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Polyphasic approach for identification and characterization of *Colletotrichum capsici* (Syd.) Butler and Bisby causing Anthracnose disease of Chilli in India

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Chilli, one of important vegetable suffers from huge loss due to the devastating disease caused by *Colletotrichum capsici.* For proper management of the disease, accurate identification of the pathogen is utmost important. In the following research various methods have been approached for the accurate diagnosis of the pathogen, which will help in the accurate disease management

Key words: Chilli, Anthracnose, *Colletotrichum*, characterization, PCR-RFLP

INTRODUCTION

Chilli peppers, belonging to family Solanaceae, are one of the very popular spices known for their medicinal and health benefiting properties. Chillies are excellent source of Vitamin, A, B, C and E with minerals like molybdenum, manganese, folate, potassium, thiamin, and copper. The main component in chillies is a chemical called Capsaicin, which is responsible for the intense heat sensation. Capsaicin lowers blood sugar levels, improves heart health, boost circulation, heal intestinal problems, and protects against strokes. Eating chillies can have a very positive impact on people who are overweight or suffer from diabetes because they reduce the insulin levels. Known as circulation boosters, chillies can have a major impact on human health by boosting circulation and also act as a blood thinner to help protect against strokes. Symptoms of chilli anthracnose disease normally appears on leaves, stem and fruits of which, disease on fruits are economically important. Under favourable conditions the escalation of crop loss was found upto 80% (Prasanna *et al.* 2009). Anthracnose of chilli is caused by more than one species of *Colletotrichum* and of these the most predominant species are *C. capsici* and *C. gloeosporioides* (Sheu and Wang, 2007). Accurate identification of species has been always critical for development of resistant varieties and management of the disease. Though several studies for identification of *C. capsici* with various approaches: cultural, morphological (Sharma *et al.* 2005) and pathogenic characterizations (Than *et al.* 2008) have been made, these are inadequate for an exact and unequivocal identification of species. This is because of existence of few distinct and variable morphological characters, wide host range and variations in pathogenicity of the pathogen (Thaung, 2008). Phylogenetic analysis using nucleic acid sequences has been successfully used to differentiate species in other fungal pathogens viz. *Fusarium* (Shenoy *et al.* 2007) and *Botryo sphaeriaceae* (Slippers *et al.* 2004 a,b). However, for identification of cultures at species level and studying the diversity/cultural variation of fungi an integrated or polyphasic approach consisting of basic morphological characterisation and molecular techniques is mandatory. The present study has been envisaged to investigate the diversity of *Colletotrichum* isolates collected from different regions of the country using morphological,

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cultural and pathogenic characters supported by molecular approaches

MATERIALS AND METHODS

Collection and maintenance of isolates

Isolates of *Colletotrichum* spp. from infected tissues of red ripe chilli were collected from different places of Uttar Pradesh (Table 1) India. The isolation was done by cutting 2 x 2 mm section from leading edges of the lesion developed on the fruits. The sections were surface sterilized with 70% ethanol for 15 seconds followed by 0.1% sodium hypochlorite (NaOCl) for 60 seconds and washed three times in sterile distilled water. After drying on blotting sheets the sections were plated on potato dextrose agar (PDA) and incubated for 3-5 days at 28°C. The cultures were purified by single spore method (Ho. and Ko, 1997) and the pure cultures were maintained on PDA slants as well as on filter paper at 4°C for further studies.

Pathogenicity of C. capsici isolates

All the *C. capsici* isolates (identified among all collected isolates with species specific primers) were inoculated on chilli fruits and standard Koch's postulates test was performed to ascertain the pathogenicity of the isolates.

Morphological and cultural characterization

Cultures of all eight isolates were transferred from stock and grown on PDA for revival. A circular bit of 5 mm mycelial disc was cut out using sterilized cork borer and transferred to Petri plate containing PDA. The cultures were allowed to grow at 28oC and radial growth was observed at 6, 9 and 12 days after inoculation (DAI). The cultures were examined for colony colour, colony margin, colony texture and development of pigments on agar medium. The size of conidia was measured by using image analyser (Leica Q550 servers, Image and Peripheral Server Software, version 3.1 from Leica (Microsystems, Imaging Solutions Ltd. Cambridge, UK).

Analysis of cultural variation of selected C. capsici isolates on different controlled environment

Two isolates of *C. capsici* (Ccf and Ccc2 , collected from Varanasi, Uttar Pradesh and Raichur, Andhra Pradesh respectively) representing two different agroclimatic regions of the country were checked for their cultural variations (radial growth) on different media, temperature and pH.

Effect of different media on growth of C. capsici isolates Ccf and Ccc2

Different media *viz*., PDA, Richard's synthetic agar (RSA), Oat Meal Agar (OMA), Potato Dextrose Rose Bengal Agar (PDRBA), Czapeck's Dox agar (CDA), Pea-nut hull extract (PHA), Corn meal agar (CMA), Rose Bengal Agar (RBA) and V-8 juice were prepared. Both the cultures were revived from stock slants and the culture discs of 5 mm size were transferred on to different media and incubated at 28°C. Each treatment was replicated four times and observations on radial growth and mycelia pattern were recorded at 9 days after inoculation (DAI).

Effect of different temperature and pH on growth of C. capsici isolates Ccf and Ccc2

Cultures were inoculated on PDA plates as described earlier and incubated in BOD incubator under different temperature regimes (4°C, 15°C, 25°C, 30°C, 35°C and 40°C). Each treatment was replicated four times and observations were recorded at 9 DAI. Similarly PDA medium with different pH ranges (4.0, 6.0, 7.0, .0 and 9.0) were prepared and cultures were inoculated on them and observations were recorded at 9 DAI.

Estimation of extracellular enzyme
Qualitative analysis
Pectinase

Cultures were grown on specific medium comprising of 500 ml of mineral salt solution, (1 g of yeast extract, 15 g of agar, 5 g of apple pectin and 500 ml of distilled water) which was dispensed in Petri plates. After 5 days of growth at 28°C the plates were flooded with 1% aqueous solution of Hexadecyl-trimethyl-ammonium-bromide which precipitated the pectin in the medium and showed clear zones around a colony indicating degradation of pectin.

