

Full Length Research Paper

Genetic characterization of mango anthracnose pathogen *Colletotrichum gloeosporioides* Penz. by random amplified polymorphic DNA analysis

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Twenty-five isolates of *Colletotrichum gloeosporioides* causing mango anthracnose were collected from different agroclimatic zones of India. The isolates were evaluated for their pathogenic variability on mango seedlings and genetic characterization using random amplified polymorphic DNA (RAPD molecular techniques). The random primers OPA-1, 3, 5, 9, 11, 15, 16 and 18 were used and the twenty-five isolates were grouped into two. The amplified DNA fragments (amplicons) obtained was compared by agarose gel electrophoresis. Isolate specific RAPD fingerprints were obtained. Out of eight primers in RAPD, OPA-1, 3 and 18 were able to produce reproducible banding pattern. Each of these primers generated a short spectrum of amplicons, located between 661 and 2291-bp markers, indicative of genetic polymorphism. Dendrogram revealed more than 75% level of similarity. 4.36% polymorphism was also found in individual isolates that was not statistically significant ($P > 0.05$) among the sample, it also indicates that all the isolates tested had approximately same genetic identity. The data suggest that RAPD may be of value by virtue of its rapidity, efficiency and reproducibility in generating genetic fingerprints of *C. gloeosporioides* isolates.

Key words: *Colletotrichum gloeosporioides*, genetic identification, mango anthracnose.

INTRODUCTION

Several species belonging to the genus *Colletotrichum* cause important diseases in a wide range of crops including cereals, legumes, vegetables, and fruit crops. *Colletotrichum gloeosporioides* is reported on a wide variety of fruits, including almond, avocado, apple, arabica coffee, guava, mango and strawberry (Agwana et al., 1997; Freeman et al., 1998; Martínez-Culebras et al., 2000; Sanders and Korsten, 2003; Xiao et al., 2004; Amusa et al., 2005).

The ubiquitous fungus *C. gloeosporioides* Penz and Sacc. is the anamorph stage (asexual stage of the pathogenic

fungus). *C. gloeosporioides* is responsible for many diseases, also referred to as "anthracnose," on many tropical fruits including banana, avocado, papaya, coffee, passion fruit and others (Nelson, 2008). The use of differential hosts is a viable option for the evaluation of pathogenic variability. Combined application of molecular diagnostic tools along with the use of different isolates could be a reliable approach for studying pathological variability in *Colletotrichum* species. Molecular methods have been employed successfully to differentiate between populations of *Colletotrichum* from several hosts in general. According to the study of Agwana et al. (1997), the random amplified polymorphic DNA (RAPD) technique has been used to identify RAPD markers for resistance to coffee berry disease, caused by *Colletotrichum kahawae*, in arabica coffee. Backman et al. (1999) also used the RAPD technique to determine if *Colletotrichum graminicola* isolates from annual bluegrass and creeping bentgrass are host specific. The comparative study of capsicum

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Table 1. Sources of *C. gloeosporioides* isolates used in this study.

S/N	Location in India	Source
Isolate nos. 1 - 6	Malihabad, Lucknow, UP	Fruit
Isolate nos. 7 - 8	Bakshi ka Talab, Lucknow, UP	Leaf
Isolate nos. 9 - 11	Faizabad, Lucknow, UP	Leaf
Isolate nos. 12 - 14	Gorakhpur, Lucknow, UP	Leaf
Isolate no. 15	Chittur, Tamilnadu	Fruit
Isolate nos. 16 - 17	Dharwad, Karnataka	Fruit
Isolate no. 18	Bangalore, Karnataka	Fruit
Isolate nos. 19, 21	Bangalore, Karnataka	Leaf
Isolate nos. 20, 22	Dharwad, Karnataka	Leaf
Isolate no. 23	Chittur, Tamilnadu	Leaf
Isolate nos. 24 - 25	Chittur, Tamilnadu	Fruit

anthracnose pathogens, *C. gloeosporioides*, *Colletotrichum coccodes*, *Colletotrichum dematium*, *Glomerella cingulata* and *Colletotrichum acutatum*, have been found on capsicum in Korea and China, that used RAPD-PCR technique to detect the variation among *Colletotrichum* species isolates (Shin et al., 2000). Weeds et al. (2003) reported that using molecular markers to compare the genetic diversity in *C. gloeosporioides* infected species of the tropical forage legume *Stylosanthes* from Brazil, Australia, China and India had been clearly demonstrated. RAPD markers were used to determine genetic relationships among *C. gloeosporioides* isolates, causing crown rot of strawberry, recovered from non cultivated hosts and disease strawberry plants in Florida (Xiao et al., 2004). Furthermore, Sharma et al. (2005) studied the genetic relationship between five morphological groups recognized within *Colletotrichum capsici* by using RAPD analysis. Molecular polymorphism generated by RAPD confirmed the variation in virulence of *C. gloeosporioides*. The cause of mango anthracnose isolates from Andhra Pradesh and Tamil Nadu were grouped into three clusters (Shampatkumar et al., 2007). Thus, the objective of this study was to estimate the genetic variability in *C. gloeosporioides* populations infecting mango in India.

