorphic fragments were generated in *C. gloeosporioides* isolates. The selection of primers was based on clear, scorable and reproducible amplified banding patterns.

Each RAPD pattern was compared with other patterns and genetic similarity matrix for all the twenty isolates was constructed

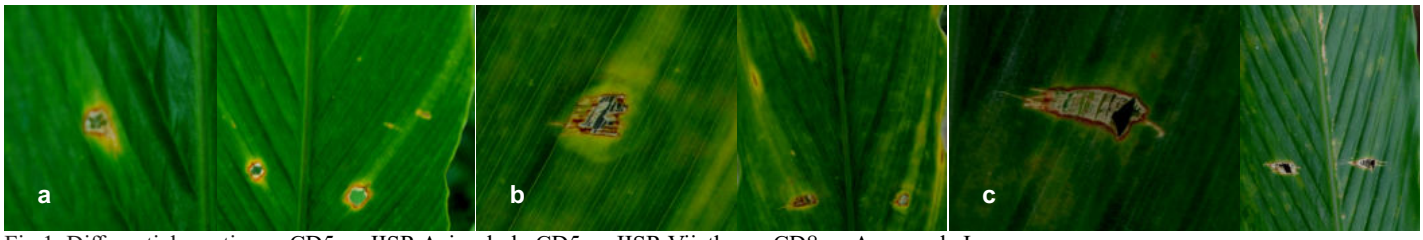

Fig 1. Differential reaction a: CD5 on IISR Avinash; b: CD5 on IISR Vijetha; c: CD8 on Appangala I

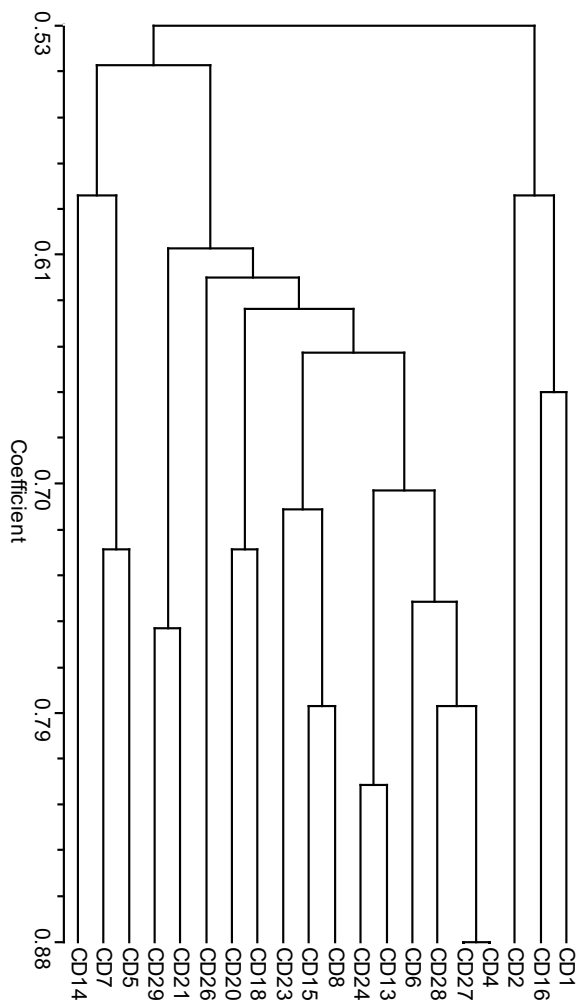

Fig. 2. Genetic variability among the *C. gloeosporioides* isolates as revealed by UPGMA cluster analysis based on RAPD primer

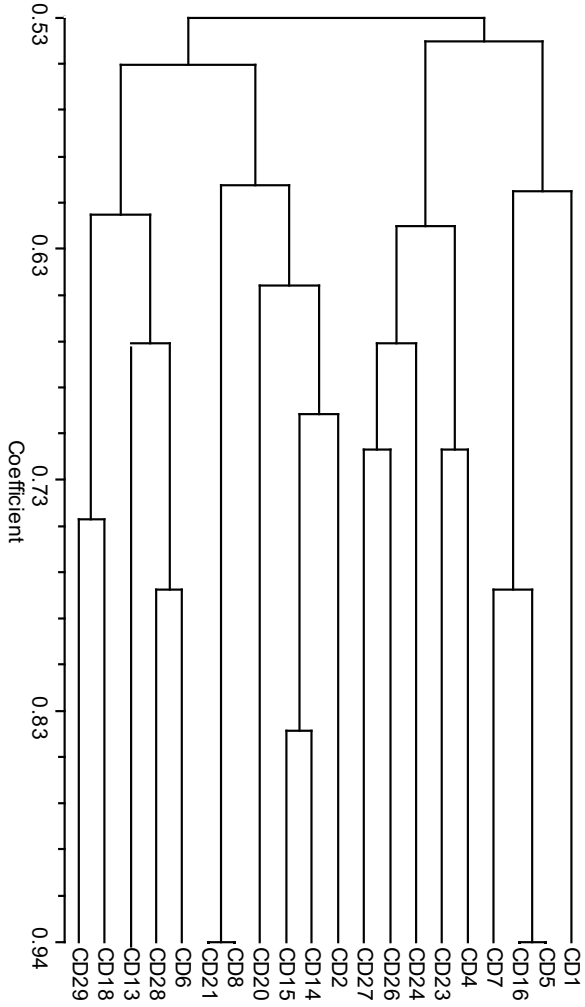

Fig. 3. Genetic variability among the *C. gloeosporioides* isolates as revealed by UPGMA cluster analysis based on SSR primers

