Virulence and Genetic Variation of Isolates of *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. on Mango (*Mangifera indica* L.) cv. Haden

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Abstract. The virulence and genetic variation shown by RAPD (Random amplified polymorphic DNA) were determined for 15 monoconidial isolates of *Colletotrichum gloeosporioides* obtained from leaves and fruits of mango (*Mangifera indica*) cv. Haden. They were classified into four groups according to their degree of virulence onto inoculated leaves: very virulent (from 19.2 to 9.0% severity, isolates F3, F4, and T5), virulent (from 8.0 to 5.0%, T4, T3, F5, and T1), moderately virulent (from 3.8 to 1.2%, H4, H2, T6, F2, and H3), and scarcely virulent (from 0.1 to 0.01%, H1, F1, and T2). F3 was the most virulent as it showed the highest anthracnose severity. Isolates H4, H1, H2, H3, T6, F2, and T2, showed greater genetic variability and were part of a group that was well separated from F3, F4, and F5. These three were outside the main groups in the dendrogram that was constructed from results of the RAPD analysis. T5 (very virulent) was associated with T3 and T4 (virulent); however, F1 (scarcely virulent) was grouped with F5 (virulent). The results from the RAPD analysis suggest the existence of great genetic variability among the 15 isolates of *C. gloeosporioides*.

Additional keywords: Anthracnose, RAPD, disease severity.

Resumen. Se determinó la virulencia y la variación genética mediante RAPD (Amplificación polimórfica del ADN al azar) de 15 aislamientos monoconidiales de *Colletotrichum gloeosporioides* obtenidos de hojas y frutos de mango (*Mangifera indica*) cv. Haden, los cuales se clasificaron en cuatro grupos de acuerdo con el grado de virulencia que mostraron en hojas inoculadas: muy virulentos (de 19.2 a 9.0% de severidad, aislamientos F3, F4 y T5), virulentos (de 8.0 a 5.0%, T4, T3, F5 y T1), moderadamente virulentos (de 3.8 a 1.2%, H4, H2, T6, F2 y H3), y levemente virulentos (de 0.1 a 0.01%, H1, F1 y T2). F3 se consideró como el más virulento, ya que presentó la severidad mayor de la antracnosis. Los aislamientos H4, H1, H2, H3, T6, F2 y T2 presentaron una variabilidad genética mayor y formaron un grupo diferente a los aislamientos F3, F4 y F5. Éstos tres estuvieron fuera de los grupos principales en el dendrograma que se construyó con los resultados del análisis de RAPD. T5 (muy virulento) se asoció con T3 y T4 (virulentos); sin embargo, F1 (levemente virulento) se agrupó con F5 (virulento). Los resultados obtenidos con la técnica RAPD sugieren que existe una gran variabilidad genética entre los 15 aislamientos de *C. gloeosporioides*.

Palabras clave adicionales: Antracnosis, RAPD, severidad de la enfermedad.

In 2001, Mexico produced 1.5 million ton of mango (*Mangifera indica* L.) on 165, 403 ha, occupying third place in world production after India and China (FAO, 2001). Mexico was the leading exporter of this fruit with a volume of 206,782 ton, and the main markets for Mexican mangoes were the USA, Canada, Europe (France, UK, Switzerland, Spain, and Holland), Japan, New Zealand, Australia, and Chile (FAO, 2000). Anthracnose is one of the diseases that affect mango and limit its production (Cook, 1975; Lim and Khoo, 1985; Ploetz *et al*., 1994). The great losses that are reported are due to the damage caused to the fruit, which is characterised by the blackening of the peel; however, there can also be serious damage to the foliage (Ploetz and Prakash, 2000). In dense plantations and in favourable conditions of humidity, considerable damage can occur to the crowns of young trees and, in extreme cases, they can be completely destroyed (Bose *et al*., 1973). Anthracnose is found in all mango production regions of Mexico, with severe attacks occurring at flowering and fruiting stages and after harvest, causing losses that vary from 15 to 50% (Becerra-Leor, 1995). In most of the mango producing regions of the world, anthracnose is caused by the fungus *Colletotrichum gloeosporioides* (Penz.) Penz. and

