Centro de Investigación Científica de Yucatán, A.C.

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ESTUDIO DE LA DIVERSIDAD GENÉTICA Y DE LA VARIABILIDAD PATOGÉNICA ENTRE AISLADOS DE *Colletotrichum truncatum* (Syn. *C. capsici*) DE DIFERENTES HOSPEDEROS

Tesis que presenta

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RECONOCIMIENTO

Por medio de la presente, hago constar que el trabajo de tesis titulado **"Estudio de la diversidad genética y de la variabilidad patogénica entre aislados de *Colletotrichum truncatum* (Syn. *C. capsici*) de diferentes hospederos"** fue realizado en el laboratorio **GeMBio** del Centro de Investigación Científica de Yucatán, A.C. bajo la dirección del **Dr. Inocencio Higuera Ciapara** y de la **Dra. Daisy Pérez Brito**, dentro de la **Opción Biotecnología**, perteneciente al Programa de **Posgrado en Ciencias Biológicas** de este Centro.

Atentamente,

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Mérida, Yucatán, México; a 19 de enero de 2015

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Claudia Guadalupe Torres Calzada

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"Me gustaría que la vida no fuera tan corta –pensó–.
Aprender un idioma toma tanto tiempo,
así como todas las cosas sobre las que uno quisiera saber".

–J. R. R. Tolkien

Para Arturo,
el mejor compañero de aventuras...

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RESUMEN

Colletotrichum truncatum (Syn. *C. capsici*) es uno de los agentes causales de la antracnosis en papaya, chile y Jatropha, en la región Sur-Sureste de México. Las pérdidas causadas por este patógeno se producen principalmente como una reducción directa en la calidad y cantidad del producto de la cosecha. Por lo general, el hongo infecta a más de una parte de la planta, provocando que la enfermedad prevalezca durante el crecimiento de la misma. En la actualidad existe poca información sobre la diversidad patogénica y molecular de esta especie. Por lo tanto, el objetivo de este estudio fue revelar la diversidad morfológica y genética del patógeno. Para esto, una combinación de caracteres morfológicos, técnicas moleculares y pruebas de patogenicidad se utilizaron para caracterizar 108 aislamientos, obtenidos de 18 localidades, en cinco estados productores de estos cultivos. Los aislamientos de *C. truncatum* formaron seis grupos morfológicos diferentes y la caracterización molecular utilizando marcadores ISSR los separó de acuerdo con su hospedero o su origen geográfico. Este es el primer estudio que revela la gran variabilidad fenotípica y genética de este patógeno. También se muestra por primera vez las diferencias en agresividad de *C. truncatum* y su falta de especificidad hacia los hospederos.

Durante años, el control químico se ha utilizado ampliamente para el tratamiento de la antracnosis. Sin embargo, la aparición de cepas resistentes a los fungicidas comúnmente empleados es cada vez más frecuente. Veinte aislamientos representativos se evaluaron *in vitro* contra cuatro fungicidas, con el fin de determinar su sensibilidad. Se encontró que los fungicidas Tecto y Amistar no fueron eficientes en el control de este patógeno. Las secuencias de nucleótidos del gen TUB-2 se caracterizaron, evidenciando una sustitución de un ácido glutámico por una alanina en la posición 198 para los aislamientos altamente resistentes. El conocimiento del desarrollo de la resistencia, así como de la diversidad genética del patógeno, tiene implicaciones importantes para el mejoramiento genético de los hospederos, en la búsqueda de resistencia a antracnosis y así mismo, pone de manifiesto la necesidad de monitorear la sensibilidad del patógeno, hacia los fungicidas como una actividad esencial para el control de la enfermedad.

ABSTRACT

Colletotrichum truncatum (Syn. *C. capsici*) is one of the causal agents of anthracnose in papaya, pepper and Jatropha in the South-Southeast region of Mexico. The losses caused by this pathogen occur mainly as a direct reduction in the quality and quantity of the harvested product. Usually, the fungus infects more than one part of the plant, causing the prevalescence of the disease during the growing season. There is currently little information concerning the pathogenic and molecular diversity of this species. Thus, the objective of this study was to reveal the morphological and genetic diversity of the pathogen. For that, a combination of morphological characters, molecular techniques and pathogenicity tests were used to characterize 108 isolates, collected from 18 fields from five producer states. *C. truncatum* isolates were divided into six different morphological groups and ISSR markers were efficient in separating the isolates according to their host or geographical origin. This is the first study that reveals the extensive phenotypic and genetic variability of this pathogen. Also we exhibit for first time the differences in aggressiveness of *C. truncatum* and its lack of host specificity.

For years, chemical control has been extensively used for the management of anthracnose. However, appearance of isolates resistant to the most commonly employed fungicides is increasingly widespread. Twenty representative isolates were tested *in vitro* against four fungicides in order to determine their sensitivity. Tecto and Amistar fungicides were proven to be inefficient in the control of the pathogen. The nucleotide sequences of the TUB-2 gene were characterized, revealing a glutamic acid to alanine substitution at position 198 for highly resistant isolates. Knowledge of the development of resistance, as well as the genetic diversity of the pathogen have important implications for the genetic improvement of the hosts, in the search for anthracnose resistance and also, highlight the need of monitoring fungicide sensitivity as an essential activity for the control of the disease.

CAPÍTULO I. ANTECEDENTES GENERALES

1.1 INTRODUCCIÓN

Dentro de las enfermedades en plantas causadas por hongos, la antracnosis, ocasionada por las especies del género *Colletotrichum*, es una de las más comunes y más importantes, debido a las pérdidas económicas que genera en diferentes cultivos alrededor del mundo, principalmente en regiones con climas tropicales y subtropicales (Freeman, 2000). Las especies de este género se caracterizan por tener un amplio rango de hospederos, que incluyen cereales, leguminosas, ornamentales y árboles frutales (Bailey y Jeger, 1992). Dentro de estos hospederos, el chile (*Capsicum* spp.), considerado uno de los cultivos más importantes en México, es afectado severamente por la antracnosis. En países como India y Tailandia, se han reportado pérdidas mayores al 50% en la producción de este cultivo (Pakdeevaraporn *et al.,* 2005) identificando a *Colletotrichum capsici* como el patógeno más importante (Montri *et al.*, 2009). Del mismo modo, de manera reciente se reportó la presencia de este hongo en cultivos de papaya en Yucatán (Tapia-Tussell *et al.,* 2008) y en Florida (Tarnowski y Ploetz, 2010), así como en cultivos de piñón (Torres-Calzada *et al.*, 2011).

La incidencia y severidad de la enfermedad pueden incrementarse si existen riesgos de infección cruzada, en la cual, ciertos cultivos, pueden actuar como hospederos alternos, sirviendo de reservorio del inóculo del patógeno. En regiones tropicales, como la de México, el riesgo de infección aumenta principalmente debido a los sistemas agrícolas que propician la proximidad de diferentes cultivos en el campo. El hecho de que algunas especies de *Colletotrichum* no muestran especificidad hacia determinados hospederos resulta potencialmente riesgoso, como se ha demostrado para la especie *C. acutatum* (Freeman *et al.*, 1998).

La correcta identificación del patógeno, es un factor importante que permite la implementación de medidas de control adecuadas para el manejo de la enfermedad, así como el establecimiento de programas de mejoramiento genético de los cultivos. Con base en lo anterior, los criterios utilizados actualmente para la identificación de patógenos fungosos, se basan tanto en las características morfológicas de la especie,

como en el empleo de diversas herramientas moleculares, que incluyen técnicas como RAPD, PCR-RFLP, AP-PCR y análisis de la secuencia de nucleótidos de diferentes regiones y genes (Johnston y Jones, 1997; Sreenivasaprasad y Talhinhas, 2005). Los estudios que combinan el uso de las técnicas moleculares con la identificación tradicional, proveen información más confiable sobre el patógeno, lo que permite monitorear la enfermedad en poblaciones del (los) cultivo(s) hospedero(s) y favorece el mantenimiento de la sanidad vegetal de los cultivos afectados.

