Indian Phytopath. 58 (4) : 448-453 (2005)

Morphological and molecular characterization of five *Colletotrichum* species from India

H.T. ROHANA WIJESEKARA, R. AGGARWAL and D.K. AGARWAL
Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi 110 012

ABSTRACT : Twenty *Colletotrichum* isolates comprising of five species *C. capsici. C. dematium, C. falcatum, C. gloeosporioides* and *C. lindemuthianum* from different geographical locations of India (New Delhi 4, Sikkim 2, Solan 3, Pantnagar 3, Daurala 2, Culcutta 1, Lucknow 1, Navasari 1, Pune 1, Dapoli 1 and Karnal 1) isolated from different crops (sugarcane, bell pepper, lal mirich, *Gomphrena*, *Melia*, bottle brush, *Passiflora foetida*, ashwagandha, egg plant, grape, cattleya, cashew nut, French bean, and soybean) were analyzed with RAPD markers. Morphological and cultural characters of these different species were correlated with the RAPD data. PCR amplification of total genomic DNA with 16 random primers generated unique banding patterns depending upon primer and the isolate. Sugarcane isolates 4800 and 4803 produced identical banding pattern while other isolates produced dissimilar bands within the particular species indicating the genetic diversity among the isolates within a species. Morphological characters were also different from each other even in sugarcane isolates 4800 and 4803, which shared identical bands. Cluster analysis also indicated a very high degree of genetic variability within a given *Colletotrichum* species. *C. capsici* and *C. dematium* isolates clustered together in the dendrogram showing their genetic relatedness. Morphological characters also supported this clustering. RAPD markers were better suited for differentiating isolates within a species rather than species.

Key words: *Colletotrichum*, molecular characterization of fungi, RAPD, PCR amplification

The genus *Colletotrichum* comprises many plant and animal pathogens. It causes anthracnose, dieback, leaf and fruit spots, damping off and root rots in many economically important crop species worldwide. Leaf spot and canker caused by *Colletotrichum gloeosporioides* on important forage legume *Stylosanthes* spp. is becoming a constraint in improving cattle production in tropical and sub tropical regions. In yam cultivation onset of epidemic before the onset of tuber formation can result in yield losses exceeding 85% (Abang *et al*., 2002). Red rot disease caused by *Colletotrichum falcatum* caused yield losses up to 50% in Indian sugarcane variety during 1939 – 40 (Chona and Hingorani, 1950). Identification and differentiation of *Colletotrichum* species based on morphological characteristics have often been inadequate, as some fungal isolates appeared similar to both *C. gloeosporioides* and *C. acutatum* (Brown *et al.,* 1996). The species of *Colletotrichum* are highly variable in morphology in culture (Bajaj *et al.*, 1964; Sutton, 1980; 1992; Waller *et al*., 1993; Canon and Kirk, 2000). The lack of distinguishable characters between *Colletotrichum* spp. has led to considerable uncertainty regarding the anthracnose fungi (Sreenivasaprasad *et al.,* 1992).

The use of molecular biological techniques has become popular in solving taxonomical problems. DNA polymorphism assay based on the amplification of random DNA fragments flanked by 10-mer of arbitrary nucleotide sequence (RAPD) has been used in the detection of variation among individuals (Crowhurst *et al*., 1991; Sherriff *et al.*, 1994; Thakur *et al*., 1998;). Therefore, an attempt was made to use RAPD markers to differentiate five species of *Colletotrichum* viz. *C. capsici, C. dematium, C. falcatum, C. gloeosporioides* and *C. lindemuthianum*.