Cellulase

To detect cellulolytic activity. specific medium containing 0.2 g sodium nitrate ($NaNO_3$), 0.1 g

Potassium dihydrogen phosphate (KH_2PO_4); 0.05 g potassium chloride (KCl); 0.02 g magnesium sulphate ($MgSO_4$); 0.01 g Calcium chloride ($CaCl_2$) and 0.05 g Yeast extract in 100 ml distilled water was poured in Petri plate,inoculated with the cultures and incubated for 4 days at 28°C. The surface was then flooded with 1% aqueous Hexadecyl-trimethyl-ammonium-bromide. Undegraded cellulose was precipitated with this reagent making a clear zone where cellulose had been degraded.

Protease

Casein soluble medium containing 40 g dextrose; 10 g peptone; 15 g agar; 3 g yeast extract; 8 g casein; 1000 ml distilled H_2O; pH 7.0 was used. Inoculated plates containing the medium were incubated at 28°C for 5-7 days. Formation of clear zones around the colonies showed protease activity. These zone formationswere enhanced by flooding the plates with 10% glacial acetic acid.

Lipase

To detect lipolytic enzyme activity the medium containing10 g difco peptone; 5 g sodium chloride (NaCl) ; 0.1 g calcium chloride ($CaCl_2$, $2H_2O$); 20 g agar; 1000 ml distilled H_2O at pH 6.0 was used. Olive oil was used as the lipid substrate. Plates were incubated at 28°C for 4 days. Clear zones which formed around the colonies indicated degradation of substrate due to lipolytic activity.

Quantitative analysis
Cellulase

1 ml suitably diluted enzyme filtrate, 1 ml of citrate acetate buffer 0.5 M (pH 5) and 2.5 ml of 1% carboxymethylcellulose (CMC) as a substrate was incubated for 1 hr. at 37°C. Presence of glucose was determined by DNS (Dinitrosalicylic acid) method. One unit of cellulose was defined as 1.0 mg of glucose released from1% CMC hr^{-1} at 37°C and pH 5. The reaction mixture was cooled and optical density was measured at 540 nm against blank.

Protease

Samples were grown in 250 ml flasks containing 100 ml dextrose and yeast extract medium containing 40 g dextrose; 10 g peptone; 15 g agar; 3 g yeast extract; 1000 ml distilled H_20 adjusted at pH 7.0 on a rotary shaker at 110 rpm at 28°C for 4 days. Ten ml samples were taken after 24, 48, 72 and 96 hrs. by filtration andthe filtrate was used to determine the proteolytic activity. 1 ml of 1% casein solution was taken with one ml of enzyme extract and then incubated for one hr. at 45°C. After incubation 3 ml of 5% Trichloroaceticacid (TCA) was added and kept in ice for 15 minutes. These samples were centrifuged at 5000 rpm for 30 min., at 4°C. Blank was prepared in the same way and 3 ml of TCA was added before incubation. O.D. was measured under UV 240 at 280 nm.

Pectinase

The reaction mixture (1ml) containing equal amount of substrate (1%) prepared in citrate buffer (0.05M) pH 4.4 and suitable diluted enzyme was incubated at 50°C for 30 min. in water bath. After incubation 3ml DNS solution was added to stop the reaction and tubes kept in boiling water for 10 min. The developed colourwas read at 575 nm.

Lipase

Cultures were grown in 250 ml flasks containing 100 ml of autoclaved medium containing 3.0 g $(NH_4)2SO_4$; 0.7 g $MgSO_4$; 0.5 g NaCl; 0.4 g $Ca(NO_3)_2$; 1.0 g KH_2PO_4; 0.1 g K_2HPO_4; 5.0 g glucose; 1.0 g yeast extract , at pH 5 and kept on a rotary shaker at 30°C and 110 rpm for 4 days.1 ml of olive oil was added per 100 ml of sterile medium. Ten ml culture medium was taken, centrifuged at 5000 rpm for 20 min, and the supernatant was used for determining lipase activity. For this 1.3 ml of olive oil, 1 ml of phosphate buffer 0.066 M at pH 7.0. 3ml of supernatant and 1.5 ml of distilled H_2O were shaken for 3mins., thenplaced in an incubator shaker at 30°C and 150 rpm for 5 hrs. After incubation 15ml of ethanol was added to the reaction mixture, followed by 12.5 ml of diethyl ether (commercial ether) to destroy the emulsion. The mixture obtained was titrated against 0.1N NaOH solution using Thymolphthalein as an indicator. The control samples were treated in the same manner but without incubation. The results of the titration of the control samples were subtracted from the results of the test samples. Lipase activity was expressed in ml of 0.1N NaOH used in the titration of the fatty acid formed where 1 ml of alkali corresponds to 500 arbitrary units of lipase activity.

Total protein profiling by Polyacrylamide Gel Electrophoresis (PAGE)
Total protein isolation

Cultures were inoculated in Potato Dextrose broth (PDB) After 4 days of inoculation, the mycelial mat of six (6) isolates (two isolates were not taken as they were morphologically very similar and geographically also not very distinct.) was extracted and dried in filter paper. Dried mycelial mats were ground in mortar andpestle with the help of liq. Nitrogen The samples were mixed in 10% of TCA equal vol. and left for 10 min. at room temperature. Then it was centrifuged at 12000g for 10 min. The supernatant was discarded and the pellet obtained was mixed in 2.5 M NaOH.