En trabajos anteriores (Tapia-Tussell *et al.,* 2008; Torres-Calzada *et al.*, 2011), se detectó que *Colletotrichum truncatum* (Syn. *C. capsici*) se encontraba en cultivos que no han sido reportados como hospederos, sin embargo es necesario realizar estudios más profundos enfocados a la caracterización de este patógeno en esos cultivos. Por todo lo anterior, el objetivo de este trabajo fue estudiar la diversidad genética de esta especie y determinar si existe variabilidad patogénica entre los aislados que fueron colectados en los diferentes hospederos.

1.2 ANTECEDENTES

Colletotrichum es uno de los géneros de hongos fitopatógenos más importantes en todo el mundo, especialmente en regiones tropicales y subtropicales, ya que causa pérdidas económicas significativas en los cultivos que afecta. La enfermedad es conocida comúnmente como antracnosis y puede encontrarse en diferentes partes de la planta durante cualquier etapa de su crecimiento (Roberts *et al.*, 2004); sin embargo, las lesiones del fruto son las más comunes y las que generan las mayores pérdidas económicas (Than *et al.*, 2008; Ventura *et al.*, 2004; Freeman y Katan, 1997). Las pérdidas son causadas por los efectos de la enfermedad en las diferentes etapas del desarrollo del hospedero, ocasionando por ejemplo, daño en las inflorescencias y la caída prematura del fruto, disminución de la tasa fotosintética y otros trastornos fisiológicos. Del mismo modo, su capacidad para causar infecciones latentes o quiescentes lo ubican dentro de los patógenos poscosecha más importantes (Jeffries *et al.*, 1990).

1.2.1 Características morfológicas del género *Colletotrichum*

Las especies de este género presentan un micelio enramado, septado, con una coloración que puede ser desde hialina hasta castaño pálido. Tienen acérvulos separados o confluentes en forma de disco, cerosos, subepidermales, epidermales y subcuticulares, típicamente con setas no ramificadas, de ápice agudo y pared gruesa, localizadas en los bordes o entre los conidióforos, los cuales son simples, elongados, con conidios hialinos, ovoides o falcados. Los conidios se producen en masas mucilaginosas, a menudo rosadas, bastante conspicuas y típicamente hundidas, con un contorno irregular (Barnett y Hunter, 1998).

1.2.2 Síntomas de la enfermedad

Los síntomas de la antracnosis involucran la aparición de lesiones húmedas, blandas, ligeramente hundidas que se tornan oscuras y que pueden cubrir la superficie del tejido, volviéndose de color salmón-naranja debido a la presencia del cuerpo fructífero del hongo. Comúnmente se observan anillos concéntricos de acérvulos y en algunos casos, las lesiones son de color negro debido a la formación de setas y la presencia de esclerocios (Freeman *et al.*, 2001).

Estas lesiones se pueden encontrar en cualquier tejido de la planta, como hojas, peciolos y raíces, aunque son más comunes en tallos y en frutos. En las hojas, estos síntomas se perciben inicialmente como lesiones claras que eventualmente se vuelven necróticas, provocando una disminución en la tasa fotosintética de la planta (Abang, 2003). La localización de la infección puede variar dependiendo tanto del hospedero como de la especie patógena (Peres *et al.*, 2005).

1.2.3 Proceso de infección

El proceso de infección de *Colletotrichum,* en la superficie de la planta, es esencialmente el mismo para todas las especies. En la mayoría de los casos, el inóculo llega al hospedero por medio del agua o dispersado por el aire, entonces los conidios se adhieren a la cutícula de la planta y germinan en las siguientes 24h, produciendo un tubo germinal, que crece usualmente de 10 a 20 µm antes de formar el apresorio terminal, que penetrará la cutícula. A partir de este apresorio, emerge posteriormente una estructura llamada clavija de penetración (Diéguez-Uribeondo *et al.*, 2005) que permite el paso directo a la cutícula, aunque existen reportes de penetración indirecta a través de estomas o por heridas. Las principales diferencias en el proceso de infección entre especies de *Colletotrichum* sólo pueden apreciarse después de la penetración, donde se distinguen dos tipos de estrategias: hemibiotrófica intracelular y necrotrófica intramural. Las especies de *Colletotrichum* que llevan a cabo la primera estrategia, se caracterizan por establecer inicialmente un proceso de infección mediante una fase biotrófica, en la cual se percibe la aparición de una hifa primaria intracelular, cambiando posteriormente a una fase necrotrófica en la cual se desarrolla una hifa secundaria, que se ramifica a lo largo del tejido del hospedero. Mientras que en la segunda estrategia de infección, una vez que el patógeno ha penetrado la cutícula del hospedero, crece por debajo de ella sin penetrar a las células, desarrollándose entre las regiones periclinal y anticlinal de las paredes de las células epidermales (O´Connell *et al.*, 2000).

Durante el proceso de infección, los factores ambientales son de gran importancia en el desarrollo de la enfermedad. La relación entre la intensidad de la lluvia, la geometría del fruto y la dispersión del inóculo causan diferentes grados de severidad (Than *et al.*, 2008). La infección ocurre cuando el clima es cálido y húmedo (Agrios, 2005). La temperatura óptima para esto es alrededor de los 27°C, con una humedad relativa alta

(más del 80%); sin embargo, dicha infección puede ocurrir a temperaturas entre 10 y 30°C (Roberts *et al.*, 2004).

1.2.4 Identificación y caracterización de *Colletotrichum* spp.

Las especies del género *Colletotrichum* presentan diferencias con respecto a la morfología de la colonia, el color, el tamaño y la forma de los conidios, la temperatura óptima de crecimiento, la presencia o ausencia de setas, la existencia del teleomorfo y la sensibilidad a los fungicidas (Katan, 2000). Algunas de estas diferencias pueden ser utilizadas para su identificación; sin embargo, debido a la influencia del ambiente en la estabilidad de las características morfológicas, estos criterios no siempre resultan adecuados, por lo que ha sido necesario complementar la identificación morfológica con métodos de identificación y caracterización moleculares, los cuales han sido utilizados exitosamente para diferenciar entre especies de *Colletotrichum* de diversos hospederos (Giblin *et al.*, 2009; Photita *et al.*, 2005).

Debido a las dificultades que representa identificar a las especies de este género por métodos morfológicos, el uso combinado de los métodos tradicionales con las técnicas de diagnóstico molecular proveen un enfoque más confiable para la identificación, ya que, a diferencia de los caracteres morfológicos, el ADN de la especie de interés no resulta influenciado por factores ambientales (Cannon *et al.*, 2000).

Recientemente, las herramientas moleculares han sido empleadas para inferir las relaciones evolutivas de las especies de *Colletotrichum*. Existen diferentes regiones y genes que pueden ayudar a la identificación correcta de una especie; sin embargo, no es conveniente realizar la filogenia basándose sólo en un gen o región determinada, principalmente si se desea analizar especies evolutivamente cercanas (Crouch *et al*, 2009a). Se han obtenido mejores resultados en los estudios de las secuencias de varios genes utilizando un análisis multilocus y con esto se han logrado clarificar algunas confusiones que existían en el género e incluso, esta estrategia ha servido para la introducción de seis nuevas especies a *Colletotrichum* (Crouch *et al.*, 2009b).

1.2.4.1 Análisis de la secuencia del ADN ribosomal

Los ribosomas son complejos moleculares encargados de sintetizar proteínas a partir de la información genética contenida en el ARN mensajero (ARNm). Estos organelos están formados por ARN ribosomal (ARNr) y por proteínas. Estructuralmente tienen dos subunidades.

Tanto los ARNr como las subunidades de los ribosomas se suelen nombrar por su coeficiente de sedimentación en unidades Svedberg (S).

En eucariotas los ribosomas son 80S, divididos en dos subunidades de distinto tamaño. La subunidad mayor 60S contiene tres tipos diferentes de ARNr: 5S, 5.8S y 28S y tiene 49 proteínas; la subunidad menor 40S tiene una sola molécula de ARNr, 18S y 33 proteínas.