MATERIALS AND METHODS

Isolates of *Colletotrichum*

Fourteen *Colletotrichum* isolates comprising of five species viz. *C. capsici. C. dematium, C. falcatum, C. gloeosporioides* and *C. lindemuthianum* were obtained from Indian Type Culture Collection (ITCC) (Table 1). Further six isolates of *Collectotrichum* comprising of species; *C. dematium, C. falcatum* and *C. gloeosporioides* were collected from Pantnagar, Karnal and Solan (Table 1). Field collected samples were wrapped in blotting papers and brought to the laboratory in Division of Plant Pathology and stored at 4^0C until isolation was performed. Isolations were performed from the advancing edge of the lesion after surface sterilizing the samples in 0.25% sodium hypochlorite solution for 2 minutes and rinsing twice with sterile distilled water. Sterilized samples were blotted dry on sterile blotting paper and plated under aseptic conditions on PDA plates. Plates were incubated in BOD incubator at 23±2 ^{0}C with 12-hr light and 12 hrs dark period. When the cultures were sporulating, single spore isolates were obtained by streaking spore suspension (10^6 spores /ml) on fresh PDA plates. These single spore colonies were used in further studies. Similarly isolates obtained from ITCC were also subjected to single spore colony isolations. Single spore colonies were maintained on PDA slants.

Total genomic DNA extraction

Five agar discs (0.5 cm diameter) with actively growing mycelium were inoculated into conical flasks with 100 ml of sterilized Modified Mathur's liquid medium (MMM). Flasks were incubated in a BOD incubator operating at 23±2 ^{0}C with 12 hour light and 12 hr dark period for 7 days. Mycelium was harvested by filtering through sterile Whatman No. 1 filter paper and dried with sterile blotting paper. Mycelium was transferred on to sterile aluminum foil and stored in a deep freezer until DNA was extracted. Mycelium was ground to a fine powder in liquid nitrogen with the help of sterile pestle and mortar. DNA extracted by CTAB method given by Sambrook *et al*. (1989). Total genomic DNA was dissolved in 100 ml of sterile double distilled water and purified by treating with RNase

Table 1. Origin, host plant and ITCC number of *Colletotrichum* isolates used.

Species	ITCC number	Host Plant	Location
C. capsici	4764	Lal mirich	New Delhi
	4871	Gomphrena	Sikkim
	5008	Melia leaf	New Delhi
	5107	Bottle brush	New Delhi
C. dematium	4790	*Passiflora foetida*	Kolkata
	5306	Ashwagandha leaf	Lucknow
	6088-05 (CdP)	Soybean	Pantnagar
C. falcatum	4800	Sugar cane	Daurala
	4803	Sugar cane	Daurala
	4893	Sugarcane	Navasari
	6085-05 (20)	Sugar cane	Pantnagar
	6086-05 (23)	Sugar cane	Pantnagar
	6087-05 (CfK)	Sugarcane	Karnal
C. gloeosporioides	4573	Egg plant	Solan
	5132	Grape leaves	Pune
	5213	Cattleya	Sikkim
	5255	Cashew nut	Dapoli
	6082-05 (BP1)	Bell pepper	Solan
	6083-05 (BP2)	Bell pepper	Solan
C. lindemuthianum	4765	French Bean	New Delhi

A solution. Purity and the quality of the DNA were assessed by measuring the UV absorbance at 260 and 280nm in a spectrophotometer (Model UVICON 931). The concentration of the DNA was adjusted to 100 ng/ ml with sterile double distilled water.

RAPD analysis

Twenty nine random primers from OPD and OPN (Operon Technologies, Inc. USA) were screened taking DNA isolates obtained from ITCC and sixteen were selected for further studies (Table 2). The reaction mixture consisted of 25 ng of template DNA, 0.5 ml of Taq DNA polymerase, 2.5 ml of reaction buffer (10 mM Tris-HCl pH 8.3 and 50 mM KCl), 5mM $MgCl_2$, 10mM each dATP, dCTP, dGTP and dTTP and 1 mM of 10- mer primer. The volume was adjusted with sterile double distilled water. Reaction was performed in a Bio-RAD Gene Cycler (Bio-RAD, USA) thermocycler with initial denaturation of 2 minutes at 94^0C, another 35 cycles of 94^0c for 5 seconds, 37^0C for 15 seconds, 72^0C for 1 minute and final extension step of 72^0C for 7 minutes.