from binary data of markers using the Jaccards algorithm. The coefficient of genetic similarity ranged from 53-88 percent. A maximum similarity of 88 percent was noticed between CD4 and CD27. Further, the information generated out of the RAPD banding pattern was used for clustering through the unweighted mean pair group arithmetic mean method (UPGMA). The data from RAPD distinguished the isolates into two main clusters at 16 percent similarity (Fig. 2). Among the twenty isolates, seventeen isolates come under Cluster I and three isolates namely CD1, CD16 and CD2 accounts in Cluster II. Cluster I is further subdivided into two clusters as A and B with 56 percent similarity. Cluster A, has three isolates comprising two sub-clusters *viz*., C and D with 68 percent similarity. Cluster B has 15 isolates and maximum similarity falls in this cluster

UPGMA analysis based on SSR markers was carried out to group twenty isolates of *C. gloeosporioides*. A dendrogram resulting from a cluster analysis showed two main distinct clusters, designated as I and II exhibiting overall genetic relationship among the isolates (Fig. 3). Cluster I sub divided into sub clusters as A and B with 58 percent similarity coefficient, having nine isolates in A and eleven isolates in cluster B. In SSR marker system also the grouping was not associated with the geographical locations not even to virulent nature.

Whereas the dendrogram resulting from the cluster analysis of ISSR primers showed two main distinct clusters, designated as I and II exhibiting overall genetic relationships among the isolates (Fig. 4). Cluster I is sub-divided into sub-clusters as A and B, having one isolate in A and 19 isolates in cluster B. In ISSR marker system grouping was not associated with the geographical locations but CD5, CD8 and CD20 were associated closely about 95 percent which were found to be highly virulent according to the lesion area calculation (Table 4, 5, 6).

Random Amplified Polymorphic DNA (RAPD) markers are used widely to detect genetic variation. In the present study, the data

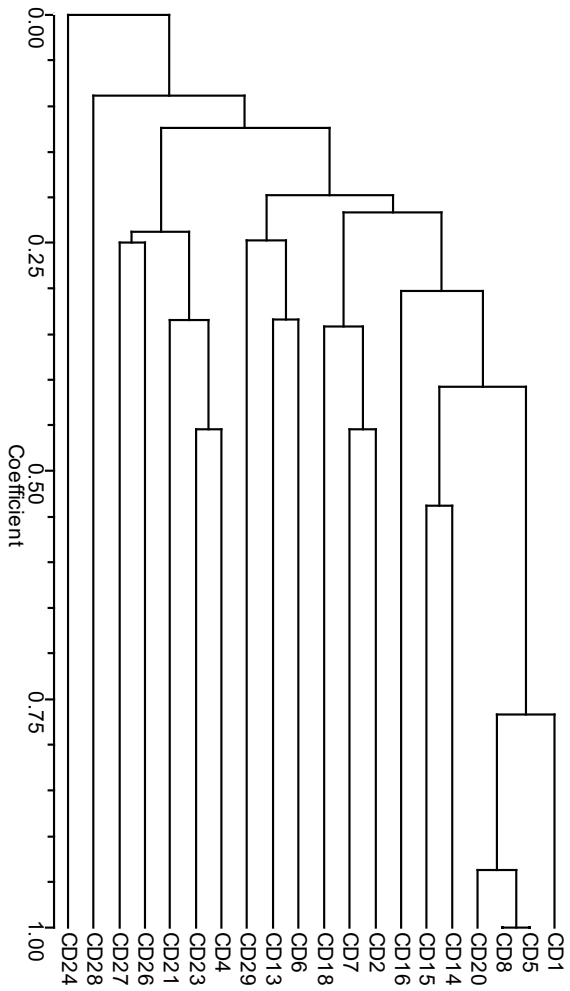

Fig. 4. Genetic variability among the *C. gloeosporioides* isolates as revealed by UPGMA cluster analysis based on ISSR primers

from RAPD distinguished the coefficient of genetic similarity ranging from 16 - 57 percent. Sharma *et al.* (2005) reported that in the phylogenetic grouping of *C. capsici* causing fruit rot/ die back or anthracnose of chillies in India, RAPD data did not appear to be congruent with morphological and virulence patterns. Several studies have demonstrated the genetic diversity of *C. gloeosporioides* isolates (Figueiredo *et al.,* 2012; Faisal *et al.,* 2013). This genetic heterogeneity could be able to the presence of the teleomorph phase.

Simple Sequence Repeats and Inter simple sequence repeat (ISSR) markers are powerful tools that can be utilized as molecular tools to access the variation around the diverse microsatellite regions that are dispersed throughout all genomes (Zietkiewicz *et al.,* 1994). In the present study, seven simple sequence repeats (SSR) and five ISSR (inter simple sequence repeat) primers were used to screen the linkage of markers to any traits. SSR markers like the RAPD markers had no linkages with any trait in our study, interestingly the ISSR markers were closely linked to virulence of the fungi but not to avirulence. In ISSR marker system grouping was not associated with the geographical locations but CD5, CD8 and CD20 were associated closely about 95 percent which were found to be highly virulent according to the lesion area calculation. Archana *et al.* (2017, 2020) showed UPGMA analysis using ISSR markers made it possible to discriminate the isolates of *C. gloeosporioides* to their virulent nature

It is concluded from the study that *C. gloeosporioides* and *C. siamense* are highly virulent as described through the ISSR primers and hence these isolates can be used for screening the germplasm against the leaf blight disease.

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