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Survey, Phenotypic and Genetic Characterization of *Colletotrichum capsici*, Incitant of Turmeric Leafspot

P. Adhipathi*, S. Nakkeeran and A. Chandrasekaran
Department of Plant Pathology, Centre for Plant Protection Studies,
Tamil Nadu Agricultural University, Coimbatore - 641003, India.

An extensive survey was conducted in major turmeric growing areas of Andhra Pradesh, Karnataka, Kerala and Tamil Nadu. The maximum severity of leaf spot recorded was 48.25% and 43.85% during 2011-12 and 2012-13, respectively. Twenty isolates of *Colletotrichum capsici* causing leaf spot of turmeric were evaluated for their phenotypic, pathogenic and virulence characters. The isolates differed in colony colour, shape and size of conidia and were categorized into four groups. The pathogen produced cottony colonies with zigzag to ring or circular pattern of growth. PCR amplification of internal transcribed spacer (ITS) rDNA conserved region, result revealed that all the twenty isolates yielded an amplicon size of 590 base pair and the sequence has been registered in NCBI genbank data base.

Key words: Leaf spot, Turmeric, *Colletotrichum capsici,* Virulence, Genetic characterization

Leaf spot of turmeric (*Curcuma longa* L), incited by *Colletotrichum capsici* (Butler and Bisby) causes considerable yield losses in major turmeric-growing regions of India. Most of the turmeric cultivars available presently are equally susceptible to leaf spot inciting extensive yield losses to turmeric production. Besides, the monoculture has resulted in severe epidemics. Several *Colletotrichum* species or biotypes are associated with a single host (Peres *et al*., 2002) and identification by morphological and physiological methods is very difficult. The use of molecular marker based tools has improved the accuracy of identification of *Colletotrichum* spp (Cai *et al*., 2009). The amplification of internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) is a precise and reliable technique for easy detection of pathogens. Hence, the present study was planned to investigate the occurrence of disease severity, phenotypic and genetic diversity the pathogen.

Materials and Methods

Survey for the occurrence of leaf spot disease of turmeric

An extensive survey was conducted in major turmeric growing areas of Andhra Pradesh, Karnataka, Kerala and Tamil Nadu to assess the severity of leaf spot during 2011- 12 and 2012 -13. The disease rating was recorded by adopting 0-6 scale (Palarpawar and Ghurde, 1989). The disease intensity (PDI) was calculated according to the formula suggested by Datar and Mayee (1981) as given below:

$$PDI = \frac{\text{Sum of all numerical rating of infected leaves on plant}}{\text{No. of leaves observed} \times \text{Maximum disease score}} \times 100$$

Collection and isolation of pathogen

Turmeric leaf spot samples were collected from different turmeric growing states of southern India during 2011- 12. The pathogen was isolated from infected leaf sample using potato dextrose agar (PDA) medium and it was incubated at 28±2$_o$C for 7 to 10 days. The pure cultures of the fungus were obtained by single spore isolation method following Choi *et al*. (1999).

Phenotypic characterization

C. capsici isolates were identified based on morphological and cultural charactertics (Than *et al*., 2008a). For the induction of sporulation, the cultures were maintained for 12 h under fluorescent light and 12 h dark alternatively. The conidia were harvested from each isolate and mounted in water. The size and shape of twenty five conidia and acervuli were measured and photographed under an image analyzer (LABOMED iVu5100, Labo America Inc, USA. Scope image 9.0 exe, software 9.1v).

Pathogenicity and virulence

Pure cultures of each isolate were grown on PDA for 10–14 days at 28±2°C under alternating 12 h fluorescent light and dark to induce sporulation (Than *et al.*, 2008b). The conidial suspension was harvested, filtered and centrifuged at 5000 rpm. The mass of spore sedimentation was collected,

*Corresponding author email :adhiagri@gmail.com

resuspended with sterilized distilled water and density of the spore was adjusted to $1\times10_6$ spores/ ml using a haemocytometer. The virulence of twenty isolates of *C. capsici* was tested on detached turmeric leaves from one month old plants of susceptible variety Erode local-8. The conidial suspension was spotted on detached leaf, placed in sterilized Petri dish, lined with moist cotton under aseptic condition and incubated for 5 days to record the lesion diameter. The conidial suspension was sprayed on plants at 3-4 leaf stage and maintained at 28±2°C and 88% RH under glass house condition. The appearance and severity of symptoms as well as virulence of pathogen was observed and recorded PDI (Than *et al*., 2008a).