MATERIALS AND METHODS

Fungal isolates and DNA extraction

Samples were collected from *M. indica* cv. *desheri* variety of mango cultivar of different agroclimatic zones of India and subjected to isolate the pathogen. A total of 25 isolates were used in this study (Table 1). All isolates were grown as single spore culture on potato-dextrose agar (PDA, HiMedia) and cultural studies of all selected isolates were carried out and *C. gloeosporioides* were identified (Mullar, 1940). Pure cultures of the isolates were maintained on PDA slants and incubated at 28±2°C for 6 days under controlled temperature and mycelia were aseptically transferred to flasks of potato-dextrose broth (PDB, HiMedia) and incubated for 5 days at 28±2°C without shaking. Total DNA was extracted according to the

protocol of Abd-Elsalam et al. (2003).

Pathogenicity assay

Pathogenicity tests with 25 *C. gloeosporioides* isolates were performed on the mango seedlings of Desheri under greenhouse condition. Pin prick inoculation technique using spore suspension solution was employed to prove pathogenicity, and for each isolates of *C. gloeosporioides* three replicate were maintained. The temperature ranged from 15 to 30°C during the test.

RAPD- primers

Eight oligodecamers OPA 01- 5' CAGGCCCTTC 3' ; OPA 03- 5' AGTCAGCCAC 3'; OPA 05- 5' AGGGGTCTTG3'; OPA 09 - 5' GGGTAACGCC 3'; OPA 11- 5' CAATCGCCGT 3'; OPA 15- 5' TTCCGAACCC 3'; OPA 16- 5' AGCCAGCGAA 3'; OPA 18- 5' AGGTGACCGT 3' (custom synthesized from Life Technologies, India) were used for RAPD marker studies.

Reactions and conditions of RAPD-polymerase chain reaction (PCR)

RAPD primer sets were used in Eppendrof Master Cycler. RAPD-PCR was performed in 25 µl reaction volume containing 25 ng genomic DNA, 0.4 µl (5 pmole) primer, 1.5 µl dNTPs (25 mM), 3 µl of 10 X assay buffer with MgCl₂ (15 mM), 0.5 µl (3 U/µl) of Taq DNA polymerase (Bangalore Genei Pvt.Ltd.). DNA was amplified by Eppendrof Master Cycler programme to provide first denaturation for 5 min at 94°C followed by 35 cycles of 1 min each at 94 and 35°C followed by 2 min at 72°C and final extension for 5 min at same temperature. PCR products were resolved by horizontal electrophoresis using agarose gel (1.2%) with TAE buffer (1%) containing ethidium bromide.

Cluster analysis

The genetic similarity of isolates was assessed, based on RAPD, by using Jaccard's coefficient (Jaccard, 1908) and the data was used to construct a dendrogram following the methodology of Sneath and Sokal (1973). The computations were carried out using the NTSYS-software (Rohlf, 1998).

