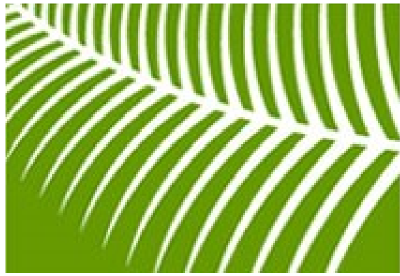

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PROCEEDINGS

OF THE

33rd ANNUAL MEETING

6-12 July 1997

Proceedings Edited
by
Nelson Semidey and Lucas N. Aviles

Published by the Caribbean Food Crops Society

Proceedings of the Caribbean Food Crops Society. 33:406-413. 1997

GENETIC AND PATHOLOGICAL DIVERSITY OF THE MANGO ANTHRACNOSE PATHOGEN IN FLORIDA

M. J. Davis, University of Florida, Tropical Research and Education Center, 18905 SW 280 Street, Homestead, FL 33031, USA.

ABSTRACT. The mango (*Mangifera indica* L.) is a major fruit crop grown in the tropics and subtropics around the world including the Caribbean region. Anthracnose, caused by *Colletotrichum gloeosporioides* Penz., is considered the most important disease of mango in the humid tropics. Randomly amplified polymorphic DNA (RAPD) analyses, pathogenicity tests on inflorescences and detached leaves and fruits, susceptibility to benomyl, and pectic zymogram analysis were used to examine diversity among isolates of the pathogen from naturally infected leaves, peduncles, flowers, and immature, green and mature fruits. Although considerable genetic diversity was found, cluster analysis of the pathogenicity and RAPD data indicated several groups of isolates. Based on spore shape, one group might be classified as *C. acutatum* and not *C. gloeosporioides*. Isolates of the "*C. acutatum*" group tended to be slightly more virulent on inflorescences and less virulent on leaves and mature fruits. Such distinctions among *Colletotrichum* isolates from mango might be significant with respect to anthracnose control. The diversity of *Colletotrichum* associated with anthracnose of mango in Florida may help to explain why some mango cultivars with reported resistance to anthracnose elsewhere are susceptible in Florida.

INTRODUCTION

The mango (*Mangifera indica* L.) is a major fruit crop grown in the tropics and subtropics around the world including the Caribbean region. Anthracnose, caused by *Colletotrichum gloeosporioides* Penz., is considered the most important disease of mango in the humid tropics. Although organic fungicides have provided an adequate means of control of anthracnose, government regulation has greatly limited the number of fungicides that can be used, and benomyl, one of the most effective fungicides used for anthracnose control, is rapidly becoming ineffectual due to the development of resistance in pathogen populations. To augment or replace chemical control measures, additional measures need to be developed that are based on improved cultural practices, the use of biological agents, or possibly other advanced technologies. However, implementation of such measures would be difficult to develop without a thorough understanding of pathogen diversity as related to the etiology and epidemiology of mango anthracnose.

Colletotrichum populations on mango and other crop plants are heterogeneous. Morphological and cultural characters have proven inadequate for distinguishing strains in respect to their host specificity, tissue specificity or ability to incite disease in the same tissues at different developmental stages; however, inconsistencies in the development of mango anthracnose suggest that such diversity within pathogen populations might exist. Molecular approaches are increasingly being successfully applied to overcome limitations imposed by morphological and cultural similarities among strains of *C.gloeosporiodes* and to provide new information about these organisms. In this study, the results of randomly amplified

polymorphic DNA (RAPD) analyses were compared with those of pathogenicity assays to examine the population diversity of *C. gloeosporiodes* on mango in Florida.