Sacc.; however, in Australia *C. gloeosporioides* var. *minor* Wollenw. has been reported to be responsible for this disease (Ploetz and Prakash, 2000; Prusky, 1994). Likewise, Fitzell (1979) indicated that *C. acutatum* Simmonds also plays a small role as a causal agent of anthracnose in Australia and India. The initial symptoms of anthracnose in the leaves are small dark brown patches, which can coalesce and form irregular lesions more than 1 cm in diameter. The center of old lesions can dry up and fall away so that the leaf becomes perforated (Prusky, 1994); from this appearance, the condition is given the name "shotholing". In extreme cases, anthracnose can cause progressive death of the tree's branches (Lim and Khoo, 1985; Prusky, 1994). However, differences in pathogenicity, aggressiveness, severity and incubation period have been reported by Katan (2000). Nowadays, there are not mango cultivars available to be used as differentials to detect variation among *C. gloeosporioides* isolates; even, if they were available, it would be faster to detect variation through molecular methods. Thus, the objective of this study was to determine genetic variation by means of molecular analysis using RAPD (random amplified polymorphic DNA), of 15 isolates of *C. gloeosporioides* obtained from leaves and fruits that showed differences in symptoms of blight and anthracnose.

MATERIALS AND METHODS

Isolation of *C. gloeosporioides*. Isolates were obtained from leaves and fruits of 15 mango (cv. Haden) trees with symptoms of blight (leaves) and anthracnose (leaves and fruit) in a commercial orchard in Iguala, Guerrero, Mexico. Fragments of tissue from leaves or fruits were surface sterilized in sodium hypochlorite solution (1.5 %) for 2 min, and a total of 300 were plated out on potato dextrose agar (PDA) in Petri dishes (four to a dish). Dishes were incubated at 24 ± 1ºC under white light. The 15 isolates that were finally selected were not obtained from the same tree or the same leaf or fruit.

Selection and production of monoconidial isolates of *C. gloeosporioides*. Subcultures were prepared on PDA from the mycelial tissue growing from samples with symptoms of anthracnose (leaves and fruit) or blight (leaves) in order to obtain monoconidial cultures by taking a single conidium under the stereoscopic microscope. Monoconidial cultures were allowed to grow for 8 days at 24 ± 2ºC under white light. Different isolates of *C. gloeosporioides* were selected on the basis of the following characteristics: a) color and growth habit of colonies, b) formation of mycelium and acervuli (Gunnell and Gubler, 1992; Katan, 2000), c) production of conidia, and d) origin of samples (type of symptom and organ affected). Fifteen isolates of *C. gloeosporioides* were selected: four from anthracnose of leaves (H1, H2, H3, and H4), five from anthracnose of fruits (F1, F2, F3, F4, and F5), and six from severe symptoms of anthracnose referred to as "blight" (T1, T2, T3, T4, T5, and T6) (Table 1).

Preparation of inoculum. The biomass of each isolate of *C. gloeosporioides* was increased in five Petri dishes on PDA medium, under the conditions described previously. After 8 days, 10 mL of sterile distilled water containing Tween 20 (0.1%) were added to each dish, which was then shaken to dislodge conidia. The number of conidia of each isolate was estimated from three sub-samples in a haemocytometer (Table 1) and conidial suspensions of each isolate were prepared by adjusting the concentrations with sterile distilled water to 1 x 10^5 conidia per mL for subsequent inoculation onto 15 day-old leaves of mango in the laboratory.

Inoculation of isolates of *C. gloeosporioides* onto leaves of mango in the laboratory. Fifteen day-old leaves of mango cv. Haden of orange-green color were removed from shoots of mango trees and taken for treatment to the laboratory in December, 2001. There, they were washed in sterile distilled water and placed in plastic boxes. Afterwards, each of the 15 isolates was used separately to inoculate a half leaf by deposition of 0.1 mL of a conidial suspension (1 x 10^5 per mL) at four points; the uninoculated halves of each leaf served as controls. To encourage the development of the fungus, paper dampened with sterile water was added to the boxes, which were then sealed in plastic bags to maintain relative humidity above 95%. A datalogger was placed inside one of the boxes to monitor the temperature and relative humidity during the bioassay. Each mango leaf was considered as an experimental unit and there were five replicates of each treatment, although only three were used for digital evaluation of disease severity by computer.

Variables assessed. The main parameter considered was

Table 1. Variation in conidia production and disease severity caused by 15 isolates of *Colletotrichum gloeosporioides* inoculated onto leaves of mango (*Mangifera indica*) cv. Haden under controlled conditions in the laboratory.