PAGE

The casting stand, two glass sheets along with spacers and the screws were placed and adjusted glass sheets were sealed. Resolving gel (10%; for 60 ml of gel, H_2O (27.6 ml), 30% bis-acrylamide mix <1>(16.2 ml), 1.5 M Tris (pH-8.8) (15 ml), 10% ammonium persulfate <1> (0.6), 0.6 ml TEMED) was prepared and poured slowly in the space between the two glass sheets avoiding bubbles. After solidification of resolving gel, stacking gel (5%; For 30 ml of gel H_2O (20.4 ml), 30% bis-acrylamide mix <1> (5.1 ml) 1.5 M Tris (pH-8.8) (3.6 ml) 10% ammonium persulfate <1> (300µl)., Tetramethylethylenediamine (TEMED).) was prepared and poured carefully avoiding bubbles and the comb was placed carefully with the help of clip. The shape of the comb was marked from back side to demarcate the position of wells. After solidification combs were removed carefully. The glass sheet containing gel was placed in the tank andthe screws were equally adjusted accordingly. The tank was filled with Tris-Glycine electrophoresis buffer and the sample was loaded carefully (100-150 µl, in each well) and subsequently run at 60-90V for 5-6 hrs.

Staining of gels

Gel was stained with Coomassie Brilliant Blue -250 for 2-4 hrs (prepared in methanol and acetic acid solution with glacial acetic acid) and then destained in methanol and acetic acid solution. After that gel was visualized in image analyser under white light.

Aggressiveness of C. capsici isolates (Ccf and Ccc2) on chilli

Reaction of *C. capsici* isolates (Ccf and Ccc2) on chilli varieties/germplasm/wild lines was observed under artificial inoculated conditions. The cultures were grown on PDA medium and after 9 days, the entire mycelial mat was gently scrapped from the agar surface in sterile distilled water followed by filtration of mycelia and conidial suspension through four layers of cheese cloth. The conidial concentration in filtrate was counted using a haemocytometer and adjusted to $2x10^5$ conidia per ml. of water. Mature ripe fruits of test varieties were collected and surface sterilized with 0.1% Mercuric chloride ($HgCl_2$) solution for one minute and rinsed three times in sterilized distilled water. After wiping the fruits to remove excess moisture, the fruits were kept in an indigenously designed small moist chamber prepared from sterilized plastic tray, cotton, filter paper and polythene sheet to maintain high relative humidity (> 98%). Each fruit was inoculated with 5 *µ*l inoculum by pin-prick method using a sterilized needle except control. Each treatment was replicated thrice and the trays were incubated at 25±2°C temperature. Lesion development on chilli fruits were recorded at 9 DAI. The disease resistance reaction was categorized on the basis of lesion size.

Assessment of genetic diversity and Polymerase Chain Reaction (PCR) based identification of C. capsici isolates
Extraction of DNA

The total genomic DNA of *Colletotrichum* spp. was isolated from mycelia. Mycelia were incubated at 28°C for 4 days in tubes containing 20 ml of potato dextrose broth and agitated at 180 rpm. Mycelia were harvested by filtration, dried and stored at -80°C for further use. Dried mycelium was ground to fine powder with sterilized pestle and mortar using liquid nitrogen and the contents were transferred to 1.5 ml eppendorf tube. Added 600 µl cetyl trimethyl ammonium bromide (CTAB) to the tube and incubated at 65°C for 30 min and tubes were vortexed after every 10 min. After cooling at room temperature, equal volume (600 µl) of chloroform: isoamyl alcohol (CIA) (24:1. v/v) was added in fume hood cabinet , gently mixed for 20-30 min and centrifuged at 7000 rpm for 5 min at 4°C. The aqueous phase was transferred to new tubes and repeated the CIA extraction. After the second CIA wash, the DNA was precipitated by adding 300 µl isopropanol in tubes and gently mixed and incubated at room temperature for 30 min. Subsequently samples were centrifuged at 12000 rpm

for 10 min and supernatant was decanted. The DNA pellet was dissolved in 50 µl of double distilled H_2O.

Identification of C. capsici isolates

PCR Species specific primer CcINT (5'-TCTCCCCGTCCGCGGGTGG-3') for *C. capsici* was used in conjunction with the conserved primer ITS4. A reaction mixture (25 µl) consisting of 2.5 µl of 10X PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 0.5 µl dNTPs mix (10 mM each of dATP, dCTP, dGTP, dTTP), 0.4 µl Taq DNA polymerase, 2.0 µl of primer 10 pmol/µl, 1.0 µ of genomic DNA (30 ng/µl) and 16.1 µl of sterilized double-distilled water was prepared. Amplification was carried out in a thermal cycler (Thermal cycler, Biorad) by using three temperature profiles programmed for initial DNA denaturation at 94°C for 3 min, followed by 40 cycles consisting of DNA denaturation for 1min at 94°C, primer annealing at 58°C for 45 sec and extension for 2 min at 72°C and additionally at the end, a final extension period of 10 min at 72°C was also given.

Electrophoresis

Amplified products were separated on 1.5% agarose gel in 1X TAE subjected to 70V for 2.5 hr. Gels were stained with ethidium bromide for 30 min. The gels were visualized with UV light and photographed.

Molecular characterization/ Genetic variation i) *Random Amplified Polymorphic DNA (RAPD)*

Genomic DNA of all the isolates were isolated as described above and subjected to RAPD-PCR. Random primers used are listed below.

Description of random primers used in analysing *C. capsici* isolates

Primers	5' to 3' sequence
OPS-06	GATACCTCGG
OPS-12	CTGGGTGAGT
OPU-01	ACGGACGTCA
OPQ-09	GGCTAACCGA
OPQ-15	GGGTAACGTG
OPT-04	CACAGAGGGA
OPT-05	GGGTTTGGCA
OPT-17	CCAACGTCGT
OPY-18	GTGGAGTCAG
OPR-02	CACAGCTGCC
OPX-08	CAGGGGTGGA
OPX-19	TGGCAAGGCA

PCR

A reaction mixture (25 µl) consisting of 2.5 µl of 10X PCR buffer, 2.5 µl of 25 mM $MgCl_2$, 0.7 µl dNTPs mix (10 mM each of dATP, dCTP, dGTP, dTTP), 0.3 µl Taq DNA polymerase (5U/µl), 2.0 µl of primer (10 pmol/µl), 1.0 µl of genomic DNA (30 ng/µl) was prepared and the volume was made upto 25 µl by adding sterilized double-distilled water. Amplification was carried out in a thermal cycler by using three temperature profiles programmed for initial DNA denaturation at 94°C for 3 min, followed by 35 cycles consisting of DNA denaturation for 30 sec at 94°C, primer annealing at 35°C for 30 sec and extension for 1 min at 72°C and additional 10 min at 72°C was also given at the end. Amplified products were separated on 1.2% agarose gel in 1X TAE and the gels were stained with ethidium bromide for 30 min, visualized with UV light and photographed.