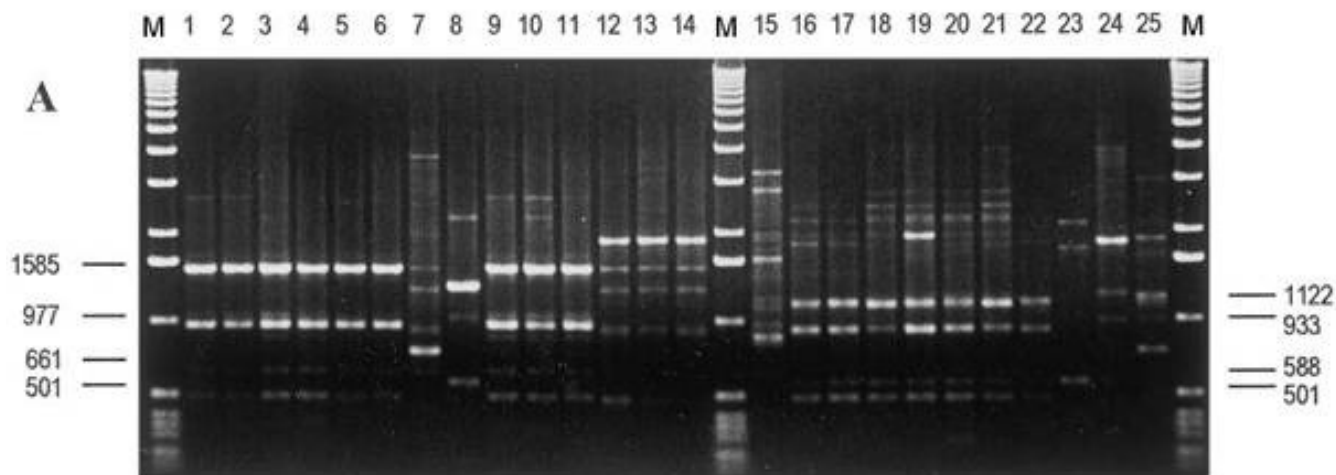


Figure 1A. RAPD fingerprints obtained with the arbitrarily selected primers OPA-1.

RESULTS

Pathogenicity assay

All 25 isolates of *C. gloeosporioides* tested were pathogenic to mango seedlings of Desheri under greenhouse conditions. Isolates were highly pathogenic causing 85 - 90% mortality in 2 - 3 months while control plants did not develop any symptoms.

RAPD-PCR analysis

Eight primers were tested in the genome of *C. gloeosporioides* isolates collected from different locations. The generated fingerprints were evaluated for overall clearness of the banding pattern. Primers OPA-1, OPA-3 and OPA-18 were chosen because they reliably and reproducibly detected polymorphisms among the selected isolates. Figure 1 shows a typical profile and polymorphic bands generated with the primer OPA-1, OPA-3 and OPA-18. All of the profiles generated were examined visually, and polymorphic bands were scored (as present or absent).

Each of the RAPD patterns obtained with *C. gloeosporioides* reference isolates with primers OPA-1, OPA-3 and OPA-18 contained a set of major amplicons that were shared by almost all the isolates; the three predominant banding patterns thus obtained were clearly distinct (Figures 1A, B, C). However, the RAPD fingerprints contained only a limited subset of amplicons that are indicative of genetic polymorphism within the species. Use of primer OPA-1 yielded the simplest fingerprints, composed of two major amplicons at 1585 and 977 bp and two minor ones (Figure 1A). This set of amplicons was common to all the reference isolates of *C. gloeosporioides* tested, except for isolates 7 and 8. This

resemblance to *C. gloeosporioides* was also demonstrated with primers OPA-18 (Figure 1C) and OPA-9 (data not shown), although a perfect match for the typical *C. gloeosporioides* RAPD patterns was not obtained. The RAPD profiles of isolate Cg8 was also quite distinct from the characteristic pattern of *C. gloeosporioides* (Figure 1). Use of primers OPA-3 and OPA-18 also yielded a specific fingerprint for *C. gloeosporioides* isolates so far tested. Each of these primers generated a short spectrum of amplicons, located between 661 and 2291-bp markers, indicative of genetic polymorphism. A set of major amplicons at 2138, 1202 and 955 bp as generated by OPA-3, and at 2291 and 1995 bp as obtained with OPA-18 (Figure 1 A, B, C), were shared by all isolates, except for isolates 7 and 8, as for the set of amplicons generated by primer OPA-1. Overall, the fingerprints obtained from *C. gloeosporioides* with the selected primers were unique and species-specific.

RAPD cluster analysis

The variable banding patterns of RAPD primers were summarized by cluster analysis which showed all isolates having extensive homology, which may therefore restrict the use of this technique for typing individual isolates. In RAPD 75% level of similarity was recorded among the tested isolates of *C. gloeosporioides* and two clusters were visible (Figure 2). 4.36% polymorphism was also found in individual isolates that was not statistically significant ($P > 0.05$) among the samples, indicating that all isolates used in this study have approximately similar genetic identity.