Isolate	Conidia per mL	Severity (%)
T1	652,000 d[z]	4.94 d[z]
T2	4,548,000 a	0.01 g
T3	1,796,000 c	5.52 de
T4	1,720,000 c	7.96 cd
T5	680,000 d	9.04 c
T6	16,000 e	2.39 efg
F1	6,000 e	0.02 g
F2	22,000 e	1.17 fg
F3	1,928,000 c	19.16 a
F4	2,156,000 c	13.55 b
F5	34,000 e	5.47 de
H1	294,000 de	0.11 g
H2	2,764,000 b	3.76 ef
H3	576,000 d	1.16 fg
H4	598,000 d	3.80 ef
r^2	0.98	0.97
LSD	535911	3.39

[z]Values with the same letter in the same column are not significantly different (Tukey, $p < 0.05$). r^2 = Coefficient of determination. LSD = Least significant difference.

severity (lesion size relative to that of the leaf) and this was used to classify the degree of virulence of each isolate. The severity was evaluated by means of digital images and the isolate that showed the greatest severity of anthracnose was considered the most virulent.

Digital evaluation of severity. Six days after inoculation, three mango leaves showing symptoms of anthracnose were scanned for each of the 15 isolates of *C. gloeosporioides*. JPEG images of each leaf were captured individually. The program Photo Magic was used to transform images from a format of 256 colors to one of only three. Red was used to color disease symptoms, light blue for healthy areas of leaves, and white for the background of the images. Once transformed, images were saved with the extension *.BMP to avoid any contamination with other colors, and thus permit a more exact interpretation of severity. The program Imagen Tool was used to modify the three colors generated (red, blue, and white), first to grey, black, and white, and then to just two colors (black and white) by combining the black and grey. This same program was used to measure the areas of black, grey, and white in the images by counting the number of pixels of each area. Then, by difference, the percentage of diseased tissue was obtained as a proportion of the total area of each leaf inoculated with each of the 15 isolates.

Statistical analysis. The experimental unit was one leaf with three replicates and a randomized block design was used. Data from the test was compared by means of analysis of variance followed by a Tukey test ($p = 0.05$).

Preparation of isolates for RAPD analysis. Monoconidial cultures were prepared from the 15 isolates that had been evaluated and classified as: very virulent (VV, more than 8% anthracnose severity), virulent (V, 5-8% anthracnose severity), moderately virulent (MV, 1-5% anthracnose severity) and scarcely virulent (SV, less than 1% anthracnose severity). Cultures were transferred to flasks containing 200 mL of liquid medium (potato-dextrose) and agitated constantly under white light at 24 ± 2ºC for 8 days.

Extraction of DNA and polymerase chain reaction (PCR). Total DNA was extracted by the method of Kelly and Alcalá-Jimenez (1994) slightly modified. A 0.1 g (fresh weight) sample of filtered mycelium was macerated in liquid nitrogen. The pulverized mycelium was transferred to an Eppendorf tube and 600 µL of lysis solution (10 mM Tris-HCl pH 8.0; 250 mM EDTA pH 8.0; proteinase K (Sigma) 200 µg mL^{-1}; Triton-X100 0.5% v/v) was added. The tube was then sealed with parafilm and incubated overnight at 37ºC with constant agitation. To each tube then was added 0.15 vol. of sodium chloride (1.5 M), 0.7 vol. of phenol (equilibrated with Tris pH 8.0) and 0.3 vol. of chloroform. Contents of the tubes were mixed by inversion and centrifuged in an Eppendorf microcentrifuge at 1,431 g at 4ºC for 20 min. The supernatant was transferred to a clean tube, to which 4 µL of RNAase was added, and incubated at 37ºC for one hour. Afterwards, 1 vol. of chloroform was added and the mixture homogenized by inversion. It was centrifuged at 1,431 g at 4ºC for 40 min, and the supernatant was decanted. The pellet obtained was washed with ethanol (70%) and evaporated for 30 min at 37ºC. Once dried, it was dissolved in 80 µL of TE (10 mM Tris-HCl pH 7.6, 1 mM EDTA pH 8.0) for subsequent quantification. The quantification of the DNA was performed in a spectrophotometer (model Lambda BIO 10, Perkin-Elmer). The reading was made at a wavelength of 260 nm, which permitted calculation of the concentration of nucleic acids.

Establishment of the conditions for RAPD analysis. The work was based on the standardization of conditions for the PCR reaction by Cámara (2001). The amplification was performed in a final volume of 25 µL, which contained 80 ng of genomic DNA of *C. gloeosporioides*, 20 pmol of primer, 200 µM of dNTPs and 2 Units of DNA polymerase in buffer solution ($MgCl_2$ mM, Tris-HCl pH 8.0), plus 25 µL of mineral oil to avoid evaporation of the sample. Amplifications were done in a thermocycler (Perkin-Elmer), using the following conditions: 5 min at 94°C, 45 cycles of 1 min at 94°C, 1 min at 37°C, and 1 min at 72°C. The reaction was completed with an extension period of 7 min at 72°C. The 10-mer primers used corresponded to series A of Operon Technologies, Inc. After completion of the PCR, the amplified products were separated on an agarose gel (1.4%) in TAE buffer (Tris-HCl 0.1 M, EDTA 0.5 M, pH 8.0). Electrophoresis was at 85 volts for 2 h. At the conclusion of the electrophoresis, the gel was stained with ethidium bromide and visualized in a UV-transilluminator (Gel Doc 2000, Bio-Rad).