Genetic characterization

Genomic DNA was extracted from different isolates of *Colletotrichum* spp as per the protocol described by Than *et al*. (2008a) and subjected to PCR amplification of ITS-rDNA region. The complete ITS-rDNA, forward primer ITS1F 5'-GTCCTAACAAGGTTTCCGTA-3' and reverse primer ITS4R 5' TTCTCCGCTTATTGATAT GC -3' (Tapia-Tussell *et al*., 2008) were amplified for molecular detection. PCR amplification of ITS-rDNA region was performed using Eppendorf - Master Cycler nexus gradient (Eppendorf, A G, Hamburg, Germany) with an initial denaturation of 94°C for 5 min, followed by 35 cycles of one min at 94°C, one min at 46°C and one min at 72°C, with a final extension for 10 min at 72°C. The PCR products were electrophorized and the DNA banding pattern was photographed using gel documentation unit. DNA sequence was submitted to obtain accession number from National Centre for Biotechnology Information (USA).

Results

Survey for the occurrence of leaf spot disease of turmeric

Survey on severity of turmeric leaf spot revealed the young crops (2 to 2.5 months old) mostly, remained free from the infectivity of leaf spot. The PDI of leaf spot varied from 18.65 to 48.25 (2011-12) and 24.15 to 43.85 (2012-13) in different turmeric growing areas. The maximum disease severity was 48.25 and 43.85 per cent in Erode district of Tamil Nadu, during 2011-12 and 2012-13, respectively. The PDI of 44.60 and 41.40 was recorded in Nizamabad district of Andhra Pradesh during 2011-12 and 2012-13, respectively. Whereas in Palakadu district of Kerala, maximum PDI of 35.80 per cent was recorded. The severity of disease was minimum (18.65%) in Gulberga district of Karnataka and Karur (24.15%) district of Tamil Nadu (Table 1).

Table 1. Survey for the occurrence and severity of turmeric leaf spot

Name of the isolate	Location	State	PDI	
			2011-12	2012-13
Cc1	Coimbatore	Tami Nadu	28.90$_k$	33.90$_f$
Cc2	Erode	Tami Nadu	48.25$_a$	43.85$_a$
Cg1	Salem	Tami Nadu	36.50$_d$	38.50$_c$
Cc3	Dharmapuri	Tami Nadu	38.40$_c$	31.68$_{gh}$
Cc4	Karur	Tami Nadu	22.50$_o$	24.15$_m$
Cc5	Namakkal	Tami Nadu	30.75$_h$	28.60$_j$
Cc6	Krishanagiri	Tami Nadu	34.50$_f$	27.95$_k$
Cg2	Perumbalur	Tami Nadu	29.45$_{ij}$	31.50$_{hi}$
Cc7	Villupuram	Tami Nadu	30.40$_h$	24.35$_m$
Cg3	Trichy	Tami Nadu	29.70$_i$	34.84$_e$
Cc8	Nizamabad	Andhra Pradesh	44.60$_b$	41.40$_b$
Cc9	Guntur	Andhra Pradesh	23.70$_n$	34.50$_e$
Cc10	Warangal	Andhra Pradesh	27.20$_l$	31.95$_g$
Cc11	Kozhikode	Kerala	23.50$_n$	28.40$_j$
Cc12	Palakkadu	Kerala	24.40$_m$	35.80$_d$
Cc13	Wayanad	Kerala	32.67$_g$	33.65$_f$
Cc14	Belgaum	Karnataka	34.30$_f$	31.33$_i$
Cc15	Mysore	Karnataka	35.80$_e$	34.60$_e$
Cc16	Chamarajnagar	Karnataka	29.20$_{jk}$	35.60$_d$
Cc17	Gulbarga	Karnataka	18.65$_p$	27.60$_l$

*Values are the mean of three replications. In a column, means followed by common letters are not significantly different at the 5% level by DMRT. PDI - Per cent Disease Index

Examination phenotypic characteristics

Twenty isolates of *Colletotrichum* spp were identified based on morphological, conidial and acervuli characters. The colonies were zigzag, cottony, ring or circular with zonation and greyish-white to dark grey or light brown on the upper surface whereas, in the reverse side it was mostly black. They were designated into four group *viz*., CC-I