MATERIALS AND METHODS

For RAPD analysis, a simplified procedure for DNA extraction from *C. gloeosporioides* was developed. Isolates of *C. gloeosporioides* were obtained as described previously (Gantotti and Davis, 1991). Mycelia of different isolates were scraped from the surface of potato dextrose agar plates to get a pellet ca. 5 mm in diameter, suspended in 3-ml buffer (8-mm TRIS-HCl, pH 8.5), placed in a boiling water bath for 15 minutes, cooled to room temperature, and stored at -20° C. For PCR, the DNA extracts were thawed, vortexed and the clear liquid added as 50% of PCR reaction mixture as the final component. Three different 10-base primers as described by Guthrie et al. (1992) were used in separate PCR reactions. All reactions were performed in a Coy ThermoCycler II Model 110S with the in sample temperature probe. A standard 50 µl reaction mixture (20 mM TRIS, pH 8.5, 1.5 mM $MgCl_2$, 25 mM KCl, 0.01% DIFCO Gelatin, 0.05% Tween 20, 100 µM of the four dNTPs, 0.8-mM primer and 1.5 U *Täq* polymerase (Promega, Madison, WI)) in 0.6-ml tubes was used for all amplifications. All mixture components were precooled to 4° C and kept on ice while mixed and prepared for amplification. Each reaction mixture was overlaid with one drop (ca. 50 µl) of certified DNAse/RNAse free mineral oil (Sigma Chemical Co., St. Louis, MO). The temperature cycling schedule was 4 min predenaturation at 94° C, followed by 40 cycles of 1 min at 94° C, 1 min at at 43° C, and a 2.5 min ramp to 94° C, and then a followed by a final extension at 72° C for 2 min. The reaction mixtures were then held at 4° C. Amplified DNA was separated by gel electrophoresis and stained with ethidium bromide. Molecular weight markers were used for size determination of amplified DNA. Stained DNA in gels was visualized using a UV transilluminator and photographed. Each reaction was repeated at least once.

An image analysis system using the Optimus computer program (Bioscan, Inc., Edmonds, WA) was used to capture images of gels from Polaroid black and white prints. For each electrophoretic separation (lane) representing a primer by isolate combination, the BIOMED computer program (Advanced American Biotechnology, Fullerton, CA) was used to assign normalized molecular weights as nucleotide base pairs (bp) and band intensity as optical density (OD) to each detected band of DNA. Bands were filtered for number of base pairs between 200 and 2000 and optical density threshold to approximately one standard deviation below the mean. The remaining filtered bands were then grouped by similar number of base pairs using the nonparametric clustering algorithm in the SAS Modeclus program (SAS Institute, Cary, NC). This statistical technique was necessary due to the accumulation of experimental error and resolution limits which precluded the simple matching of bands with identical number of base pair parameters. Bands falling within an optimum statistically valid cluster were considered genetically similar. Clustering parameters were optimized to maximize the density of members within each cluster and minimize the overlap between clusters and members outside clusters.

For analysis of similarity among banding patterns among isolates, a binary data matrix was constructed indicating the presence or absence of each DNA band cluster for each isolate and RAPD primer as identified by the Modeclus procedure. The binary scores were used to

calculate the pair-wise similarity among banding patterns using the Dice coincidence index: Similarity between paired haplotypes (S_{xy}) was the number of common bands in both DNA profiles (n_{xy}) divided by the average number of bands for the two haplotypes [$S_{xy} = 2n_{xy}/(n_x + n_y)$]. Dissimilarity or distance between haplotypes (D_{xy}) was one minus the similarity ($D_{xy} = 1 - S_{xy}$).

Cluster analysis of the distance matrix was conducted by the unweighted pair-group method with arithmetic means (UPGMA) using the Cluster procedure (SAS). To evaluate the robustness of the groupings obtained by cluster analysis, the binary data was subjected to bootstrap analysis using the Winboot computer program (Yap, I. V. and Nelson, R. J. Winboot: A program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. International Rice Research Institute, Discussion paper series no. 14. Manila, Philippines). Bootstrap values were calculated as the frequency with which a particular group appears among 1000 dendrograms constructed by bootstrapping..

Detached leaf and fruit assays were used to test the pathogenicity of *C. gloeosporioides* isolates from mango. Mature fruit of the 'Tommy Atkins' cultivar were obtained from J. R. Brooks and Son, Homestead, FL, after post-harvest heat treatment and importation from Central or South America (locally produced mangoes were unavailable due to Hurricane Andrew). Fresh leaves of the 'Kent' cultivar were harvested locally on the date of inoculation from a commercial grove. Both leaves and fruit were surface disinfected with 0.5% sodium hypochlorate, rinsed with running tap water, and allowed to dry before inoculation. Spore suspensions (5 x10^7 spores/ml) in sterile water were prepared from 14 day old cultures growing on one-half strength potato dextrose agar at room temperature under fluorescent lighting and then used as inoculum. Ten filter paper disks (6-cm in diameter) were placed separately on the surface of each of the leaves (upper surface) and fruits to be inoculated. Fifteen microliters of inoculum was deposited on each disk. Inoculations with each isolate were replicated randomly five times for both leaves and fruits. The inoculated leaves and fruit were incubated in moist chambers at room temperature. To prevent accumulation of CO_2 in moist chambers which prevented lesion formation on fruits, a 0.1-N KOH solution was placed on the bottom of the chambers containing fruit and the chamber opened briefly 1-2 times daily for ventilation. The lesion width outward from each disk was measured after 5 days of incubation. The each experiment was repeated three times.