El ADN ribosomal es una secuencia de ADN de longitud variable, que codifica para el ARN ribosómico (Scorzetti *et al.*, 2002). El ADNr consiste en un segmento de ADN repetido en tándem compuesto por IGS, 18S, ITS1, 5.8S, ITS2, 28S y 5S.

Los espacios internos transcritos (ITS, por sus siglas en inglés) son fragmentos de ARN no funcionales situados entre los ARNr. Los espacios intergénicos (IGS, por sus siglas en inglés) son regiones de ADN que no son transcritas y que separan las múltiples copias que existen del ADNr. Durante la maduración del ARNr, los fragmentos ITS son eliminados y degradados, obteniéndose de este modo los ARNr 18S, 5.8S y 28S.

El bajo nivel de polimorfismo intraespecífico en la unidad de transcripción de ADNr, permite la caracterización de las especies usando sólo dicha secuencia y hace que este ADN sea útil para la comparación interespecífica. Además, las repeticiones de las diferentes regiones de codificación del ADNr muestran distintas tasas de evolución. Como resultado de ello, este ADN puede proporcionar información sobre casi cualquier nivel sistemático. De la misma manera, los ITS son, al momento, la región más utilizada para la identificación en el reino fungi, debido al grado de variación que presenta en su secuencia, a nivel interespecífico.

El ADN ribosomal ha servido en estudios de identificación de especies por sus numerosas ventajas, como su amplia distribución en el genoma y su variabilidad. Debido a esto, la región ITS, localizada entre las subunidades pequeña y grande del ADNr, se han empleado exitosamente en estudios de filogenia y caracterización en el género *Colletotrichum* (Van Hemelrijck *et al.*, 2010; Lotter y Berger, 2005).

Del mismo modo, el análisis de esta secuencia ha permitido la identificación de varias especies de *Colletotrichum* como *C. lindemuthianum, C. fragarie, C. boninense, C. gloeosporioides* y *C. capsici* (Masyahit *et al.*, 2009; Marulanda *et al.*, 2007; Wang *et al.*,

2008; Moriwaki *et al.*, 2002) a partir de diferentes hospederos, de una manera más rápida y sencilla que mediante el empleo de otras técnicas moleculares o morfológicas.

1.2.4.2 Marcadores moleculares SSR e ISSR

Los marcadores moleculares son ampliamente utilizados en genética porque permiten evidenciar polimorfismos en la secuencia del ADN entre dos individuos, sin importar que éstas modifiquen o no su fenotipo (Ribaut y Hoisington, 1998). Entre sus principales aplicaciones se incluyen la obtención de huellas genéticas de individuos, variedades y poblaciones, el análisis de la estructura y diversidad genética en poblaciones naturales y de mejoramiento (Prince *et al.*, 1992; Azurdia *et* al., 1995) y el establecimiento de relaciones filogenéticas entre diferentes individuos y especies.

Los marcadores moleculares más usados son los basados en la reacción en cadena de la polimerasa (PCR), dentro de los cuales destacan los microsatélites y los intermicrosatélites (Ribaut y Hoisington, 1998).

Las Secuencias Simples Repetidas (SSRs) o microsatélites, son regiones hipervariables, dispersas a través del genoma eucariótico, que se componen de secuencias de unos pocos pares de bases (uno a seis) repetidas muchas veces en "motivos" específicos como son (AT)n, (GT)n, (ATT)n ó (GACA)n, etc. Las secuencias de ADN que flanquean a los microsatélites son generalmente conservadas entre individuos de la misma especie, esto ha permitido la selección de oligonucleótidos iniciadores que a través de la reacción de PCR, pueden amplificar los SSRs presentes en todos los genotipos. La variación en el número de repeticiones en tándem resulta en diferentes longitudes de los productos de amplificación, por lo que dichas repeticiones son altamente polimórficas. Su herencia es codominante (Aravanopoulos 2003; Pica *et al.* 2004).

Por otra parte, las Intersecuencias Simples Repetidas (ISSR) son marcadores en los que se emplea un único iniciador, compuesto de una secuencia microsatélite que puede o no estar anclada en el extremo 3' ó 5', por dos a cuatro nucleótidos que son a menudo arbitrarios y degenerados. No se requiere previo conocimiento de la secuencia del genoma para el diseño del iniciador, lo que facilita su uso en estudios de diversidad genética (Reddy *et al.*, 2002; Zhao *et al.*, 2006; Meng *et al.*, 2011).

1.2.4.3 Marcadores moleculares AP-PCR y RAPDS

Welsh y McClelland (1990) describieron la técnica denominada Arbitrary Primed (AP-PCR) que se basa en la amplificación por PCR del ADN genómico, con iniciadores que tienen secuencias repetitivas y adyacentes denominadas micro (2 a 9 nucleótidos) o minisatélites (de 15 a 30 nucleótidos), distribuidas a lo largo del genoma del organismo de interés. Estas repeticiones arbitrarias revelan polimorfismos entre individuos de la misma especie, por lo tanto proporcionan resultados que permiten discriminar especies estrechamente relacionadas e identificar organismos hasta el nivel de subespecie (Libkind, 2007; Orberá, 2004; Ralph y McClelland, 1998; William, 1996).

La técnica de RAPDs (Random Amplified Polymorphism DNA, por sus siglas en inglés), ha sido una de las metodologías más utilizadas en la PCR para diferentes propósitos; se basa en el uso de pequeños iniciadores de secuencias aleatorias que se "pegan" a distintos sitios del genoma, si es que existen diferentes "blancos" para ellos. El producto de la amplificación podrá ser visualizado con electroforesis. Los marcadores de RAPDs proporcionan un método rápido para generar mapas genéticos y analizar poblaciones, especies de un género o diferentes géneros de una familia (Valadez y Kahl, 2000).

Las técnicas de AP-PCR y RAPDs han sido muy usadas para la identificación y caracterización de especies de hongos. Freeman *et al.* (2000) utilizaron la técnica de AP-PCR para diferenciar *C. acutatum* de *C. gloeosporioides* en anémona coronaria. La técnica de RAPD ha sido empleada para estudios de variabilidad en *C. capsici* (Sharma *et al.*, 2005) y para la detección de *C. lindemuthianum* en frijol (Wang *et al.*, 2008). Estas técnicas, dentro del género *Colletotrichum*, han sido empleadas principalmente para diferenciar a nivel de subespecies; sin embargo, a pesar de su confiabilidad, requieren mucho tiempo para su optimización y para el análisis de los resultados, lo cual es complicado al trabajar con un gran número de muestras.

1.2.5 *Colletotrichum truncatum* (Syn. *C. capsici*)

Colletotrichum capsici (Syd.) Butler and Bisby fue descrito por primera vez como *Vermicularia capsici* en 1913, por Sydow cuando fue aislado de frutos de chile (*Capsicuum frutescens* L.) colectados por W. McRae en India en 1912, y desde

entonces ha sido reportado como patógeno en 121 géneros de plantas (Farr *et al.*, 2009) en diferentes partes del mundo.

Esta especie se caracteriza por presentar acérvulos elípticos o circulares, de 85 a 245 µm de diámetro, distribuidos concéntricamente alrededor de la lesión, subepidermales, con masas de esporas de color gris. Posee setas rígidas, abundantes, de color café oscuro, con un tamaño de 70-135 µm de longitud por 5 µm de ancho, las cuales presentan de 1 a 5 septos. Los conidios alcanzan tamaños de 17-26 µm de longitud por 3-4 µm de ancho, son hialinos y falcados con ápices en forma de flecha (Shenoy *et al.*, 2007).

Se reportó que existen tres patotipos de *C. capsici*, los cuales mostraron diferentes niveles de agresividad al realizar los estudios de patogenicidad en nueve genotipos de chile (Montri *et al.*, 2009). Sin embargo, este estudio se limitó a diferenciar entre aislamientos de *C. capsici* obtenidos de sólo uno de sus hospederos (*Capsicum* spp.). Hasta el momento, se desconoce si existe variabilidad patogénica entre los aislamientos de esta especie que se encuentran infectando otros cultivos hospederos, como ha sido reportado previamente para *C. gloeosporioides* y *C. acutatum* (Denoyes-Rothan *et al.*, 2003).