(Cc1, Cc3, Cc6, Cc13) produced zigzag cottony, smooth surface with grey colonies; CC-II (Cc2, Cc4, Cc12, Cc16) produced circular cottony colonies with white to grey colour, whereas the isolate in group CC-III (Cg1, Cg2, Cc5, Cc8, Cc15, Cc17) and CC-IV (Cc7, Cg3, Cc9, Cc10, Cc11, Cc14) possessed ring like or circular growth with zigzag zonation of colonies. The colony diameter of different isolates varied from 74 to 90 mm on PDA. The shape of conidia produced by all the isolates was fusiform with round oil- globule at the centre with curved, pointed tips at both the ends. The average mean length of conidia recorded was 21.60 µm. The maximum and minimum length of conidia was recorded in Cc9 (24.61 µm) and Cc3 (17.38 µm), respectively. The average width of conidia was observed as 4.26 µm. However, the maximum and minimum width of conidia was noticed in Cc2 (4.92µm) and (Cc12) 3.68 µm, respectively. All the isolates were separated into three groups *viz*., small, medium and large based on length of conidia. The average length of conidia in the group of small, medium and large ranged from 17.38 - 18.39, 19.23 - 21.49 and 21.50 - 24.41 µm, respectively. The average length of setae in the acervuli was 103.96 µm. The highest length was recorded in Cc6 (122.75µm). Whereas, the least length was recorded in Cc14 (89.25µm). The length and width of conidia and acervuli varied significantly varied with each of the isolates of *C. capsici* (Table 2).

Table 2. Phenotypic characteristics of *C. capsici*

Isolate	Colony morphology	Colony color	Colony diameter (mm)*	Conidia shape	Conidia Length (µm)*	Conidia Width (µm)*	Setae length of acervuli (µm)*
Cc1	Zigzag cottony colonies, smooth	Grey	88.0$_{ab}$	Fusiform, medium	21.49$_{bc}$	4.75$_{ab}$	83.35$_{k}$
Cc2	Circular cottony colonies	Grey	90.0$_{a}$	Fusiform, medium	20.07$_{cde}$	4.92$_{a}$	119.54$_{ab}$
Cg1	Ring like zonation, smooth	White	84.0$_{a-d}$	Fusiform, large	23.08$_{ab}$	4.66$_{ab}$	106.80$_{c-g}$
Cc3	Zigzag cottony colonies, smooth	Grey	85.0$_{a-d}$	Fusiform, small	17.38$_{f}$	4.71$_{ab}$	115.65$_{abc}$
Cc4	Circular cottony colonies, rough	White	87.0$_{abc}$	Fusiform, medium	20.78$_{cd}$	4.51$_{abc}$	98.21$_{g-j}$
Cc5	Ring like zonation, rough	Grey	90.0$_{a}$	Fusiform, small	18.29$_{ef}$	3.88$_{ef}$	90.56$_{jk}$
Cc6	Zigzag cottony colonies, smooth	Grey	82.0$_{b-e}$	Fusiform, medium	19.40$_{de}$	3.85$_{ef}$	122.75$_{a}$
Cg2	Round cottony colonies	White	75.0$_{e}$	Fusiform, medium	22.98$_{ab}$	4.02$_{def}$	103.50$_{e-h}$
Cc7	Zigzag colonies	Grey	77.0$_{de}$	Fusiform, large	23.77$_{a}$	3.82$_{ef}$	108.56$_{c-f}$
Cg3	Circular, Smooth	White	83.0$_{a-e}$	Fusiform, large	24.05$_{a}$	3.81$_{ef}$	104.50$_{d-h}$
Cc8	Ring like growth,	Grey	89.0$_{ab}$	Fusiform, large	23.91$_{a}$	4.74$_{ab}$	113.65$_{a-d}$
Cc9	Ring like growth, rough	Black	74.0$_{e}$	Fusiform, Large	24.61$_{a}$	4.76$_{ab}$	114.56$_{a-d}$
Cc10	Zigzag colonies, Smooth	Grey	87.0$_{abc}$	Fusiform, Large	24.21$_{a}$	3.95$_{ef}$	92.40$_{ij}$
Cc11	Zigzag zonation, rough	White	85.0$_{a-d}$	Fusiform, Large	24.41$_{a}$	4.21$_{cde}$	100.50$_{f-i}$
Cc12	Cicular zonations, rough	White	90.0$_{a}$	Fusiform, large	22.87$_{ab}$	3.68$_{f}$	112.50$_{a-e}$
Cc13	Zigzag cottony colonies, smooth	Light brown	75.0$_{e}$	Fusiform, Large	22.86$_{ab}$	3.88$_{ef}$	95.60$_{hij}$
Cc14	Zigzag colonies, smooth	Grey	85.0$_{a-d}$	Fusiform, small	18.39$_{ef}$	4.12$_{cde}$	89.25$_{jk}$
Cc15	Ring like zonation, rough	Grey	86.0$_{abc}$	Fusiform, medium	19.23$_{de}$	4.37$_{bcd}$	95.30$_{hij}$
Cc16	Circular colonies, rough	White	84.0$_{a-d}$	Fusiform, large	19.72$_{cde}$	4.16$_{cde}$	109.53$_{b-f}$
Cc17	Zigzag cottony colonies, smooth	White	79.0$_{cde}$	Fusiform, large	20.43$_{cd}$	4.37$_{bcd}$	102.50$_{fgh}$
Mean			83.75		21.60	4.26	103.96