RAPD analysis

Cluster analysis of RAPD products was done using the STAND module of NTSYS-pc software was used. Pair-wise distance matrix was used as an input for analysis of clusters. UPGMA (Unweighted Pair Group Method with Arithmetic Mean)-based clustering was done using SAHN module of NTSYS-pc. Each isolate was scored manually for presence or absence of a particular amplification product. Bands with negligible intensity were not taken into consideration. These data were analyzed using software NTSYS-pc version 2.02. Pair-wise combinations of isolates were employed to calculate Jaccord's similarity coefficient (GS): a/(n _ d), where a is the number of positive matches, n is the total sample size, and d is the number of negative matches. Genetic distance (GD) between pair of isolate was estimated as GD = 1 _ GS. The unweighted pair-group method with arithmetic averages (UPGMA)-based dendrogram was constructed using the SAHN module of the software. The MXCOMP module of NTSYS-pc was used to compare the Jaccard's similarity values estimated from RAPD patterns and the average taxonomic distance values were derived for the trait genetic distinctness.

ii) *ITS (Internal transcribed spacer) AND α-TUBULIN amplification*

Nucleotide sequences of the rRNA gene-ITS re-

gion and a variable region of the tub2 gene was determined for a set of 8 isolates selected. Complete ITS/5·8S rDNA and partial α-tubulin (*tub*2) sequences were amplified using fungal-specific primers ITS1 and ITS4 and Bt 2A and Bt 2B, respectively. PCR was carried out in a thermal cycler as follows: 95°C for 5 min; 40 cycles of denaturing at 95°C for 30 sec., annealing at 52°C for 30 sec and elongation at 72°C for 30 sec; and a final extension step of 72°C for 10 min. PCR products were verified by staining with ethidium bromide on 1% agarose electrophoresis gels.

iii) PCR amplification and restriction enzyme digestion of rDNA ITS regions

All the isolates were used for PCR restriction fragment analysis. Primers ITS1 and ITS4, located in the conserved 18S and 28S rDNA genes and flanking the entire ITS1-5·8S ITS2 region, were used for PCR amplification. Using the two universal primers, ITS1 and ITS4, PCR products of about 500–600 bp were obtained from amplification of the ITS regions of the *Colletotrichum* isolates, using total DNA preparations as templates. PCR fragments were separated by electrophoresis in 1% agarose gels in TAE (0·04 mTris–acetate, 0·001 mEDTA) pH 8·0 buffer. Eight restriction enzymes *Alu*I, *Hae*III, *Msp*I, *Rsa*I, *Xba1,Xha1, EcoR1* and *Pst1* (Genetix biotech. Asia Pvt. Ltd.) were used to study polymorphisms in the ITS regions of the isolates. Enzyme treatments were made by taking a 5 µl aliquot of the PCR reaction and incubating it with 0.06 µl of the respective enzyme, buffer 1µl, 0.1µl BSA and H_2O for overnight at 37°C using the specification by the manufacturer. The restriction fragments generated were analysed by electrophoresis in 2% agarose gels in TAE buffer.

Statistical analysis

Data were analyzed using IRRISTAT programme developed by International Rice Research Institute (IRRI), Philippines.

RESULTS AND DISCUSSION

Isolation and pathogenicity of the isolates

Fifteen different isolates (VC-1, VC-2, VC-3, VC-4, VC-5, S-1, S-2, S-3, S-4, S-5, S-6, S-7, S-8, Ccc-2, and Ccf) were isolated from the infected chillies and purified through single spore isolation (Table 1). Isolates produced typical cultural and morphological characters of *c. capsici* in Petri plates and spore structures were verified by microscopy. All the isolates were preserved on PDA slants and on filter paper for further experiment. Among 15 isolates tested for PCR amplification of *C. capsici* species specific primers, 8 isolates (Ccf, Ccc-2, VC-1, VC-4, VC-5, S-5, S-6 and S-7) showed expected amplification of 450 bp.Which was in conjunction that *C. capsici* is the predominant species causing anthracnose of chilli in India. It indicated that these eight isolates were belonging to *C. capsici* and hence only these selected 8 isolates were used for further characterization studies.

Cultural and morphological characterization of C. capsici isolates

Among different isolates tested for their maximum growth on different days of incubation, isolates Ccc-2 and Ccf exhibited a maximum radial growth at 9 DAI whereas VC-4, S-5, VC-5, S7, S6 and VC-1 attained the maximum growth after 12 days of inoculation (Table 2). Hence, the *C. capsici* isolates were classified into two groups i.e. fast growing (Ccc-2 and Ccf) and slow growing (VC-4, S-5, VC-5, S7, S6 and VC-1) groups.

Classification of *C. capsici* isolates based on morphological characters *viz*., culture appearance, margin, pigmentation, and fruiting body indicated that there was no critical difference among the characters but a significant difference was observed in the size of the conidia of isolates (Table 3). Based on conidial size, cultures were divided into four groups *viz*., group I (S-5, S-7 and Ccf), group II (Ccc-2), group III (S-6 and VC-1) and group IV (VC-4 and VC-5) (Fig.1). Cultural and morphological grouping of isolates showed the completely dissimilarity among these two grouping because based on cultural characteristics the selected eight isolates were grouped into two groups (fast growing and slow growing), whereas based on conidial morphology they were divided into four (4) groups. The results showed that identification based solely on morphological characteristics can lead to misidentification, since morphological characters are easily influenced by environmental factors such as humidity, temperature and rainfall.