Damm *et al.* (2009) hicieron una reclasificación de las especies de *Colletotrichum* con conidios falcados. A partir de este trabajo, *Colletotrichum truncatum* se estableció como basiónimo de las especies *Colletotrichum dematium* f. sp. *truncatum*, *Vermicularia capsici*, *Colletotrichum capsici*, *Steirochaete capsici* y *Colletotrichum curvatum*. Debido a que *Vermicularia truncata* (= *C. truncatum*) fue descrita antes que *C. capsici* y *C. curvatum*, ambas especies son consideradas actualmente como sinonimias de la primera.

1.2.6 Hospederos de *C. truncatum*

Las enfermedades causadas por *Colletotrichum* spp. se presentan en un amplio rango de hospederos distribuidos en todo el mundo. Cereales, pastos, plantas ornamentales, leguminosas y árboles frutales pueden ser afectados seriamente por este patógeno. Las especies de *Colletotrichum* causan síntomas tales como necrosis del tejido, hundimiento del fruto en el sitio de la lesión y formación de anillos concéntricos. La enfermedad se presenta en todos los estados de crecimiento, desde la germinación de las semillas hasta las plantas adultas, afectando el desarrollo de los cultivos en el

campo. O bien, puede presentarse en los frutos en maduración durante su almacenamiento en la etapa poscosecha (Roberts *et al.*, 2004).

En el campo, el hongo puede permanecer en ausencia de su hospedero sobreviviendo como saprófito en los restos de las cosechas. Su actividad como patógeno inicia otra vez con el establecimiento de los nuevos cultivos, infectando a las plántulas después de su germinación.

A pesar de que algunos taxones parecen estar un poco más restringidos en cuanto a sus hospederos, limitándose a ciertas familias, géneros, especies, o incluso cultivares dentro de éstas, muchos otros son capaces de infectar a varias especies. Por lo tanto, la diferenciación entre especies basada en el hospedero del cual es aislado no es confiable, sobre todo cuando se trata de patógenos tan importantes como *C. gloeosporioides* y *C. graminicola*, que infectan un amplio rango de plantas (Howard *et al.*, 1992).

El potencial para causar infección cruzada se ha reportado en diferentes especies del género, en una variedad de hospederos vegetales, bajo condiciones artificiales de inoculación (Freeman *et al.*, 1998). Por ejemplo, *C. gloeosporioides* aislado de siete cultivos diferentes, fue capaz de realizar la infección cruzada en todos ellos, siendo la única limitante la densidad del inóculo (Alahakoon *et al.*, 1994). Otra de las especies que ha sido reportada como patógena de un gran número de hospederos es *C. capsici*. En México, se ha encontrado a esta especie en cultivos de chile, papaya y Jatropha (Tapia-Tussell *et al.*, 2008; Torres-Calzada *et al.*, 2011), ocasionando pérdidas que van desde un 10% hasta un 60% dependiendo del cultivo y la época del año.

1.2.6.1 *C. truncatum* en *Capsicum* spp.

Capsicum es un género de plantas nativo de las regiones tropicales de América, perteneciente a la familia de las Solanáceas. El género está constituido por aproximadamente 27 especies, de las cuales cinco han sido domesticadas: *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens* y *C. pubescens* y son cultivadas en diferentes partes del mundo. Las otras 22 especies, son silvestres y endémicas de los trópicos de América (Csilléry, 2006).

México es el primer exportador de chile verde a nivel mundial, mientras que Estados Unidos, Japón, Canadá, Reino Unido y Alemania son los principales consumidores. En el año 2013, se cultivaron 136 053.46 hectáreas, las cuales produjeron 2 294 399.97 toneladas de chile (SIAP, 2014).

Muchas enfermedades y desórdenes (bióticos y abióticos) pueden interferir en la producción del chile. Los hongos, nematodos y virus son los factores bióticos que más afectan al cultivo. Entre éstos, la antracnosis es una enfermedad fúngica muy común en el género *Capsicum*. La enfermedad fue reportada por primera vez en Nueva Jersey, E.U.A., en 1890 y actualmente se encuentra distribuida en América, Asia, Australia y África. Las especies de *Colletotrichum*, típicamente tienen un amplio rango de hospederos y pueden infectar otros cultivos de solanáceas, como el tomate y la papa. La incidencia de la antracnosis en chile se estima entre un 5 y un 75% (Hadden, 1989).

En chile, el patógeno causa ahorcamiento, muerte descendente y manchas en las hojas, además de los síntomas característicos del fruto. *Colletotrichum capsici* (Syd.) Butler & Bisby y *C. gloeosporioides* (Penz.) Penz. & Sacc. In Penz. son las dos especies que se reportan con mayor frecuencia. Sin embargo, también se ha reportado la presencia de *C. coccodes* (Wallr) Hughes y *C. acutatum* Simmonds (Manandhar *et al.*, 1995).

1.2.6.2 *C. truncatum* en *Carica papaya*

Carica papaya es una planta tropical herbácea y perenne, que pertenece a la familia *Caricacea*. Es originaria del sur de México y Centroamérica (Manshardt, 1992), y se cree que surgió como producto de la hibridación natural entre *Carica peltate* y otras especies silvestres (Purseglove, 1968). Los datos históricos indican que las semillas del fruto fueron llevadas hacia República Dominicana y las costas de Panamá, aproximadamente en 1525, y que a partir de entonces el cultivo se dispersó hacia otras islas del Caribe y hacia otras partes del mundo (Morton, 1987).

Actualmente, la papaya se cultiva en todas las regiones tropicales y subtropicales del planeta. En el 2014, India, Brasil y República Dominicana fueron los principales países productores de este cultivo (FAOSTAT 2014).

En México, la papaya es uno de los cultivos más importantes económicamente; es exportado a varios países, principalmente a Estados Unidos y Canadá. En el año 2013, el área cultivada de papaya fue de 16,367.50 hectáreas, con una producción de 764,514.40 toneladas (SIAP, 2014), posicionando a México como el sexto productor a nivel mundial de dicho cultivo en ese año (FAOSTAT, 2014). De todo esto, Yucatán ocupa el noveno lugar en la producción nacional, mientras que Oaxaca, Chiapas y Veracruz son los principales estados productores (SIAP, 2014).

Uno de los problemas más comunes que enfrentan los agricultores es la pérdida de los frutos a causa de la antracnosis. Debido a la aparición de las lesiones en la superficie de los frutos, éstos dejan de ser aptos para la comercialización, ocasionando pérdidas de hasta un 50% en la producción (Arias, 1992).

En México, el patógeno más común asociado con esta enfermedad es *Colletotrichum gloeosporioides* (Penz) Penz y Sacc. Sin embargo, existen reportes de la presencia de *C. capsici* en este hospedero, tanto en México (Santamaría-Basulto *et al.*, 2008; Tapia-Tussell *et al.*, 2008), como en Estados Unidos (Tarnowski & Ploetz, 2010), Trinidad y Tobago (Rampersad, 2011) y Malasia (Rahmad *et al.*, 2008). Los síntomas característicos de *C. capsici* en el fruto son: manchas circulares, grises o negras, hundidas, con abundantes masas de conidios, y pueden diferenciarse de los síntomas típicos ocasionados por *C. gloeosporioides*, debido a que este último provoca lesiones de color café y apariencia húmeda, con masas de conidios color salmón-naranja.

1.2.6.3 *C. truncatum* en *Jatropha curcas*

Jatropha curcas L. –comúnmente conocida como piñón- es un cultivo que en los últimos años ha despertado gran interés, debido a que el alto contenido de aceite de sus semillas lo convierte en un cultivo atractivo para la producción de biodiesel (Ovando-Medina *et al.*, 2010).