*Values are the mean of three replications. In a column, means followed by common letters are not significantly different at the 5% level by DMRT.

Pathogenicity and virulence test

Wide variation in pathogenicity was observed upon inoculation of *C. capsici* on the leaves of turmeric. All the isolates were pathogenic and produced leaf spot symptoms. Virulence test revealed that all the isolates were capable of producing symptoms on turmeric leaves at five days after inoculation. The maximum diameter of 15 mm lesion was recorded with Cc2 isolate followed by 14 mm with Cc7 isolate. The virulence of Cc5, Cc11, Cc12 and Cg2 were statistically on par with each other. The least lesion diameter level was observed in Cc17. The pattern of virulence was recorded based on the disease progress and PDI recorded under glass house condition. The isolates Cc2 caused the highest disease severity (39.85%) whereas, the least disease severity was recorded in Cg2 (11.55%). Based on the severity, the Cc2 isolate from Erode was found to be highly virulent. The least disease severity was recorded with Cg2 isolate from Perumbalur. The isolates were grouped into three groups based on virulence pattern. Six isolates were highly virulent (Cc2, Cc4, Cc5, Cg2, Cc9 and Cc12), five isolates were moderately virulent (Cc1, Cc8, Cc10, Cc13 and Cc17) and all others were less virulent. The disease severity caused by high, moderate and less virulent isolates ranged ranging from 31.33 - 39.85 %, 24.25 - 30.60 % and 11.55 - 22.50 %, respectively. The disease severity caused by each of the isolate was statistically significant as compared to with control (Table 3).

Molecular detection of pathogen using genotypic characterization

PCR amplification of 5.8S-rDNA region of *Colletotrichum* spp revealed that the DNA amplicon size was of 590 base pair. All the twenty isolates used in this investigation showed specific amplification site of 590 base pair, which confirms the pathogen identity. The purified DNA samples were sequenced and Nucleotide BLAST analysis

was performed in the NCBI. The results revealed that the DNA sequences were 97-100% homologous *viz*.,with *C. capsici* data base in the GenBank. However, the BLAST analysis of three isolates matched 99-100% homology with *C. gloeosporioides* strains registered in the NCBI. Based on the nucleotide BLAST results of ITS-rDNA sequences, the pathogens responsible for turmeric leaf spot were confirmed as *C. capsici* and *C. gloeosporioides*. All the DNA sequences were submitted to NCBI GenBank database and assigned with accession numbers *viz*., Cc1 KC565714, Cc2-KC565717, Cg1 -KC565723, Cc3-KC565724, Cc4-KC565725, Cc5 KC565726, Cc6-KC565727, Cg2 -KC565728, Cc7-KC565729, Cg3-KC565730, Cc8 KC565731, Cc9-KC565732, Cc10-KC565733, Cc11-KC565734, Cc12-KC565735 and Cc13-KC565736.

Discussion

C. capsici causing leaf spot of turmeric is reported to be responsible for major economic loss of turmeric production in India. In the present study, the maximum disease severity was recorded in Erode district of Tamil Nadu. The severity of disease was least in Gulberga district of Karnataka and Karur district of Tamil Nadu. The cultivation of local varieties in a large area coupled with favourable environment renders disease outbreak and disease severity (Uma Devi, 2008). The coincidences of high rainfall, relative humidity and low night temperature with mono-cropping prevailed in Erode, might have been responsible for the occurrence of maximum severity. The leaf spot severity varied from place to place,

Table 3. Virulence diversity of *C. capsici* isolates causing leaf spot disease of turmeric