The amplified products were size separated on 0.7% agarose gel stained with 0.2% ethidium bromide in 1x TE buffer. Electrophoresis was carried out at constant voltage of 65 for 3 hours. Separated RAPD products were photographed using a digital camera. The bands were scored as 1 (if present) or 0 (if absent). The data was analyzed using the software NTSYS-PC, version 1.80.

RESULTS

Genetic variability

Each primer tested, produced a specific banding pattern displaying 1 – 12 bands per isolate. The size of the amplified products ranged from 200 bp to 5.2 kb. From 20 isolates tested *C. falcatum* isolates 4800 and 4803 from Daurala were same in RAPD profile with respect to 16 primers tested. *C. gloeosporioides* isolates obtained from ITCC grouped together in a distinct sub cluster. *Colletotrichum gloeosporioides* bell pepper isolates BP1 and BP2 from Solan clustered with falcate conidia bearing isolates CfK, CdP and isolate 20 respectively. There was no relationship among the isolates with respect to geographical origin of the isolates.

C. capsici isolates and *C. dematium* isolates shared three main bands of 1.9 kb, approximately 2.2 kb and 4.2 kb while isolate CdP from soybean had bands less than 1.4 kb in size. Four *C. falcatum* isolates (except isolates 20 and CfK) shared a band about 2.8 kb in size. Isolates 23 from Pantnagar and CfK from Karnal shared only one band in common with the size of approximately

Table 2. Primers used for RAPD analysis of *Colletotrichum* species.

Code	Sequence (5’ to 3’)	Code	Sequence (5’ to 3’)
OPD-01*	ACCGCGAAGG	OPN-05*	ACTGAACGCC
OPD-02*	GGACCCAACC	OPN-06*	GAGACGCACA
OPD-03	GTCGCCGTCA	OPN-08*	ACCTCAGCTC
OPD-04*	TCTGGTGAGG	OPN-09	TGCCGGCTTG
OPD-05*	TGAGCGGACA	OPN-10*	ACAACTGGGG
OPD-06*	ACCTGAACGG	OPN-11*	TCGCCGCAAA
OPD-07	TTGGCACGGG	OPN-12*	CACAGACACC
OPD-08*	GTGTGCCCCA	OPN-13	AGCGTCACTC
OPD-09	CTCTGGAGAC	OPN-14	TCGTGCGGGT
OPD-10*	GGTCTACACC	OPN-15	CAGCGACTGT
OPD-11	AGCGCCATTG	OPN-16	AAGCGACCTG
OPD-13	GGGGTGACGA	OPN-17	CATTGGGGAG
OPN-01*	CTCACGTTGG	OPN-18	GGTGAGGTCA
OPN-02*	ACCAGGGGCA	OPN-19	GTCCGTACTG
OPN-03*	GGTACTCCCC		

* primers selected for further study

1.6 kb. Isolates 4800, 4803, 4893, 20 and CfK shared a band of approximately 1.58 kb. *C. gloeosporioides* isolates 5132, 5213, 5255 and BP2 shared about 2.3 kb band in common. Isolates 5132, 5255, BP1 and BP2 shared approximately a band of 1.58 in common. Bands produced by *C. lindemuthianum* isolate 4756 more or less were similar to that produced by *C. capsici* isolates.

Isolates formed two main clusters with all the 16 primers tested and there were 57% similarity between these two clusters. Sugarcane isolate 20 and bell pepper isolate BP2 formed this small cluster sharing 61.76% similarity with each other. With all the primers tested, the falcate conidia bearing species *C. capsici* and *C. dematium* formed a distinct sub cluster, with exception of *C. dematium* isolate CdP from Karnal, which, clustered with *C. falcatum* isolates 23 and CfK. *C. gloeosporioides* isolates also clustered in a distinct sub group with the exception that two bell pepper isolates BP1 and BP2 grouped with isolates CdP and 20. *C. falcatum* isolates 4800 and 4803 from Daurala were 100% similar. Isolates 4893 from Navasari, 23 from Pantnagar and CfK from Karnal formed another group. Isolate 4893 shared 66% similarity with