Esta planta es originaria de Centroamérica (Woods y Estrin, 2007), y según Heller (1996), México es su centro de distribución más probable. En este país, *J. curcas* puede ser encontrada en más de 15 estados, entre los que destacan Puebla, Hidalgo, Veracruz, Michoacán, Quintana Roo y Yucatán (Martinez *et al.*, 2010; Makkar *et. al.*, 1998). Este último es el principal estado productor de Jatropha, con un área cultivada de 2944.50 hectáreas y una producción de 1705.00 toneladas, en el año 2013 (SIAP 2014).

Es evidente el impulso que con el paso de los años se ha dado al cultivo, pues para el 2008, tan sólo en Yucatán se contaban ya con más de 300 hectáreas sembradas, estimándose que para el 2016 la cifra aumente a 10,000 hectáreas (KUO, 2010).

Como es de esperarse, *J. curcas* no es un cultivo que esté exento de plagas y enfermedades (Banjo *et al.*, 2006). Y, aunque es considerada una planta resistente, se ha reportado la presencia de diversos patógenos virales, bacterianos y fúngicos.

Las enfermedades fúngicas más frecuentes son causadas por los hongos de los géneros *Colletotrichum, Pythium, Alternaria*, *Fusarium, Rhizoctonia* y *Phytophthora.* Dentro del género *Colletotrichum*, las especies *C. gloeoeosporiodes* y *C. capsici* son los causantes de la antracnosis en el cultivo.

El primer reporte de *C. capsici* en *J. curcas* en México se hizo en el 2011 (Torres-Calzada *et al.*, 2011), aunque algunos reportes previos ya señalaban la presencia de esta misma especie en este cultivo en Brasil (Desiqueira y Gabriel, 2008; Freire y Parente, 2006); sin embargo, la información que se tiene sobre el patosistema, así como las pérdidas que genera en campo, todavía no se conocen con claridad. Por lo que, resulta necesario estudiar con mayor detalle aspectos relacionados con el patógeno, con la finalidad de generar conocimientos que pudieran ser aplicados en el control de la enfermedad.

1.2.7 Manejo de antracnosis

El manejo y el control de la antracnosis involucra la combinación del control cultural, el biológico, el químico y la resistencia intrínseca del hospedero (Wharton y Diéguez-Uribeondo, 2004). A pesar de los riesgos ambientales que implica, el control químico es aún la estrategia más utilizada en el manejo fitosanitario de esta enfermedad.

Se ha visto que algunas especies de *Colletotrichum* reaccionan diferente ante el empleo del mismo fungicida (Peres *et al.*, 2004), lo cual resulta importante para la caracterización de especies de este género. Asimismo, la identificación de especies de *Colletotrichum* resistentes a fungicidas, es un aspecto importante en la implementación de estrategias de control de la enfermedad.

Además del empleo de fungicidas que resulten eficientes en el control del patógeno, es necesario considerar las regulaciones de exportación impuestas por los países que consumen nuestros cultivos, principalmente Estados Unidos, e incluir en las

evaluaciones de resistencia los productos aprobados por la Food and Drug Administration (FDA).

En la búsqueda de alternativas que puedan ser empleadas para el control de la antracnosis, se han desarrollado diferentes métodos de manejo de los cultivos en campo, entre los que destacan los métodos de control biológico. Comparado con el control químico, el control biológico de la antracnosis no ha sido estudiado tan ampliamente. Se ha considerado la posibilidad de utilizar algunas levaduras con capacidad antagonista contra *C. capsici*, entre las que destacan *Pichia guillermondii*, *Candida musae* e *Issatchenkia orientalis*, las cuales han logrado inhibir el crecimiento del hongo hasta un 93% en estudios realizados *in vitro* (Chanchaichaovivat *et al.*, 2007). Otra estrategia ha sido dirigida hacia la inducción de la respuesta de defensa de la planta ocasionada por *Pichia guillermondii* (Nantawanit *et al.*, 2009). Sin embargo, hasta el momento, la actividad de estos organismos como agentes de biocontrol no ha sido analizada en campo.

Por otra parte, también existen casos exitosos de control biológico poscosecha en diversos cultivos (Jeffries *et al.*, 1990), los cuales se han ido mejorando en las últimas décadas. Sin embargo, el principal reto al que se enfrentan los métodos de control biológico es que el uso continuo de agroquímicos está firmemente arraigado en las prácticas de cultivo. Ambos sistemas de control deben ser combinados e integrados de manera balanceada con la finalidad de proveer un control más eficiente de la enfermedad, propiciando así el desarrollo de una agricultura sustentable.

1.3 HIPÓTESIS

Las cepas de *Colletotrichum truncatum* (syn. *C. capsici*) aisladas de diferentes hospederos vegetales presentan diferencias a nivel genético, en su capacidad infectiva y en la sensibilidad *in vitro* hacia los fungicidas empleados para su control.

1.4 OBJETIVOS

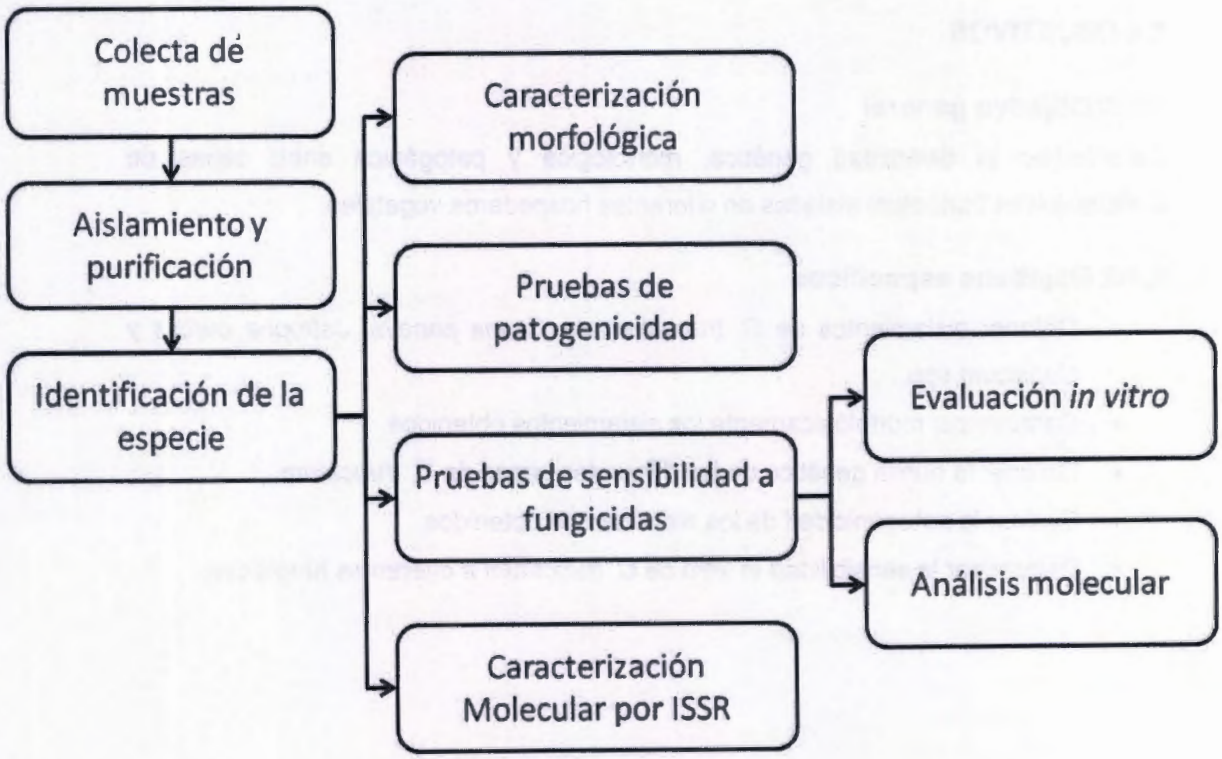
1.4.1 Objetivo general

Caracterizar la diversidad genética, morfológica y patogénica entre cepas de *Colletotrichum truncatum* aisladas en diferentes hospederos vegetales.