Statistical analysis. From the band patterns obtained by electrophoresis, a binary matrix was constructed; values were assigned to indicate the presence (1) or absence (0) of bands. Using the program NTSyS pc. 2.0, a similarity matrix was constructed using the coefficient of Dice. Finally, a cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA). To show the genetic relationships between isolates graphically and to evaluate the quality of the cluster analysis, a matrix of cophenetic values was constructed from the similarity matrix.

RESULTS AND DISCUSSION

Six days after inoculation, the isolates of *C. gloeosporioides* were classified on the basis of the severity of symptoms they caused, as: very virulent (VV), virulent (V), moderately virulent (MV), and scarcely virulent (SV) (Fig. 1). Isolates F3, F4, and T5 showed the greatest severity of anthracnose with 19.2, 13.5, and 9.0% damage, respectively, and showing a, b, and c grouping in the statistical analysis; accordingly, these were classified as very virulent. T4, T3, F5, and T1 had statistical grouping c and d, and damage severities of 8.0, 5.5, 5.5, and 5.0%, respectively, so they were classified as virulent. The isolates classified as moderately virulent were H4 (3.8%), H2 (3.8%), T6 (2.4%), F2 (1.2%), and H3 (1.2%), whose statistical groupings were ef, efg and fg. Due to the low disease severity and a statistical grouping of g for isolates H1 (0.1%), F1 (0.02%), and T2 (0.01%), these were classified as scarcely virulent (Table 1). Genetic variation among isolates was also

evident when the reproductive potential was compared; from the epidemiological point of view and management of the disease, such differences could be relevant since the reproductive potential might influence the competitive capability of the pathogen (Martens and Dyck, 1989; Shaner *et al*., 1992). The DNA banding patterns obtained with the various primers in RAPD analysis showed polymorphism between the 15 isolates of *C. gloeosporioides* (Fig. 2 and Table 2). Isolates F1 and F5 showed most of the fragments amplified by the primers used, and more than the other isolates. The dendrogram obtained from the cluster analysis (Fig. 3) showed evidence of a correlation between the data on pathogenicity and virulence, and the separation into four groups. The most virulent group was clearly separated from the main groups. The value of the coefficient of co-phenetic correlation was r = 0.84533, which indicates a good fit. With the exception of isolate F2, it was also evident that all isolates obtained from leaves were separated from those of fruits, and the isolates causing foliar blight were separated from those causing foliar anthracnose; also, there was a separation between isolates causing anthracnose on leaves from those of fruits. The differences in virulence obtained in this study agree with that reported by Takustu and Rego (1976) and Katan (2000) for *C. gloeosporioides*. Alahakoon *et al*. (1994) indicated that the virulence of isolates of *C. gloeosporioides* seems to depend on inoculum density; however, in this work the concentration of inoculum was kept constant, so the differences in virulence found in this investigation are probably due to the existence of more than one race or special form of *Colletotrichum* (Fitzell, 1979; Ploetz and Prakash, 2000; Prusky, 1994). Such behaviour could be due to the

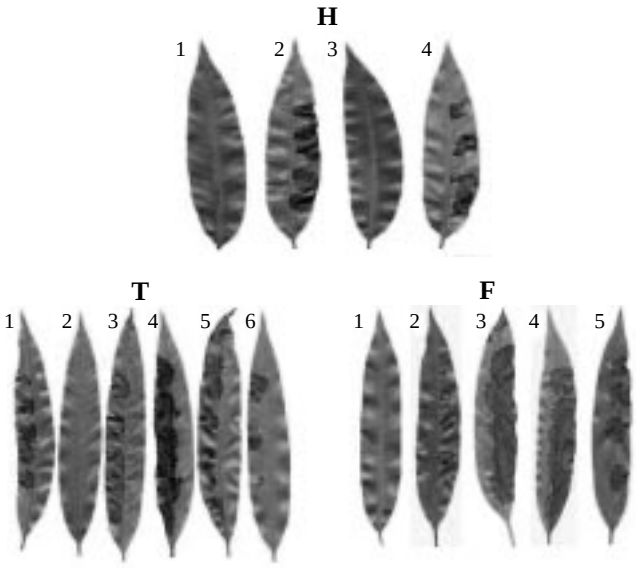

Fig. 1. Symptoms induced by isolates of *Colletotrichum gloeosporioides* on mango (*Mangifera indica*) leaves six days after inoculation. In general, isolates of group H were less virulent than T and F, being the last group the most virulent.