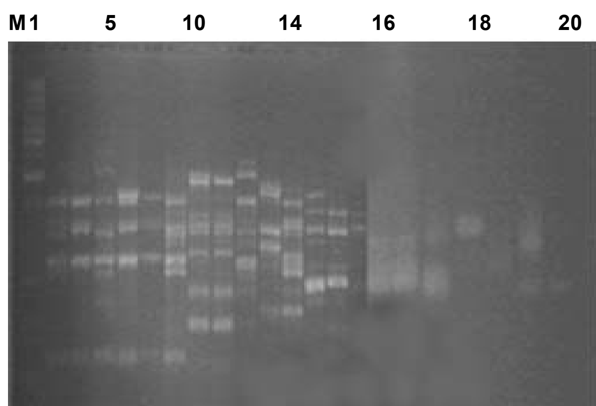

Fig. 1. RAPD profiles of *Colletotrichum* species using random primers OPN3

Lanes: M- I DNA Hind III/EcoRI marker, 1 – 4 *C. capsici*, 5-6 *C. dematium*, 7-9 *C. falcatum*, 10-13 *C. gloeosporioides*, 14 *C. lindemuthianum,* 15-17 *C. falcatum*, 18 *C. dematium*, 19-20 *C. gloeosporioides.*

Isolates 1- 4764, 2- 4871, 3- 5008, 4- 5107, 5- 4970, 6- 5306, 7- 4800, 8- 4803, 9- 4893, 10- 4573, 11- 5132, 12- 5213, 13- 5255, 14- 4765, 15- 20, 16- 23, 17- CfK, 18- CdP, 19- BP1, 20- BP2

isolates 4800 and 4803. Isolates 4800, 4803 and 4893 shared 61.8% similarity with isolates 23 and CfK while isolate 20 were more distantly related (57.0% similarity) to all other *C. falcatum* isolates. *C. lindemuthianum* isolate 4765 from New Delhi was 67.2% similar to falcate conidia bearing *C. capsici* and *C. dematium* isolates than straight conidial *C. gloeosporioides* isolates showing 66.2% similarity. Isolate 4765 formed a solitary sub group in the main sub cluster, which contained species *C. capsici* and *C. dematium.*

DISCUSSION

The present investigation was undertaken to study the morphological characters of five *Colletotrichum* species *C. capsici*, *C. dematium, C. falcatum, C. gloeosporioides* and *C. lindemuthianum* and to differentiate these five species by polymerase chain reaction (PCR) amplification of polymorphic DNA using random primers (RAPD). Each primer tested generated a specific pattern of bands displaying 1 – 12 bands per isolate. The sixteen primers differentiated species *C. falcatum, C. gloeosporioides, C. capsici* and *C. dematium* but were unable to differentiate *C. capsici* isolates from *C. dematium* isolates. This could be due to the fact that these two species are very much similar in their morphology. von Arx (1957), Sutton (1980) and Kulshrestha *et al*., (1976) accepted both species are same, but Sutton (1992) later distinguished *C. dematium* species having broader conidia. He further, stated that *C. dematium* is saprophytic while *C. capsici* is pathogenic. Within each species, the genetic variability was high. This could be due to the fact the isolates have been obtained from different host plants. Even the isolates BP1 and BP2 from same host plant species were hyper variable indicating the polymorphic nature of isolates of *C. gloeosporioides.* Morphological characters of *C. gloeosporioides* isolates also supported this hyper variability. Similarly, isolates 4893, 20, 23 and CfK obtained from sugarcane of different places (Navasari, Pantnagar and Karnal respectively) also showed hyper variability. This could be partly due to the effect of the geographical region in which they were adapted. *C. capsici*, and *C. dematium* isolates also expressed similar variability among isolates. Latha *et al*., (2002), Thakur *et al*., (1998) and Sreenivasaprasad *et al*., (1993) have also observed similar genetic variability among isolates of *C. graminicola, C. sublineolum*