Isolate	Location	Virulence test	
		Lesion diameter*(mm) - *in vitro*	PDI* - glass house
Cc1	Coimbatore	8.00	30.50$_{cd}$
Cc2	Erode	15.0$_{a}$	39.85$_{a}$
Cg1	Salem	6.0$_{j}$	22.50$_{fg}$
Cc3	Dharmapuri	7.0$_{i}$	20.68$_{gh}$
Cc4	Karur	9.0$_{g}$	31.33$_{cc}$
Cc5	Namakkal	12.0$_{d}$	34.60$_{b}$
Cc6	Krishanagiri	8.0$_{h}$	21.80$_{g}$
Cg2	Perumbalur	10.0$_{f}$	35.60$_{b}$
Cc7	Villupuram	14.0$_{b}$	19.35$_{hi}$
Cg3	Trichy	6.0$_{j}$	13.50$_{j}$
Cc8	Nizamabad	7.0$_{i}$	28.40$_{de}$
Cc9	Guntur	5.0$_{k}$	31.50$_{c}$
Cc10	Warangal	9.0$_{g}$	24.25$_{f}$
Cc11	Kozhikode	11.0$_{e}$	17.58$_{i}$
Cc12	Palakkadu	13.0$_{c}$	35.80$_{b}$
Cc13	Wayanad	8.0$_{h}$	30.60$_{cd}$
Cc14	Belgaum	6.0$_{j}$	18.15$_{i}$
Cc15	Mysore	10.0$_{f}$	24.60$_{f}$
Cc16	Chamarajnagar	9.0$_{g}$	11.55$_{k}$
Cc17	Gulbarga	4.0$_{l}$	27.60$_{e}$
Control		0.0	0.0

*Values are the mean of three replications. In a column, means followed by common letters are not significantly different at the 5% level by DMRT.

because of varied agro-climatological situations, cropping pattern, cultivars and cultural practices. It may also be attributed to the existence of variability or pathogenic diversity (Chawda *et al*., 2012). The pathogenicity tests confirmed that the species *C. capsici* was mainly responsible for leaf spot disease of turmeric in India. The expression of disease symptoms was homogeneous among the isolates of *C. capsici*. However, the degree of disease severity, virulence and aggressiveness varied among the isolates, which were measured quantitatively under *in vitro* and glasshouse conditions.

The investigation on morphological characterization of *C. capsici* based on cultural characters, spore shape and size showed an overlap in colony colour, conidial shape and size. This result is in agreement with a Sandgee *et al*. (2011), who found a morphometric overlap of conidial size within the isolates of *Colletotrichum* species. Moreover, Cai *et al* . (2009) observed differences in colony colour of *Colletotrichum* spp. Morphological groups and pathological groups did not show any clear cut relationship among isolates of *C. capsici*. The combination of these two characteristics has been successfully used to categorize *Colletotrichum* spp (Thind and Jhooty, 1990). All the twenty isolates examined showed hyaline and short conidiophores bearing hyaline fusiform conidia. In the present study three groups of virulent pathotypes of *C. capsici* were found to infect turmeric. Sharma *et al*. (2005) reported 15 pathotypes of *C. capsici* among 37 isolates collected from different chilli growing regions of India. However, pathotype differences were mainly based on quantitative differences in host reaction, i.e., level of virulence and aggressiveness.

This study presents the first report of molecular detection of *C. capsici* affecting leaves of turmeric in southern states of India. For several years molecular techniques have been widely used to differentiate the *Colletotrichum* genus at species or race level (Bardas *et al*., 2007). The precise detection is difficult due to combination of leaf spot and leaf blotch disease in turmeric. In this study, twenty isolates of *Colletotrichum* spp were collected for molecular detection based on 5.8S rDNA- ITS regions were confirmed the identification of pathogen as *C. capsici.* These results are in agreement with Tapia-Tussell *et al*. (2008), who used amplification of ITS region and sequencing for detection of *C. capsici* and *C. gloeosporioides*, causing anthracnose in papaya. The ITS region is the most widely sequenced region, but still there are some concerns as to whether ITS sequence data can provide adequate resolution to determine and differentiate *Colletotrichum* species.

Pathogenic variability plays a major role in disease dynamics and consequently, in the success of disease management strategies including the development of cultivars resistant to diseases. The

results of the present study demonstrate that there exists certain levels of morphological, pathotypes and virulence diversity among isolates of *C. capsici*, causing leaf spot disease of turmeric. Pathogenicity tests revealed that these isolates expressed different levels of virulence. The molecular detection of pathogen using ITS-rDNA region could be a reliable technique for precise and rapid identification of the pathogen.

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