Growth pattern of C. capsici isolates (Ccf and Ccc-2) under different media, pH, and temperature

Radial growth of both Ccf and Ccc-2 isolates on

Table 1 : Characterization of ORF11 suppress

Name of place	
Varanasi	VC-1
(25.2800° N, 82.9600° E)	
	VC-2
	VC-3
	VC-4
	VC-5
Azamgarh	S-1
(26.0600° N, 83.1900° E)	
	S-2
	S-3
Deoria	S-4
(26.5100° N, 83.7800° E)	
Shanshahpur	S-5
(25.3176582° N, 82.9739014° E)	
	S-6
	S-7
Ballia	S-8
(28.2000° N, 79.3667° E)	
IIVR farm	Ccf
(25.10 ^{0}N 82.52 ^{0}E)	
Raichur	Ccc-2
(16.2000° N, 77.3700° E)	

different media indicated that PDA was superior to PHA, RBC, PDRBA, RSA, CDA, oatmeal agar, corn meal agar and V-8 (Table 4) (Fig.2A). Hence further experiments with different pH and temperatures were conducted using PDA as a base medium. Both the isolates were able to grow significantly at pH 8 and pH 9 (Table 5) (Fig. 2B) and attained maximum growth at 30°C (Table 6). The two isolates (Ccf and Ccc-2) were able to survive high pH (9.0) and temperature (40°C) indicating that the pathogen might have evolved to adjust extreme environmental conditions as earlier reports indicated that the pathogen culture was grown best in pH 6.5 and temperature 30°C

Estimation of extracellular enzyme
Qualitative assay

In case of cellulose and pectinase the maximum halo region was observed in Ccc-2, followed by S6. In lipase since the media in plate was not in its solid (firm) state, the region developed could not be measured but the isolates S6, S7 and Ccc-2 showed the maximum halo region. Significant difference was not visible among isolates in protease assay (Table 7a), (Fig. 3).

Quantitative assay

In quantitative assay for cellulase, pectinase and Protease maximum absorbance was recorded in Ccc-2 followed by S6, but in lipase estimation, Ccc-2 recorded maximum absorbance followed by Ccf. (Table 7b),(Fig. 4).

PAGE

All the six isolates showed the similar band pattern, with band of higher molecular weight in one of the isolate (Ccc-2).(Fig. 5).

Aggressiveness of *C. capsici* isolates (Ccf and Ccc-2) on different chilli genotype

Ccf and Ccc-2 were used to check their pathogenic variation on the basis of their genetic distinctness and the results indicated difference in the aggressiveness of both the pathogen when checked on chilli germplasm lines (Table 8). Both the isolates proved to be virulent but the qualitative difference in lesion size clearly indicated that isolate Ccf was more aggressive than Ccc2 as the lesion length ranged 11.4-37.90 mm in case of the former and 8.23-17.20 mm in case of the latter respectively.

For studying the aggressiveness of the culture and identification of pathotype, quantitative difference in the infection level was considered as per Taylor (2007). Quantitative difference in infection reflects the natural distribution of aggressiveness within a population, ranging from low to high whereas a pathotype is defined as a group of isolates distinguished from others of the same species by a qualitative difference in disease severity. On the basis of qualitative difference Ccf was observed

Table 2 : Cultural characterization of *Colletotrichum capsici* isolates based on radial growth

Name of the isolate	Observations on radial growth at different day after inoculation (DAI)		
	6 DAI	9 DAI	12 DAI
VC-4	69.5^{efg}	71.3^{def}	78.5^{abc}
S5	71.7^{def}	77.7^{bc}	81.1^{ab}
VC-5	64.1^{hi}	75.2^{cd}	78.6^{abc}
S7	67.7^{fgh}	72.2^{de}	78.0^{abc}
S6	60.3^{i}	68.1^{efgh}	77.8^{bc}
Ccc-2	67.2^{efgh}	81.8^{ab}	82.3^{a}
Ccf	66.5^{gh}	80.6^{ab}	82.1^{ab}
VC-1	69.2^{efg}	74.5^{cd}	82.3^{a}
CD (5%)			
Isolate 2.5			
Days 1.5			
Isolate x days 4.4			

In a column a mean followed by common letters are not significantly different at 5% level by DMRT

Table 3 : Morphological characteristics of different isolates of *C.capsici* on PDA

Group	Isolate	Conidia (μm)	Culture appearance	Margin	Pigmentation	Fruiting body
I	S-5	22.50^{a}	Fluffy	Smooth	Colourless	Abundant acervuli
	S-7	21.91^{a}	Adherent	Smooth	Colourless	Sparse acervuli
	Ccf	22.53^{a}	Adherent	Smooth	Colourless	Abundant acervuli
II	Ccc-2	20.40^{ab}	Adherent	Smooth	Colourless	Sparse acervuli
III	S-6	18.66^{bc}	Adherent	Smooth	Colourless	Abundant acervuli
	VC-1	18.09^{bc}	Adherent	Smooth	Colourless	Abundant acervuli
IV	VC-4	18.00^{c}	Adherent	Smooth	Colourless	Abundant acervuli
	VC-5	17.33^{c}	Adherent	Smooth	Colourless	Sparse acervuli
	CD (5%)	2.32				

In a column a mean followed by common letters are not significantly different at 5% level by DMRT

Table 4 : Effect of different media on radial growth of *C. capsici* isolates Cef and Cec-2

Media	Radial growth (mm)	
	Ccf	Ccc2
PDA	65.8 [a]	65.0 [ab]
PHA	59.0 [fg]	59.4 [efg]
RBC	51.7 [j]	54.8 [hi]
PDRBA	62.2 [cd]	59.7 [defg]
RSA	59.1 [efg]	63.1 [abc]
CDA	62.8 [bc]	61.6 [cdef]
Oat meal agar	63.8 [abc]	61.8 [cde]
Corn meal agar	54.0 [ij]	57.5 [gh]
V-8 agar	63.1 [abc]	59.7 [defg]
CD 5%		
Media	1.9	
Isolate	1.2	
Media x isolate	2.8	

In a column a mean followed by common letters are not significantly different at 5% level by DMRT

Table 5 : Effect of different media on radial growth of *C. capsici* isolates Cef and Cec-2

pH of the medium	Radial growth(mm)	
	Ccf	Ccc-2
9	75.5 [ab]	78.1 [a]
8	72.9 [ab]	77.3 [ab]
7	56.5 [d]	64.0 [c]
6	61.5 [cd]	64.8 [c]
5	48.9 [d]	71.8 [b]
4	46.3 [d]	56.9 [d]
CD 5%		
pH	4.08	
Isolate	2.3	
pH x isolate	5.7	