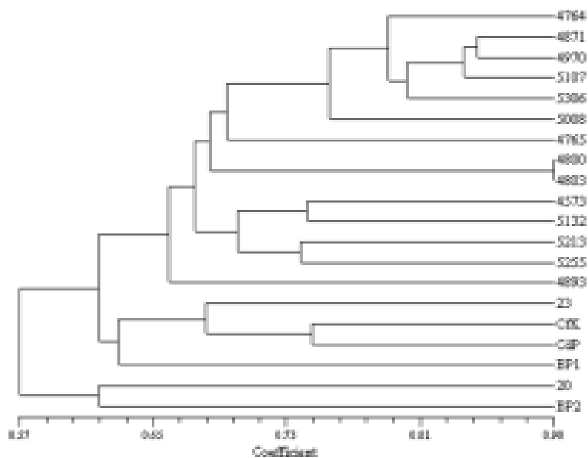

Fig. 2. Dendrogram of RAPD analysis of Colletotrichum isolates. Scale is similarity given by UPGMA implemented in NTSYS-PC.

ITCC Nos.: 4764, 4871, 5008, 5107 – *C. capsici*
4970, 5306, 6088-05 CdP – *C. dematium*
4800, 4803, 4893, 6085-05 (20), 6086-05 (23), 6087-05 (CfK) – *C. falcatum*
4573, 5132, 5213, 5255, 6082-05 (BP1), 6083-05 (BP2) – *C. gloeosporioides*
4765 – *C. lindemuthianum*

Sources of Isolates :
4764 - lal mirich, New Delhi
4871 - *Gomphrena*, Sikkim
5008 - *Melia* leaf, New Delhi
5107 - bottle brush
4970 - *Passiflora foetida*, Culcutta
5306 - ashwagandha leaf, Lucknow
CdP - soybean, Pantnagar
4803 - sugarcane, Daurala
4893 - sugarcane, Navasari
20,23 - sugarcane, Pantnagar
CfK - sugarcane, Karnal
4573 - eggplant, Solan
5132 - grape leaves, Pune
5213 - cattleya, Sikkim
5255 - cashew nut, Dapoli (MH)
BP1, BP2- bell pepper, Solan

and *C. gloeosporioides* respectively in their studies.Several pathotypes are known to exist in populations of different pathogens (Latha *et al*., 2002) and influence their survival and pathogenic fitness (Liu *et al*., 1996; Park *et al.,* 1995; Tapsoba and Wilson, 1996).

According to the results of this study, it is clear that RAPD markers could be used in differentiation of individuals better than differentiation of *Colletotrichum* species.

ACKNOWLEDGEMENTS

We gratefully acknowledge partial funding for this work from the Council for Agricultural Research Policy, Sri Lanka.

REFERENCES

Abang, M.M., Winter, S. Green, K.R., Hoffmann, P., Mignouna, H.D. and **Wolf, G.A.** (2002). Molecular identification of *Colletotrichum gloeosporioides* causing yam anthracnose in Nigeria. *Plant Pathol.* **51**: 63-71.

Bajaj, B.S., Chatrath, M.S. and **Vasudeva, R.S.** (1964). Mutation in *Colletotrichum falcatum* Went. The causal organism of red rot of sugarcane. *Indian Phytopath.* **17**: 296-303.

Brown, A.E., Sreenivasaprasad, S. and **Timmer, L.W.** (1996). Molecular characterization of slow growing orange and key lime anthracnose strains of *Colletotrichum* from *Citrus as C. acutatum*. *Phytopathol.* **86**: 523-27.

Chona, B.L. and **Hingorani, M.K.** (1950). Comparative studies of certain isolates of *Colletotrichum falcatum* Went. *Indian Phytopath.* **3**: 196-202.

Canon, P.F. and **Kirk, P.M.** (2000). The philosophy and practicalities of amalgamating anamorph and teleomorph concepts. *Studies in Mycology.* **45**: 19-25.