To test pathogenicity on inflorescences, entire panicles on trees of cultivars 'Tommy Atkins' and 'Kent' were inoculated by spraying with spore suspensions (5 x10^7 spores/ml) of individual Colletotrichum isolates and covered in plastic bags for 24 hr . After four days, the severity of overall panicle blight and lesion development on immature fruits was rated on a scale of zero to three as follows: None to slight (0% to 15%) = 0; slight to moderate (16% to 50%) = 1; moderate to severe (51% to 85%) = 2; severe (86% to 100%) = 3. Four panicles were inoculated for each treatment and the entire experiment was repeated once. Analysis of variance was conducted on the ranks of the severity ratings for each experiment using the SAS GLM procedure.

Table 1. Characteristics of 85 isolates of *Colletotrichum gloeosprioides* from mango in Florida.

Isolate	Plant tissue	Location	RAPD cluster	Pathogenicity cluster	Pectin zymogram group	Benomyl suscep-tibility
2-1FL04	Flower	Grove 2	1	1	ND	R
1-10F01	Immature fruit	Grove 1	1	1	ND	S
1-4F01	Immature fruit	Grove 1	1	1	ND	R
1-6F03	Immature fruit	Grove 1	1	1	ND	R
MGRS10	Immature fruit	TREC	1	1	1	R
MIF03	Immature fruit	TREC	1	1	ND	R
MIF04	Immature fruit	TREC	1	1	ND	R
MIF07	Immature fruit	TREC	1	1	1	R
MIF08	Immature fruit	TREC	1	1	1	S
MIF09	Immature fruit	TREC	1	1	ND	R
MIF11	Immature fruit	TREC	1	1	ND	R
MIF13	Immature fruit	TREC	1	1	1	S
MIF14	Immature fruit	TREC	1	1	2	R
MIF16	Immature fruit	TREC	1	1	1	R
MMFTD06	Mature fruit	Grove 3	1	1	ND	R
MMFTD15	Mature fruit	Grove 3	1	1	ND	R
MMFTD15	Mature fruit	Grove 3	1	1	ND	R
MMKS08	Mature fruit	Grove 3	1	1	1	S
MMF05	Mature fruit	Haiti	1	1	1	S
MMFHD01	Mature fruit	TREC	1	1	1	R
MMFHD03	Mature fruit	TREC	1	1	ND	R
TMMF01	Mature fruit	TREC	1	1	ND	R
TMMF04	Mature fruit	TREC	1	1	1	R
TMMF05	Mature fruit	TREC	1	1	1	R
TMMF06	Mature fruit	TREC	1	1	1	R
TMMF08	Mature fruit	TREC	1	1	ND	R
1-10L01	Leaf	Grove 1	1	1	ND	S
1-1L02	Leaf	Grove 1	1	1	ND	R
1-3L01	Leaf	Grove 1	1	1	ND	S
1-3L03	Leaf	Grove 1	1	1	ND	S
1-8L02	Leaf	Grove 1	1	1	1	S
MLL46-9	Leaf	TREC	1	1	ND	S
TML10	Leaf	TREC	1	1	1	R
TML12	Leaf	TREC	1	1	ND	R
TML13	Leaf	TREC	1	1	ND	S
TML18	Leaf	TREC	1	1	1	R
TML19	Leaf	TREC	1	1	1	R
MGRL15	Green fruit	TREC	1	2	ND	R
1-10F02	Immature fruit	Grove 1	1	2	ND	R
1-4F02	Immature fruit	Grove 1	1	2	ND	ND
MMFS02	Mature fruit	Grove 3	1	2	ND	R
MMFS09	Mature fruit	Grove 3	1	2	ND	R
TMMF02	Mature fruit	TREC	1	2	ND	R
1-8L03	Leaf	Grove 1	1	2	1	S
MLL46-8	Leaf	TREC	1	2	ND	S

(Continued)

Table 1. (continued)