DISCUSSION

On mango, *C. gloeosporioides* anthracnose produces

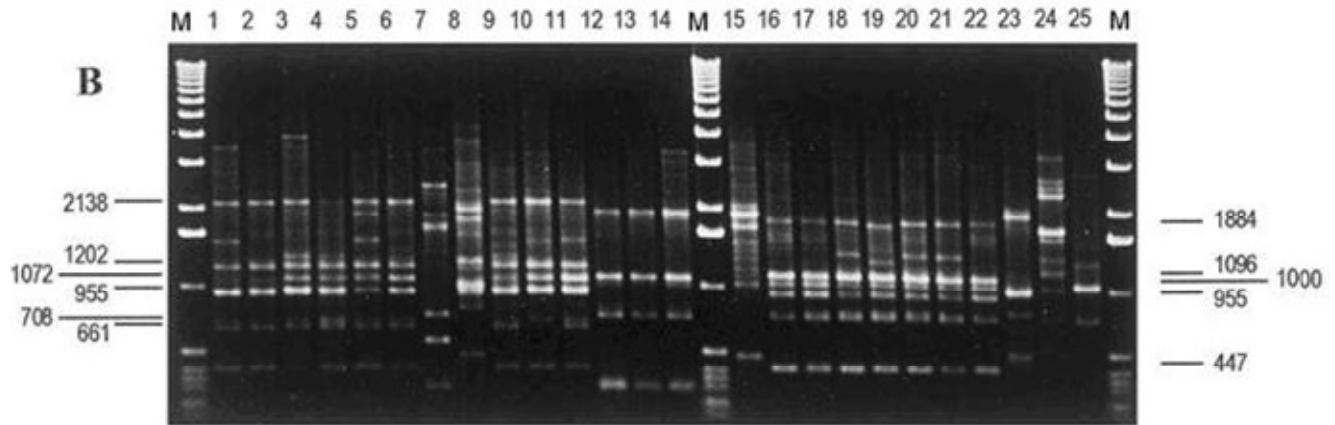


Figure 1B. RAPD fingerprints obtained with the arbitrarily selected primers OPA-3.

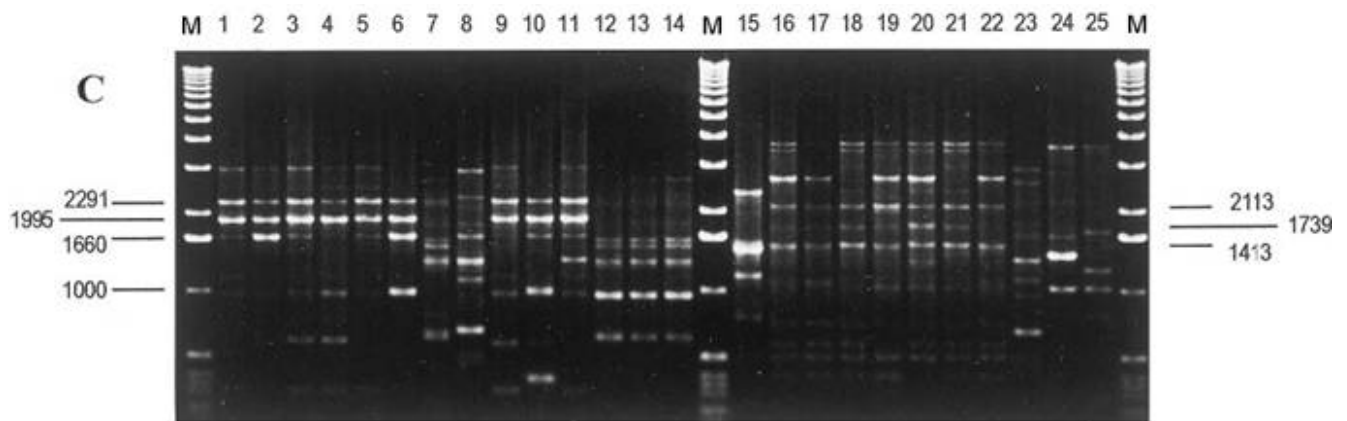


Figure 1C. RAPD fingerprints obtained with the arbitrarily selected primers OPA-18. Molecular weight markers (1 kb ladder) are in bp. Marked sizes indicate amplicons that constitute a distinctive set characteristic of the *C. gloeosporioides* isolates.