In a column a mean followed by common letters are not significantly different at 5% level by DMRT

Table 6 : Growth of C.capsici isolates Cef and Ccc-2 on PDA incubated at different

Temperature	Radial growth (mm)	
	Ccf	Ccc2
15	35.1 [d]	29.6 [e]
20	26.4 [f]	31.1 [e]
25	49.8 [c]	61.5 [b]
30	67.8 [a]	65.9 [a]
35	65.3 [a]	62.0 [ab]
40	65.5 [a]	59.3 [b]
CD 5%		
Temperature	2.2	
Isolate	1.7	
Temperature x isolate	3.1	

In a column a mean followed by common letters are not significantly different at 5% level by DMRT

Table 7a : Qualitative assay of Extra cellular enzyme

Isolate	Cellulase	Pectinase	Lipase
VC-1	6.7	6.2	++
VC-5	6.4	6.9	++
S5	4.7	7.1	+
S6	6.9	7.3	+++
S7	6.3	6.1	+++
VC-4	5.3	3	+
CcF	6.2	5.7	++
Ccc-2	7.4	7.5	+++
CONTROL	0.0	0.0	-

Table 7b : Qualitative assay of Extra cellular enzyme

Isolate	Cellulase	Pectinase	Lipase	Protease
VC-1	0.585	0.874	909.33	0.782
VC-5	0.578	1.006	2613.33	0.809
S5	0.313	1.260	4266.66	0.633
S6	0.706	1.671	4773.33	0.874
S7	0.510	0.771	3440.00	0.699
VC-4	0.415	1.232	3760.00	0.691
Ccf	0.272	1.424	5040.00	0.778
Ccc-2	0.800	1.707	5920.00	0.893
CONTROL	0	0.293	0.483	0.016

Table 8 : Aggressiveness of *C. capsici* isolates (Cef and Ccc-2) on chilli genotypes

Varieties	Ccf	Ccc-2
AC-Assam 10	18.20^{a}	10.23^{b}
PBC 535	27.66^{a}	13.60^{b}
P-1649	35.30^{a}	14.70^{b}
CCH-2	37.90^{a}	12.16^{b}
Pusa Jwala	24.93^{a}	17.20^{b}
KA-2	11.40^{a}	8.23^{b}
CD 5%		
Varieties		3.4
Isolates		1.9
Varieties x isolates		4.8

classify *Colletotrichum*, as DNA characters were not directly influenced by environmental factors. Phylogenetic analysis based on nucleic acid sequences have been successfully used to differentiate species in other difficult genera or groups of many fungal pathogens (Kvas *et al.* 2009; O'Donnell *et al.* 2009; Slippers *et al.* 2004a,b; Phillips *et al.* 2007).

Random amplified polymorphic DNA (RAPD)

RAPD profiling *C. capsici* isolates were given in Fig.6. According to RAPD based analysis using

Table 9 : TTS length and restriction fragments from ITS products generated

Isolate	ITS	Β- tubulin	Xba I	XhaI	Eco R1	Pst 1	Alu	Hae III	MspI	Rsa1
VC-1	600	680, 600, 480	580	600	600,300	600	600,380, 220	600	150,450, 600	600
VC-5	600	680,600, 480	580	600	300	600	600,380, 220	150,480, 600	150, 450,600	600
S5	600	680,600, 480,	580	600	300	600	600,380, 220	150,480, 600	150, 450,600	600
S6	600	680, 600, 480	580	600	300	600	600,380, 220	150,480, 600	150, 450,600	600
S7	600	680, 600, 480	580	600,480, 160	600,300	600	600,380, 220	150,480,500 600	150, 450,600	600
VC-4	600	680, 600, 480	580	600	600,300	600	600,380, 220	150,480, 600	150, 180,380, 450,600	600
Ccf	600	-	580	600,480, 160	600,300	600	600,380, 220	150,480,	150, 450,600	600
Ccc-2	600	-	580	600	300	600	600,380, 220	150,480, 600	150, 450,600	600

to be the most aggressive among both the isolates and further experiments were carried out with the same isolate

Molecular identification/ characterisation of C.capsici isolates using specific primers

Recent studies have shown that morphological characters should be used in conjunction with molecular data to establish species relationships within *Colletotrichum* (Crouch *et al.* 2009; Prihastuti *et al.* 2009). To overcome the inadequacies of these traditional schemes, molecular techniques have been used to characterize and identify taxa within *Colletotrichum* (Du *et al.* 2005; Shenoy *et al.* 2007). Nucleic acid analyses should provide the most reliable framework to similarity co-efficient, *C. capsici* isolates were divided into two major clusters *viz.*, cluster I (S-5, VC-1, Ccf, VC-1 and VC-1) and cluster II (Vc-4, Ccc-2, S-7 and S6) (Fig. 7). Hence based on RAPD, two isolates *viz.*, Ccf and Ccc-2 representing each major group were selected and used for further characterization studies. In the present study based on RAPD analysis all the *C. capsici* isolates were grouped in two major clusters which was not in congruence with the cultural and morphological grouping and this was in tandem with the results obtained by Sharma *et al.* (2005). Based on cluster analysis in present study, some isolates appeared to be identical but they could not be unequivocally identified as haplotype without molecular study based confirmations.