Isolate	Plant tissue	Location	RAPD cluster	Pathogenicity cluster	Pectin zymogram group	Benomyl suscep-tibilty
1-1P02	Peduncle	Grove 1	1	2	ND	R
2-4FL03	Flower	Grove 2	2	3	ND	R
2-4FL04	Flower	Grove 2	2	3	ND	R
2-2F01	Immature fruit	Grove 2	2	3	2	R
2-2F02	Immature fruit	Grove 2	2	3	2	R
MMKS02	Mature fruit	Grove 3	2	3	1	R
2-2P03	Peduncle	Grove 2	2	3	2	R
2-4P03	Peduncle	Grove 2	2	3	2	R
TML17	Leaf	TREC	3	1	ND	R
TML22	Leaf	TREC	3	1	ND	R
MGFP08	Green fruit	Grove 3	3	2	ND	R
2-8F01	Immature fruit	Grove 2	3	2	1	R
MMFD08	Mature fruit	TREC	3	2	1	R
MMFD09	Mature fruit	TREC	3	2	1	R
MMFD11	Mature fruit	TREC	3	2	1	R
MMFHD02	Mature fruit	TREC	3	2	1	R
MMFHD07	Mature fruit	TREC	3	2	1	R
MLS01	Leaf	Grove 3	3	2	1	R
TML04	Leaf	TREC	3	2	1	R
TML16	Leaf	TREC	3	2	ND	R
1-2P01	Peduncle	Grove 1	3	2	ND	R
MGFP03	Green fruit	Grove 3	3	3	ND	R
MGFP35	Green fruit	Grove 3	3	3	ND	R
1-2F01	Immature fruit	Grove 1	3	3	ND	R
MMKS06	Mature fruit	Grove 3	3	3	ND	R
MMKS07	Mature fruit	Grove 3	3	3	ND	R
MMFD10	Mature fruit	TREC	3	3	1	R
MMFD12	Mature fruit	TREC	3	3	1	R
MMFD13	Mature fruit	TREC	3	3	1	R
MMFD14	Mature fruit	TREC	3	3	1	R
MLL10	Leaf	Grove 3	3	3	1	R
MLS07	Leaf	Grove 3	3	3	1	R
TML05	Leaf	TREC	3	3	1	R
TML21	Leaf	TREC	3	3	1	R
1-1P01	Peduncle	Grove 1	3	3	ND	R
2-8F02	Immature fruit	Grove 2	4	3	1	R
BRKPLP1	Mature fruit	unknown	4	3	ND	S
MIF12	Immature fruit	TREC	5	1	1	S
TMMF03	Mature fruit	TREC	5	1	1	R
TMMF07	Mature fruit	TREC	5	1	1	R

RESULTS AND DISCUSSION

The characteristics of *C. gloeosporioides* isolates examined in this study are presented in Table 1. In addition to the RAPD and pathogenicity analyses conducted in the present study,

information is also presented for comparison on pectin zymogram groupings (Gantotti and Davis, 1991) and benomyl susceptibility (unpublished) of the isolates. Although 10 pectin zymogram patterns were originally described, subsequent analyses indicated that these patterns were inconsistent variations belonging to two major groups (unpublished). Benomyl susceptibility was based on inhibition of spore germination on one-half-strength potato dextrose agar supplemented with 2 mg/L benomyl.

Cluster analysis of the pathogenicity data from the detached leaf and mature fruit inoculation assays indicated three groups of isolates (Table 2). Pathogenicity group 1 was highly virulent on both leaves and mature fruits. Pathogenicity group 2 moderately virulent on leaves but highly virulent on fruits. And pathogenicity group 3 was weakly virulent on leaves and fruits. Although not shown, the frequency of lesion formation was directly correlated to virulence.

Table 2. Mean lesion radii for pathogenicity clusters as determined in detached leaf and mature fruit assays.

Pathogenicity cluster	Number of isolates	Mean lesion radius (mm)[a]	
		Leaf	Fruit
1	42	4.42 a	2.02 a
2	20	2.91 b	1.90 a
3	23	2.28 c	1.32 b

[a]Means in the same column followed by the same letter were not significantly different (Waller-Duncan K-ratio t-test, K=100).

Cluster analyses of RAPD data indicated five groups. Bootstrap analysis indicated that RAPD groupings were not rigorous. Thus, RAPD groupings could not be used to evaluate phylogenetic relationships among the groups, and membership of individual isolates within a particular group could be considered equivocal. The groups as a whole, however, appeared to have some validity when compared with the other characteristics of the isolates. There was strong agreement between membership in pathogenicity group 1 and RAPD group 1, although some members of RAPD group 1 belonged to pathogenicity group 2. All members of RAPD group 2 belonged to pathogenicity group 3. Members of RAPD group 3 belonged to each of the three pathogenicity groups. All members of RAPD group 4 belonged to pathogenicity group 3. All members of RAPD group 5 belonged to pathogenicity group 1. Comparison of data for the detached leaf and mature fruit pathogenicity assays with the RAPD groupings indicated that members of RAPD group 1 tended to be highly virulent on both leaves and fruits, members of RAPD group 2 were weakly virulent on both, and members of the other RAPD groups were intermediate in their pathogenicity (Table 3). All members of RAPD groups 2 and 3 were resistant to benomyl; whereas, benomyl resistance was not consistent for the other groups.