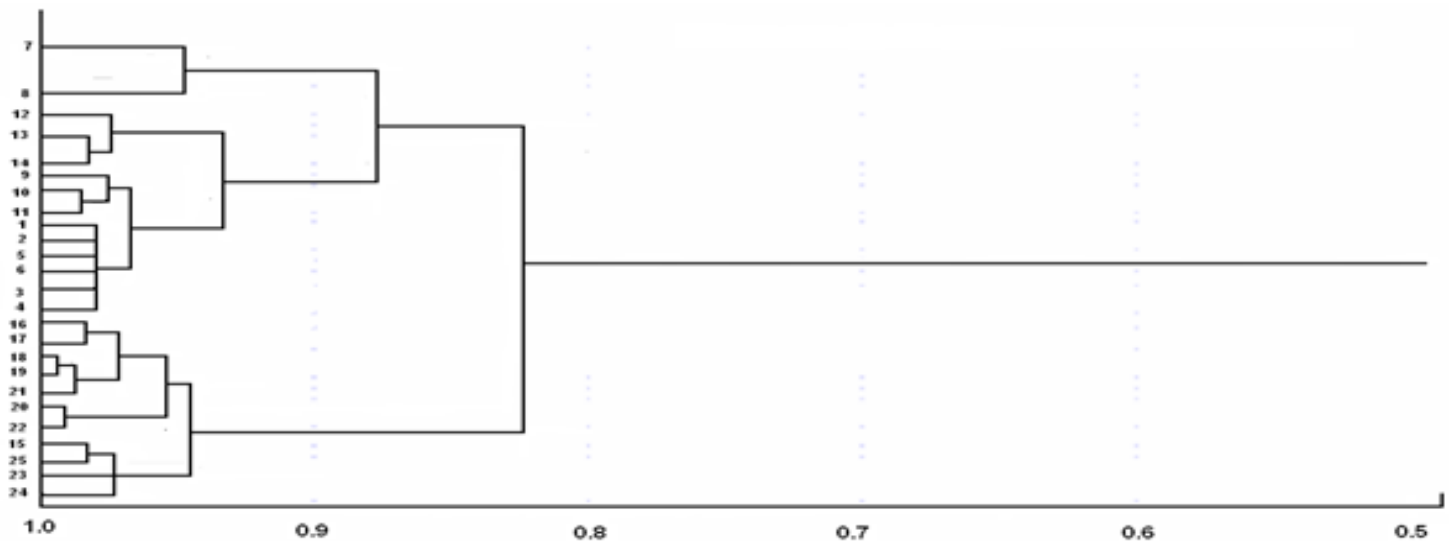


Figure 2. Based on RAPD allelic pattern, dendrogram was derived from cluster analysis (UPGMA) showing relationship among the 25 *C. gloeosporioides* isolates.

symptoms on leaves, twigs, petioles, flower cluster (panicales) and on fruits. On leaves, lesions, brown to black spots can be seen. The symptoms on panicales are small black or dark brown spots, and can kill the flowers. Petioles, twigs and stems are also susceptible by developing black, expanding lesions on mango seedlings of 2 - 3 year old Desheri mango plant (a very famous variety of mango fruit plant of India growing on the famous Malihabad mango belt of CISH, Lucknow, India). The pathogenicity study showed that the behavior of *C. gloeosporioides* isolates was homogeneous, with no variations in virulence and symptoms were same as described above. RAPD applied to fungal studies would be useful in providing markers for identification purpose. Furthermore, it revealed polymorphisms within reference isolates of *C. gloeosporioides* and established DNA fingerprints useful for race characterization (Weeds et al., 2003). Combined cluster analysis of the RAPD data placed the *C. gloeosporioides* isolates into two clusters. Xiao et al. (2004) reported that RAPD markers were used to determine genetic relationships among isolates that recovered from non cultivated hosts and diseased strawberry plants. Phylogenetic analysis using RAPD marker data divided isolates of *C. gloeosporioides* from non cultivated hosts into two separate clusters. Isolates from strawberry were interspersed within the cluster containing the isolates that were recovered from non cultivated hosts. However, Backman et al. (1999) studied anthracnose basal rot caused by *C. graminicola* (Ces.) Wils, which is a destructive disease of annual bluegrass (*Poa annua* L.) and creeping bentgrass (*Agrostis palustris* Huds.) golf course putting greens in North America and Europe. Alahakoon et al. (1994) reported that when inoculated into non-host, *C. gloeosporioides* could genetically adapt to a new host, and the resultant isolates could not be distinguished morphologically from the wild type. Thus, it requires further investigation for the cross-infection of different hosts by *Colletotrichum* species. Ratanacherdchai et al. (2010) suggested that RAPD markers can be a quick and reliable alternatives for differentiating isolates of *C. gloeosporioides* into their respective pathogenic group which corroborate the results of our study with RAPD similarity of 75%.

In this study, we assessed the suitability of RAPD techniques for rapid molecular characterization of *C. gloeosporioides* isolates. Also, the data presented here supports the idea that *C. gloeosporioides* are derived from a single clonal lineage. All isolates were found to have approximately the same RAPD type.

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