1.4.2 Objetivos específicos

- Obtener aislamientos de *C. truncatum* de *Carica papaya*, *Jatropha curcas* y *Capsicum* spp.
- Caracterizar morfológicamente los aislamientos obtenidos.
- Obtener la huella genética de las diferentes cepas de *C. truncatum*.
- Evaluar la patogenicidad de los aislamientos obtenidos.
- Determinar la sensibilidad *in vitro* de *C. truncatum* a diferentes fungicidas.

1.5 ESTRATEGIA EXPERIMENTAL



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CAPÍTULO II. MORPHOLOGICAL, PATHOLOGICAL AND GENETIC DIVERSITY OF *Colletotrichum* SPECIES RESPONSIBLE FOR ANTHRACNOSE IN PAPAYA (*Carica papaya* L.).[1]

2.1 ABSTRACT

Recently, anthracnose has become a major problem in papaya production and postharvest stages. The occurrence of both *Colletotrichum gloeosporioides* and *Colletotrichum capsici* has been demonstrated in this crop. The differential response of these pathogens to fungicides has highlighted the need to use rapid and accurate techniques to identify them. Thus, the objective of this study was to reveal the genetic diversity of *Colletotrichum* isolates in Mexican papaya fields. *C. gloeosporioides*-and *C. capsici*-specific primers were successfully used to detect the pathogens from different papaya parts. A combination of morphological characters, molecular techniques and pathogenicity tests were used to characterize 37 isolates from different localities of five papaya producing states. Analyses of the 5.8-ITS region and arbitrarily primed-PCR revealed intraspecific groups; most of the isolates within these groups have the same geographical location and morphological characteristics. Knowledge of the genetic diversity of *Colletotrichum* spp. in Mexican papaya fields will facilitate the identification of the pathogen population in this crop in order to select the appropriate fungicide to control anthracnose as well as to improve genetic resistance breeding programs.

2.2 INTRODUCTION

Anthracnose, caused by *Colletotrichum* species, is an economically important disease in tropical and subtropical areas worldwide, affecting a wide host range including vegetables, legumes, cereals and fruits (Bailey and Jeger 1992). During the last five years in Mexico, the prevalence of anthracnose in papaya production fields and postharvest has increased with subsequent losses of over 50%, even after repeated treatment with fungicides. It has been shown this disease in papaya is caused by *C. capsici* and *C. gloeosporioides* in Mexico (Tapia-Tussell et al. 2008), USA (Tarnowski and Ploetz 2010) and Trinidad and Tobago (Rampersad 2011). The importance of species differentiation is critical for control purposes, because *Colletotrichum* spp often differ in their sensitivity to fungicides, resulting in inefficient control (Freeman et al. 1998). A reliable identification method therefore, can facilitate the development of appropriate disease management strategies. Also studies on the variability of the

[1] **Torres-Calzada C.**, R. Tapia-Tussell, I. Higuera-Ciapara y D. Perez-Brito (2013). Morphological, pathological and genetic diversity of *Colletotrichum* species responsible for anthracnose in papaya (*Carica papaya* L). European Journal of Plant Pathology, 135:67-79. Doi: 10.1007/s10658-012-0065-7

pathogen populations are needed to direct breeding efforts towards long-term resistance to anthracnose as it has been seen for the development of *Phaseolus vulgaris* resistant cultivars against *Colletotrichum lindemuthianum* (Mahuku and Riascus 2004).

Traditionally, *Colletotrichum* species have been identified by a range of cultural and morphological characteristics, such as conidial morphology, presence or absence of setae, fungicide sensitivity, colony color and growth rate (Adaskaverg and Hartin 1997). Although valuable, these criteria alone are not always adequate as morphological characteristics may vary under different environmental conditions (Cannon et al. 2000).

Molecular techniques, combined with morphological studies have proven to be effective for characterization of *Colletotrichum* species (Sreenivasaprasad and Talhinhas 2005; Van Hemelrijck et al. 2010). Sequence analyses of the ribosomal DNA (rDNA) (Photita et al. 2005), PCR with species-specific primers (Mills et al. 1992), fingerprinting with UP-PCR (Schiller et al. 2006), AP-PCR (Talhinhas et al. 2005) and RAPD (Sangdee et al. 2011) have been used extensively to determine genetic diversity within the genus.

To our knowledge, molecular analyses to assess the genetic diversity among the *Colletotrichum* isolates responsible for anthracnose in papaya have not been performed. Thus, the objective of this work was to characterize the *Colletotrichum* species from Mexican papaya fields, using a range of molecular techniques including PCR with species-specific primers, ITS sequence and AP-PCR analyses, as well as morphological and pathological assays.

2.3 MATERIALS AND METHODS

2.3.1 Sampling and isolation of *Colletotrichum* spp.

Papaya fruit, leaves and flowers with characteristic symptoms of anthracnose were collected at different papaya production sites in Mexico (Table 2.1). Diseased plant tissues taken from the advanced margin of lesions were cut into small pieces (5 × 5 mm) and disinfested by immersing them in 3% NaOCl solution, followed by rinsing with sterile distilled water three times. They were then transferred onto Potato Dextrose Agar (PDA) medium and incubated at room temperature (25°C) for 7 days. Pure cultures were obtained by single spore isolation and were maintained on PDA dishes at 25°C for 7 days, before observation of cultural and morphological characteristics.

2.3.2 Morphological and cultural characterization

Each isolate was plated onto PDA at room temperature. Three 5-mm plugs were aseptically punched from actively sporulating areas near the growing edge of a 5-day-old culture of these isolates. Each plug was placed onto PDA dishes and incubated under the same conditions as starter cultures. After 7 days, colony size, shape, margin and color were recorded. Colony diameter of every culture was recorded daily for 7 days. Growth rate was calculated as the 7-day average of mean daily growth (mm per day). Three cultures of each isolate were investigated and experiments were conducted twice.

For examination of conidial morphology, all isolates were subcultered as mention above. Cultures were washed with sterile water and drops of the suspension were placed on microscope slides and mixed with lactophenol/cotton blue to stain the conidia. Length and width were measured for 30 conidia per isolate. Conidial shape (cylindrical or falcate) was also recorded.

Table 2.1 *Colletotrichum* isolates used in this study.

Species	Isolate	Tissue	Location	PCR Amplification	
				CgInt/ ITS4	CcapF/ CcapR
C. gloeosporioides	CGP1	Fruit	Morelos, Q. Roo	+	-
C. gloeosporioides	CGP2	Fruit	Quizás, Q. Roo	+	-
C. gloeosporioides	CGP3	Fruit	Quizás, Q. Roo	+	-
C. gloeosporioides	CGP4	Fruit	Quizás, Q. Roo	+	-
C. gloeosporioides	CGP5	Fruit	Quizás, Q. Roo	+	-
C. gloeosporioides	CGP6	Fruit	Quizás, Q. Roo	+	-
C. gloeosporioides	CGP7	Fruit	Quizás, Q. Roo	+	-
C. gloeosporioides	CGP8	Fruit	Quizás, Q. Roo	+	-
C. gloeosporioides	CGP9	Fruit	Soledad de doblado, Veracruz	+	-
C. gloeosporioides	CGP10	Fruit	Soledad de doblado, Veracruz	+	-
C. gloeosporioides	CGP11	Fruit	Soledad de doblado, Veracruz	+	-
C. gloeosporioides	CGP12	Fruit	Soledad de doblado, Veracruz	+	-
C. gloeosporioides	CGP13	Fruit	Soledad de doblado, Veracruz	+	-
C. gloeosporioides	CGP14	Leaf	Conkal, Yucatan	+	-
C. gloeosporioides	CGP15	Leaf	Conkal, Yucatan	+	-
C. gloeosporioides	CGP16	Leaf	Conkal, Yucatan	+	-
C. gloeosporioides	CGP17	Flower	Conkal, Yucatan	+	-
C. gloeosporioides	CGP18	Flower	Conkal, Yucatan	+	-
C. gloeosporioides	CGP19	Fruit	Quizas, Q. Roo	+	-
C. gloeosporioides	CGP20	Fruit	Quizas, Q. Roo	+	-
C. gloeosporioides	CGP21	Fruit	Quizas, Q. Roo	+	-
C. gloeosporioides	CGP22	Fruit	Quizas, Q. Roo	+	-
C. capsici	Ccg1	Fruit	Tapachula, Chiapas	-	+
C. capsici	Ccg2	Fruit	Tapachula, Chiapas	-	+
C. capsici	Ccg6	Fruit	Morelos, Q. Roo	-	+
C. capsici	Ccg7	Fruit	Morelos, Q. Roo	-	+
C. capsici	Ccg15	Leaf	Valladolid, Yucatan	-	+
C. capsici	CCP1	Fruit	Champoton, Campeche	-	+
C. capsici	CCP4	Fruit	Champoton, Campeche	-	+
C. capsici	CCP6	Fruit	Morelos, Q. Roo	-	+
C. capsici	CCP10	Fruit	Quizás, Q. Roo	-	+
C. capsici	CCP11	Fruit	Quizás, Q. Roo	-	+
C. capsici	CCP12	Fruit	Quizás, Q. Roo	-	+
C. capsici	CCP14	Fruit	Quizás, Q. Roo	-	+
C. capsici	CCP15	Fruit	Quizás, Q. Roo	-	+
C. capsici	CCP16	Fruit	Quizás, Q. Roo	-	+
C. capsici	CCP17	Fruit	Quizás, Q. Roo	-	+