Table 3. Mean lesion radii for randomly amplified polymorphic DNA (RAPD) clusters as determined in detached leaf and mature fruit assays.

RAPD cluster	Number of isolates	Mean lesion radius (mm)[a] Leaf	Fruit
1	46	4.35 a	1.95 a
2	7	2.24 c	0.54 b
3	27	2.41 bc	1.80 a
4	2	2.25 c	2.30 a
5	3	3.75 ab	2.16 a

[a]Means in the same column followed by the same letter were not significantly different (Waller-Duncan K-ratio t-test, K=100).

Table 4. Ratings[1] of overall blight and severity of fruit lesions four days after inoculation of inflorescences with *Colletotrichum* isolates representative of the major RAPD and pathogenicity clusters. Means ratings of approximately eight replications per treatment are given.

			Cultivar 'Kent		Cultivar 'Tommy Atkins'	
Isolate	RAPD cluster	Pathogenicity cluster	Fruit lesion rating	Overall blight rating	Fruit lesion rating	Overall blight rating
MIF16	1	1	2.0 ab	1.9 abc	1.6 bcd	1.5 bc
TMMF04	1	1	2.8 a	2.2 ab	1.5 d	1.3 cd
2-2F01	2	3	1.7 bc	1.9 abc	2.1 ab	1.9 abc
2-4FL04	2	3	2.1 ab	1.8 bc	2.4 ab	2.3 a
2-4P03	2	3	2.1 ab	2.4 a	2.4 a	2.4 a
MLS01	3	2	1.7 bc	1.5 c	2.1 ab	2.3 a
MMFD08	3	2	1.5 bc	1.4 cd	1.8 bcd	1.8 abc
1-1P01	3	3	1.5 bc	1.9 abc	2.0 abc	2.3 a
1-2F01	3	3	1.8 bc	2.3 ab	1.9 abcd	1.4 bcd
MLS07	3	3	1.4 bc	2.0 abc	1.8 cd	1.9 ab
H_2O-inoculated			1.3 cd	1.6 cd	1.4 cd	1.8 abc
Non-inoculated			0.3 d	0.6 d	0.1 e	0.8 d

[1]The severity of infection was rated on a scale of zero to three as follows: None to slight (0% to 15%) = 0; slight to moderate (16% to 50%) = 1; moderate to severe (51% to 85%) = 2; severe (86% to 100%) = 3. Means in the same column followed by the same letter were not significantly different according to Fisher's LSD (alpha = 0.05).

The results of this study identified several genetically and pathologically distinct populations of *C. gloeosporioides* within mango in Florida. One population corresponds to a large extent to RAPD group 2, and pathogenicity group 3 and pectin zymogram group 2. Members of this group tended to come from inflorescences and small fruitlets but not leaves and more mature fruit. They also tend to be less virulent to mango leaves and fruits than the

other *C. gloeosporioides* isolates examined. However, when intact mango inflorescences were inoculated with members of this group, they were more virulent as a whole than other representative isolates (Table 4). Members of this group tended to have spores which were somewhat pointed on both ends; whereas, the other isolates for the most part had spores that were rounded on one or both ends (data not shown). This pointed spore morphology is similar to that of *C. acutatum* which has been reported on mango in Australia (Fitzell, 1966) in addition to *C. gloeosporioides* var, *minor* (Simmonds, 1965). In Trinidad, two distinct populations of *C. gloeosporioides* on mango were identified on the basis of colony morphology; one being isolated more frequently from leaves and the other being isolated more frequently from fruits (Baker et al., 1940). Such diversity among populations of *Colletotrichum* on mango as identified in this and other studies has important implications with respect to control of mango anthracnose. The population diversity of *Colletotrichum* on mango in Florida may help to explain why some mango cultivars with reported resistance to anthracnose elsewhere are susceptible in Florida.

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ACKNOWLEDGEMENTS

The technical assistance of J. B. Kramer and C. J. Warmuth is gratefully acknowledged. This research was supported by grant no. 94-34135-7279 from the USDA Tropical/Subtropical Agriculture Grants Program and published as University of Florida Agricultural Experiment Station journal series number ________.