Crowhurst, R.N, Hawthorne, B.T., Rikkerink, E.H.A. and **Templeton, M.D.** (1991). Differentiation of *Fusarium solani* f.sp. *cucurbitae* race 1 and 2 by random amplification of polymorphic DNA. *Curr. Gene.* **20**: 391-396.

Kulshrestha, D.D., Mathur, S.B. and **Neergaard, P.** (1976). Identification of seed-borne species of *Colletotrichum. Friesia Bind XI Hafte 2.* Kobenhavn, Denmark.

Latha, J., Mathur, K., Mukherjee, P.K., Chakrabarti, A., Rao, V.P. and **Thakur, R.P.** (2002). Morphological, pathogenic and Genetic variability amongst sorghum isolates of *Colletotrichum graminicola* from India. *Indian Phytopath.* **55**: 19-25.

Liu, J.Q., Harder, D.E. and **Kolmer, J.A.** (1996). Comparative ability of races of *Puccinia graminis* f.sp. *tritici* on three barley cultivars and a susceptible wheat cultivar. *Phytopathol*. **88**: 627-632.

Park, R.E., Burdon, J.J. and **McIntosh, R.A.** (1995). Studies on the origin, spread, and evolution of an

important group of *Puccinia recondita* f.sp. *tritici* pathotypes in Australia. *Eur. J. Plant Pathol.* **101**: 613-622.

Sambrook, J., Fritsch, E. and **Maniatis, T.** (1989). *Molecular Cloning. A Laboratory Manual*, 2nd Edition. Cold Spring Harbor Laboratory Press, NY. USA. pp. 6.3-6.6.

Sherriff, C., Whelan, M.J., Arnold, G.M., Lafay, J.F., Brygoo, Y. and **Bailey, J.A.** (1994). Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. *Experimental Mycology*. **18**: 121-138.

Sreenivasaprasad, S., Brown, A.E. and **Mills, P.R.** (1992). DNA sequence variation, and inter-relationships among *Colletotrichum* species, causing strawberry anthracnose. *Physiol .Mole. Plant Pathol.* **41**: 265-281.

Sreenivasaprasad, S., Brown, A.E. and **Mills, P.R.** (1993). Coffee berry disease pathogen in Africa: genetic structure and relationship to the group species *Colletotrichum gloeosporioides*. *Mycol. Res.* **97**: 995-1000.

Sutton, B.C. (1980). *The Coelomycetes, Fungi imperfecti with pycnidia, acervuli and stromata.* Commonwealth Mycological Institute, Kew, Surrey, England. pp. 523-537.

Sutton, B.C. (1992). The genus *Glomerella* and its anamorph *Colletotrichum*. In "*Colletotrichum Biology, Pathology and Control*". (eds. Biley and Jeger) British Society for Plant Pathology, CABI, UK. pp. 1-26.

Tapsoba, H. and **Wilson, J.P.** (1996). Pathogenic variability in *Puccinia substriata* var. *indica* in the Southern United States and screening for resistance in pearl millet germplasm. *Plant Dis.* **80**: 395-397.

Thakur, R.P., Mathur, K., Rao, V.P., Chandra, S., Shivramakrishnan, S., Kannan, S., Hiremath, R.V., Tailor, H.C., Kushwa, U.S. Dwivedi, R.R. and **Indira, S.** (1998). Pathogenic and genetic characterization of six Indian populations of *Colletotrichum sublineolum,* the causal agent of sorghum anthracnose. *Indian Phytopath.* **51**: 338-348.

von Arx, J.A. (1957). Die arten der gatung *Colletotrichum* Cda. *Phytopathol. Z.* **29**: 413- 468.

Waller, J.M., Bridge, P.D., Black, R.L. and **Hakiza, G.** (1993). Differentiation of the coffee berry disease pathogen. *Mycol. Res.* **97**: 989-94.

Received for publication May 3, 2005