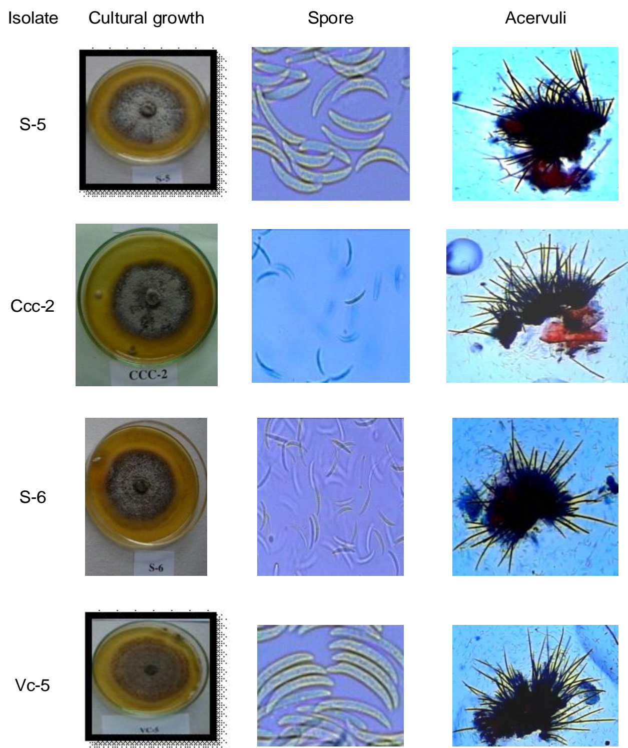

Fig 1: Morphological characters of different isolates of *C. capsici*

Fig. 2a and 2b : A. Effect of different media on radial growth *C. capsici* (Ccf). B. Effect of different pH on radial growth of *C. capsici* isolates Ccc2 and Ccf

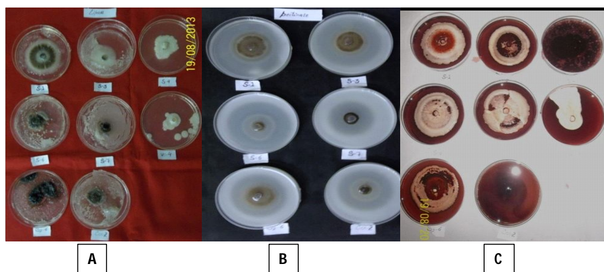

Fig. 3 : Qualitative assay of Extra cellular enzymes. A. Lipase, B. Protease, C. Cellulase

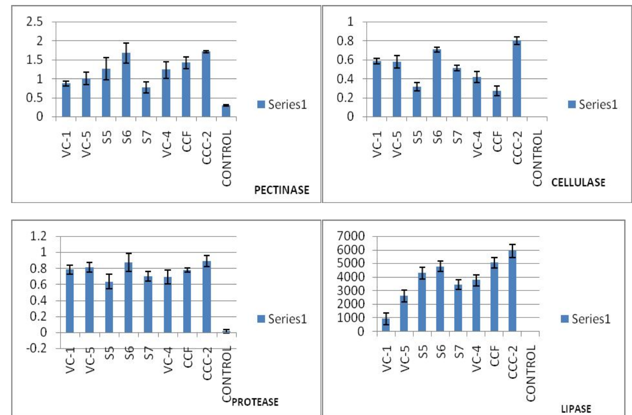

Fig. 4 : Quantitative assay for cellulase, pectinase , lipase and protease

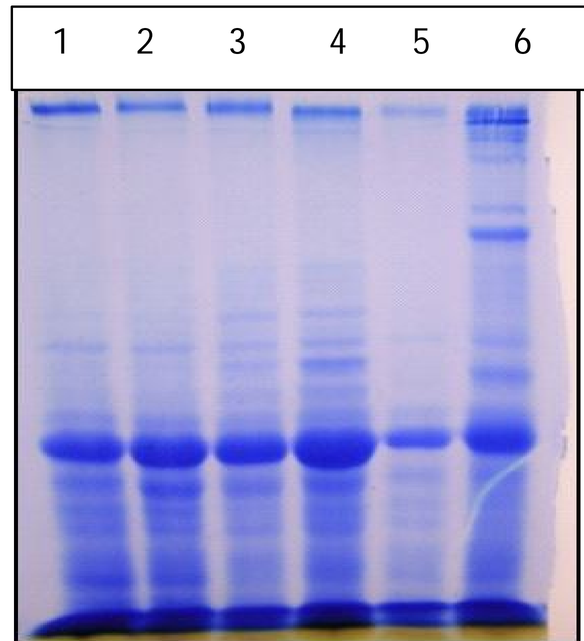

Fig. 5 : Protein profiling (Isolate number in all the fig.VC-1, VC-5, S5, S6, S7, VC-4, CCF, CCC-2

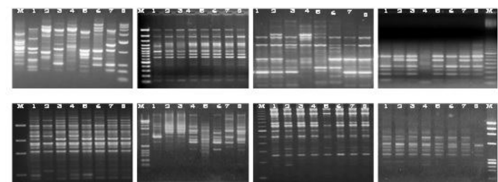

Fig.6 : RAPD profiling of *C. capsici*. Lane 1:VC-1, 2:VC-4, 3:VC-5, 4: S-5, 5:S-6, 6:S-7, 7:Ccf, 8:Ccc-2, M:100 bp molecular marker

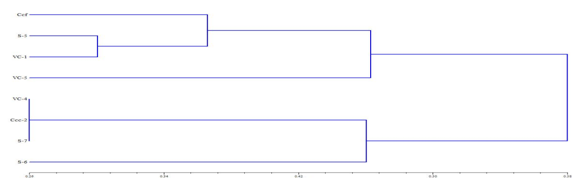

Fig.7 : Genetic relationship among different isolates of *C. capsici* using UPGMA cluster analysis of the distance matrix

Genetic groupings of *C. capsici* did not correlate with geographical distribution of the isolates as the isolates from different regions were grouped in same grouping, which could be due to large genetic variations within the population. Two isolates *viz.*, Ccf and Ccc2 representing two major clusters of RAPD were selected and further studied on different pH, temperature and hosts plants.

ITS and α-TUBULIN amplification

PCR products obtained from the ITS regions (including 5.8 S) ranged from 550 to 600 bp, whereas those from the â-tubulin gene ranged from 480 to 600 bp.(Fig. 8 A,B)

Overall comparison of the cultural, morphological, pathogenic and molecular grouping indicated that there was a correlation between RAPD and pathogenic groupings as Ccf and Ccc2 showed their belonging to two different groups, in RAPD and pathogenically on a given set of chilli lines. Once a species is accurately identified and characterised, it unlocks data that can be used for developing and implementing effective disease control strategies.