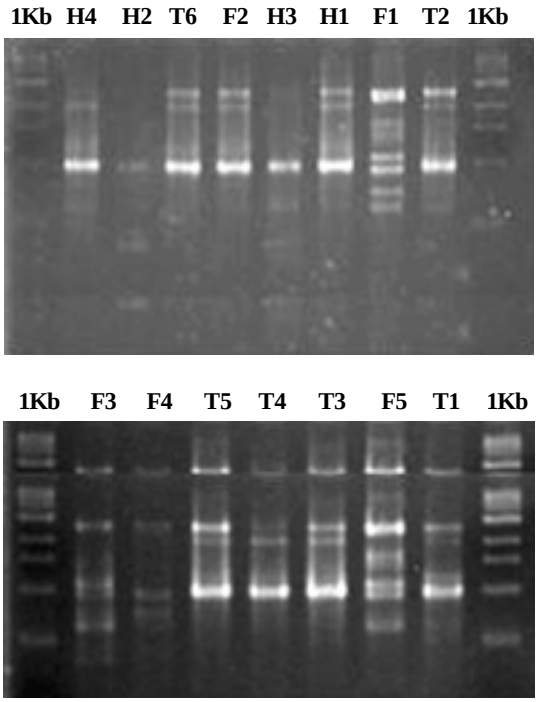

Fig. 2. RAPD patterns of 15 isolates of *Colletotrichum gloeosporioides* amplified using primer A-16.

frequent application of fungicides with a single mode of action (Verdecia, 1999), which could encourage the emergence of more aggressive races that are more difficult to control. Our results suggest the existence of considerable genetic variability among the 15 isolates of *C. gloeosporioides* that we obtained and analyzed, which was corroborated by the results of the RAPD analysis. The data support the report by Manners *et al.* (1992), who detected genetic variation between two populations of this species. Although this analysis grouped most of the isolates with scarce and moderate virulence together, there is a clear separation of isolates classified as very virulent (F3 and F4) from the rest of the isolates (Fig. 3). Isolates T5 (very virulent) and F1 (scarcely

Table 2. Fragments amplified by RAPD of differents isolates of *Colletotrichum gloeosporioides*.

Primer	Fragments amplified	Polymorphism fragments	Polymorphism (%)
A02	13	10	76.9
A03	13	9	69
A04	17	13	76.47
A05	9	6	66.6
A06	27	18	66.6
A07	19	16	84.2
A08	17	13	76.47
A09	14	8	57.14
A10	10	4	40
A16	7	9	66.6

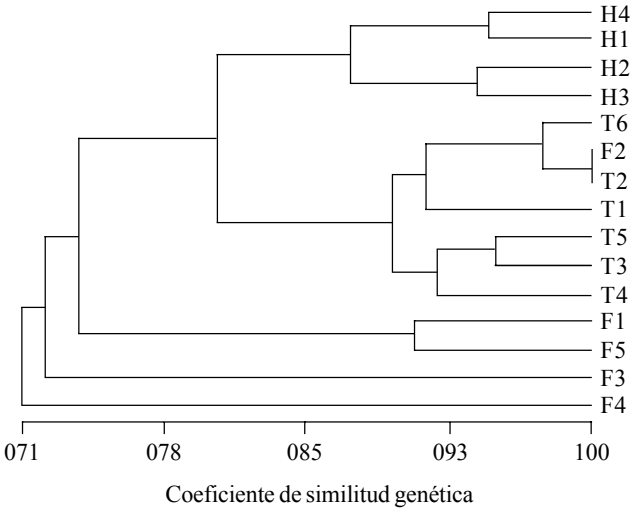

Fig. 3. Dendrogram obtained from consideration of 34 bands found in DNA patterns generated by RAPD analysis, using the UPGMA method and the coefficient of Dice.

virulent) appear to be associated with groups from others of their category. *Colletotrichum gloeosporioides* shows considerable morphological and pathogenic variation, which has been difficult to define using morphological characters (Cox and Irwing, 1988) and because it infects a wide range of hosts. Because we know so little of the genetic relations within the species (Manners *et al*., 1992; Sutton, 1992) it is necessary to complete molecular studies that show the genetic differences between races of the fungus. This could establish a base for future investigations on the mechanisms of genetic variation in this type of fungus. RAPD analysis was useful to detect genetic variation among isolates of *C. gloeosporioides* studied.

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