2.3.3 Genomic DNA extraction and identification with species-specific primers

For genomic DNA extraction, 10 pieces of agar culture (1 × 1 cm) obtained from the 7-day-old colonies grown on Richard´s V8 (RV8) medium were transferred into 250-mL Erlenmeyer flasks containing 50 mL of Nutrient Broth (NB). After incubation at 28°C on an orbital shaker (100 rpm) for 7 days, the mycelia were collected by filtration, frozen at -80°C for 2 h and lyophilized (LABCONCO 77530) until use. The total genomic DNA was extracted according to the method described by Tapia-Tussell et al. (2006) and diluted to a final concentration of 50 ng/µL.

Molecular identification was carried out using *C. gloeosporioides*-specific primers CgInt/ITS4 (Mills et al. 1992) and *C. capsici*-specific primers CcapF/CcapR (Torres-Calzada et al. 2011a). PCR reaction (25 µL final volume) contained 25 ng of DNA, 1× PCR buffer (10×: 200 mM Tris-HCl, 500 mM KCl, pH 8.4; Invitrogen), 0.20 mM of each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 1 µM primers and 1U *Taq* polymerase (Invitrogen). DNA amplification was performed in a GeneAmp 9700 DNA Thermal Cycler (Perkin-Elmer), and consisted of an initial denaturing step at 95° for 5 min, followed by 25 cycles of 30s at 94°C, 2 min at 62°C and 2 min at 72°C, and a final extension step of 5 min at 72°C. PCR products were separated by electrophoresis in 1.5% (w/v) agarose gels and visualized by ethidium bromide staining.

2.3.4 ITS-5.8S sequence analysis

The internal transcribed spacer regions, including the 5.8S rDNA, were amplified using universal primers ITS1 and ITS4 (White et al. 1990). The PCR reaction was carried out in 25 µL consisting of 25 ng of DNA, 1× PCR buffer (Invitrogen), 0.20 mM of each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 1 µM primers and 1U Taq polymerase (Invitrogen). DNA amplification was performed in a GeneAmp 9700 DNA Thermal Cycler (Perkin-Elmer), and consisted of an initial denaturing step at 95° for 1 min, followed by 30 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, and a final extension step of 7 min at 72°C. PCR products were separated by electrophoresis in 1.5% (w/v) agarose gels and visualized by ethidium bromide staining. PCR products were purified and sequenced by Macrogen Inc. Korea. Alignment and edition were carried out with the BioEdit Sequence Alignment program (Altschul et al. 1990). Sequences were then compared against *C. gloeosporioides* and *C. capsici* sequences downloaded from the

GenBank Database. Data were analyzed using the Molecular Evolutionary Genetic Analysis (MEGA) software version 5.0 (Tamura et al. 2011), distances were calculated using the Jukes-Cantor model (assumes equal probability of independent change at all sites) and a tree was produced using the UPGMA, Neighbor Joining and Maximum Likelihood methods. A bootstrap analysis using 1000 resamples of the data was done. The ITS-5.8S sequence of *Colletotrichum anthrisci* was obtained from the GenBank database and used as outgroup.

2.3.5 AP-PCR (arbitrarily- primed PCR) analysis

Five 15-bp primers derived from the repeated sequences $(GTG)_5$, $(GACAC)_3$, $(CAG)_5$, $(GAC)_5$ and $(TCC)_5$ were used for AP-PCR analysis (Nguyen et al. 2009). PCR reaction was carried out in 25 µL reaction volumes containing 50 ng of DNA, 1× PCR buffer (Invitrogen), 0.25 mM of each dNTP (Invitrogen), 2 mM $MgCl_2$, 0.8 µM primers and 1U *Taq* polymerase (Invitrogen). DNA amplification was performed in a GeneAmp 9700 DNA Thermal Cycler (Perkin-Elmer), and consisted of an initial denaturing step at 95° for 5 min, followed by 40 cycles including: denaturing at 95°C for 40 seconds, annealing for 1 min at either 42°C for $(GACAC)_3$ and $(TCC)_5$, or 50°C for $(GTG)_5$ and $(GAC)_5$, or 60°C for $(CAG)_5$, extension for 1 min at 72°C; and a final extension step of 5 min at 72°C. The amplified PCR products were loaded onto a 1.5% agarose gel and the bands were detected by ethidium bromide staining. The presence or absence of bands produced by AP-PCR was recorded and a binary matrix was generated for each marker. A similarity matrix was calculated using the Dice coefficient and employed later in a cluster analysis performed by the Unweighted Pair-Group Method with Arithmetic averaging, using PAST software (Hammer et al. 2001). Branch support of the tree was assessed by bootstrapping (Felsenstein 1985) using 1000 replications.

2.3.6 Pathogenicity tests

Pathogenicity tests were performed with a representative set of isolates, from all morphological groups and locations, using papaya fruit (*C. papaya* var. Maradol). Fruit were disinfested by immersing them in 1% NaOCl solution for 1 min, washed twice with sterile distilled water and dried at room temperature. An aqueous conidial suspension (1×10^6 spores mL^{-1}) was prepared from 7-day-old cultures of each isolate and then placed on the fruit by the wound/drop method (Kanchana-Udomkan et al. 2004). This method involved pin-pricking the surface of the fruit to a 1-mm depth and the placing 20 µL of conidial suspension over the wound. Three fruits were tested per isolate and

experiments were conducted twice. The inoculated fruit, along with appropriate controls (fruit inoculated with sterile distilled water) were incubated at room temperature (25° C) in humid chamber. Symptoms were recorded 5 days after inoculation (d.a.i.) and re-isolation, according to Koch's postulates, was made from all resulting lesions.

2.3.7 Statistical analyses

Statistical analyses for conidial morphology, growth rate and pathogenicity tests were performed using the R statistical package version 2.13.1 (Ihaka and Gentleman 1996). Data were analyzed using ANOVA in order to determine the significance of differences in conidia dimensions, growth rates and lesion diameters post inoculation with *C. capsici* and *C. gloeosporioides*. Means were compared using Tukey's multiple range test.

2.4 RESULTS

Colletotrichum isolates were obtained mainly from lesions on papaya fruit, but some isolates were obtained from leaves, petioles and flowers. In total, thirty seven were obtained from 7 different orchards located in five papaya producing states in Mexico (Fig. 2.1).