PCR amplification and Restriction fragment length polymorphism analysis

The digestion of the fragments of the ITS with *Xba I and Pst I* produced a monomorphic fragment of approximately 580-bp and 600bp respectively, for all *Colletotrichum* spp. (Fig. 8C, D). Isolates thereby posing a difficulty in differentiating the species.*Xha I* generated two distinct fragments one of 600 bp, for all of the *C.capsici* isolates and other of 480 and 160bp for two isolates(S-7 and Ccf (Fig. 8C). The restriction of the products digested with the enzyme *Eco R* I generated two fragments distinct in sizes of 300 bp for all the *C. capsici* and 600 bp for four isolates (VC-1, S-7, VC-4 and Ccf) (Fig. 8D).The restriction of the products digested with the *Alu* enzyme generated three fragments distinct in sizes of 220, 380 and 600 bp for all the *C. capsici* (Fig. 8E).The restriction of the products digested with the *Hae*III enzyme generated three fragments distinct in sizes of 150, 480 for seven isolates(except VC-1) and 600 bp for all the *C.capsici* and S7 having one more band of 500 bp (Fig. 8E). The restriction of the products amplified with the *MspI* enzyme generated four fragments distinct in sizes of 150, 450 and 600 bp for all the *C. capsici* except one isolate (VC-4) which produces two more bands of at 180 and other at 380 bp (Fig. 8F). The restriction of the products digested with the *Rsa*I enzyme monomorphic fragment of approximately 600 bp, for all *Colletotrichum* sp. isolates that made impossible to differentiate the species (Fig. 8F) (Table 9).

In this study, restriction enzymes *XhaI, Eco RI, Alu*, *Msp*I and *Hae*III showed polymorphism in the number and length of the resulting fragments, supporting the idea of the existence of intraspecific genetic diversity among different *C. capsici* isolates. The Alu, *Msp*I and *Hae*III restriction enzymes were capable of differentiating the three species of *Colletotrichum*, in comparison to the other enzymes tested; however the enzymes were not efficient in separating the *C. capsici* isolates from the geographic region. The ITS regions, including the 5·8S ribosomal RNA gene, were amplified using the universal primers ITS1 and ITS4. PCR fragments of approximately 600 bp were obtained and directly used to generate restriction fragment polymorphisms for differentiating the *Colletotrichum* species. With the exception of *XbaI, Pst* and *Rsa*I, all amplicons were digested with each of the enzymes (Table 9). Polymorphic restriction fragments were obtained with all rest of the enzymes tested, and the isolates were classified into separate rDNA RFLP (RG) groups based on the fragment patterns (Table 9). Most of the *Colletotrichum* isolates showed identical ITS RFLP patterns for all enzymes tested. Using sets of restriction enzymes in separate enzymatic digestions of PCR fragments, a preliminary identification of *C. Capsici* isolates was possible. The ITS RFLP study confirmed that none of the isolates found on chilli belonged to same species.

In this work, the ITS-RFLP technique was effective to demonstrate the intraspecific genetic characterization between the *C.capsici* isolates. The inaccuracies of identifying *C. capsici* solely by morphological criteria have been largely overcome by the use of molecular methods for differentiating *Colletotrichum* species. Ribosomal DNA sequences havebeen used extensively for species delineation within the genus *Colletotrichum* and analysis of the variable ITS regions – especially the ITS1 portion, provides sufficient information to infer phylogenetic relationships among *Colletotrichum* species. Morphologically, these isolates fit the description of *C. capsici* and this was subsequently confirmed by their reaction via biochemical characterization,

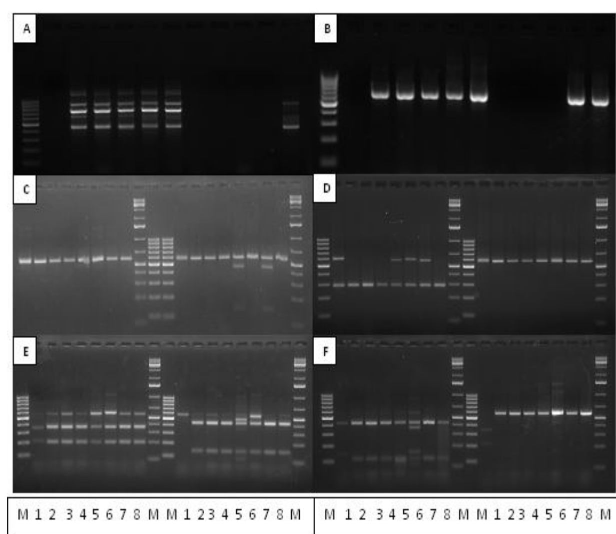

Fig. 8 : PCR amplification with **A.** ITS, B. â-TUBULIN; **C-F** ITS PCR-RFLP pattern (**C**-XbaI, XhaI, **D**-Eco R1, Pst I, **E**-Alu, Hae III,**F-**Msp1, Rsa1). Isolate VC-1, VC-5, S5, S6, S7, VC-4, Ccf, Ccc-2 are shown after ITS amplicons were digested

protien profiling and by ITS RFLP. These isolates of *Colletotrichum* have been observed to induce typical anthracnose symptoms on chilli, and hence it can be stated that isolates are virulent isolates of C. *capsici* causing anthracnose. It was also noted that isolates belonging to one cluster of dendrogram were capable of producing higher amount of extracellular enzymes in comparison to isolates of other clusters and by pathogenicity experiments that isolates producing these enzyme were virulent in nature.

Colletotrichum capsici is one of the most destructive fungal pathogens causing Anthracnose on variuos crops. The disease is more prevalent in subtropical and temperate countries.This adaption may be due to the existence of wide variability in pathogen. To understand the details of the gene functionality in pathogen under such conditions, in-depth study like characterization of internal transcriber space region, toxins and interaction of different varieties of chilli to the pathogen needs to be studied. Their survival in the off season on different alternate host is an important point of investigation. The present study is the cornerstone for studies which can be exploited to controll the disease in an effetive manner.

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