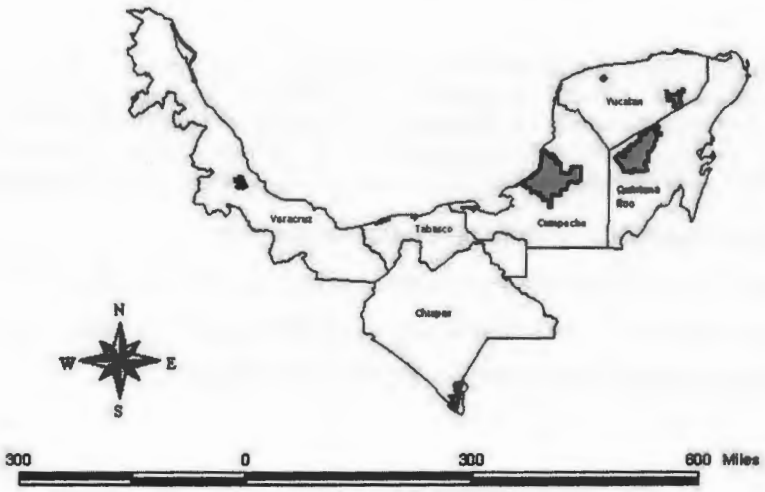

Figure 2.1 Map of Mexico, where 37 isolates of *Colletotrichum* spp. were collected from papaya plants with anthracnose symptoms.

Preliminary identification was based on the morphological description of *Colletotrichum* species (Shenoy et al. 2007; Sutton 1992). Of all isolates collected, 15 fitted the description of *C. capsici* and 22 fitted the description of *C. gloeosporioides*.

2.4.1 Identification with species-specific primers

All 37 *Colletotrichum* spp isolates collected from papaya, plus the *C. capsici* reference isolate (*C. capsici* ATCC 48574), were amplified with the species-specific primers for *C. gloeosporioides* and *C. capsici*. A 450-bp DNA fragment was amplified with the *C. gloeosporioides*- specific primers CgInt and ITS4 in 22 of the 37 isolates obtained. The rest, 15 of 37 isolates, and the reference *C. capsici* ATCC 48574 isolate, amplified a 390-bp DNA fragment when the *C. capsici*-specific primers CcapF and CcapR were used (Fig. 2.2).

Figure 2.2 Amplification products obtained using species-specific primers, (a) PCR amplification with the primer pair CgInt/ITS4 for detection of *Colletotrichum gloeosporioides* and (b) PCR amplification with the primer pair CcapF/CcapR for detection of *C. capsici*. Lane M, DNA marker (1 kb DNA ladder); lanes 1-7, representative *C. gloeosporioides* isolates; lanes 8-12, representative *C. capsici* isolates; lane 13, *C. capsici* ATCC 48574; lane 14, negative control.

2.4.2 Morphological and cultural characterization

Differences in colony characteristics among the isolates resulted in formation of morphological groups. *C. gloeosporioides* isolates formed six distinct groups and *C. capsici* isolates formed another three different groups (Table 2.2).

Table 2.2 Morphological features of the *Colletotrichum* species causing anthracnose in papaya

Colletotrichum species	Group	% isolates	Colony appearance		
			Shape	Margin	Color
C. gloeosporioides	1	5.4	Circular	Entire	White to orange, with orange conidial masses in center
C. gloeosporioides	2	8.1	Circular	Curled	White flocculose, with orange conidial masses in center
C. gloeosporioides	3	10.8	Circular	Curled	White to pale grey, with orange conidial masses produced in concentric rings
C. gloeosporioides	4	5.4	Circular	Curled	White flocculose, with no visible conidial mass
C. gloeosporioides	5	24.3	Circular	Entire	Olive to black, with no visible conidial mass
C. gloeosporioides	6	5.4	Circular	Curled	Dense, olive to black, with dark grey-colored conidial masses in center
C. capsici	7	5.4	Circular	Entire	Light salmon to pale grey, with conidial masses in center.
C. capsici	8	8.1	Circular	Undulate	White to light salmon, with conidial masses produced in concentric rings.
C. capsici	9	27.0	Irregular	Entire	Pale grey to black, with conidial masses produced in concentric rings.

Cultural characteristics

Isolates of the *C. gloeosporioides* groups 1, 2, 3 and 4 produced white colonies. In some of them, it was possible to observe orange conidial masses in the center (group 1 and 2) or distributed in concentric rings throughout the colony (group 3). Colonies produced by *C. gloeosporioides* isolates from group 5 and 6 were olive to black colored, but the presence of dark grey-colored conidia were observed only in group 6. Most of the *C. capsici* isolates (67.5%, group 9) had pale grey to black colored colonies with beige-colored conidial masses forming concentric rings around the dish. Colonies from *C. capsici* group 7 and 8 were both light salmon, but the distribution of conidial masses was different for each group (Fig. 2.3).

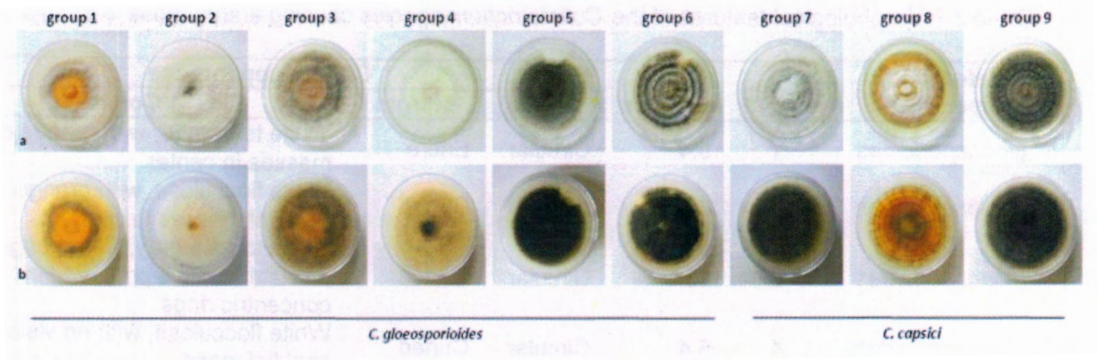

Figure 2.3 Morphotypes of *Colletotrichum gloeosporioides* and *C. capsici* isolated from papaya, (a) upper side of the colony and (b) reverse side of the colony.

Growth rate

There was no significant difference in growth rate among isolates of *C. gloeosporioides*. However, isolates from group 7 of C. capsici grew faster than those in groups 8 and 9 (P = 0.003). The *C. gloeosporioides* isolates grew significantly faster than the *C. capsici* isolates (P = 0.001), especially those belonging to group 4 (Table 2.3).

Conidial morphology

Two types of conidia were observed: cylindrical and falcate. *C. capsici* isolates had significantly longer conidia than those of *C. gloeosporioides*. *C. capsici* isolates produced one-celled, hyaline, falcate conidia with acute apex. The average length and width of the conidia were 22.8-23.8 µm and 3-3.02 µm, respectively. The *C. gloeosporioides* conidia were all cylindrical, with both ends rounded. The average length and width of the conidia were 13.56-14.24 µm and 4-4.02 µm, respectively (Table 2.3).

Table 2.3 Conidial characteristics of the morphological groups of *Colletotrichum* species.

Species	Group	Conidial characteristics			Growth rate[x] (mm day^{-1})	Lesion[y] diameter (mm)
		Shape	Length(µm)	Width(µm)		
C. gloeosporioides	1	Cylindrical, with rounded ends	13.98 ± 0.04b[z]	4.01 ± 0.02ab[z]	11.02 ± 0.69ab[z]	23.89± 0.13b[z]
C. gloeosporioides	2	Cylindrical, with rounded ends	13.97 ± 0.05b	4.02 ± 0.02ab	10.76 ± 0.68ab	24.17± 0.31b
C. gloeosporioides	3	Cylindrical, with rounded ends	14.24 ± 0.49b	4.0 ± 0.0b	11.15 ± 0.71ab	23.43± 0.49b
C. gloeosporioides	4	Cylindrical, with rounded ends	14.02 ± 0.01b	4.0 ± 0.0b	11.57 ± 0.42a	22.18± 0.28b
C. gloeosporioides	5	Cylindrical, with rounded ends	13.77 ± 0.51b	4.0 ± 0.0b	10.90 ± 0.57ab	23.76± 0.67b
C. gloeosporioides	6	Cylindrical, with rounded ends	13.56 ± 0.62b	4.0 ± 0.0b	10.93 ± 0.31ab	24.34± 0.36b
C. capsici	7	Falcate, with acute apex	23.76 ± 0.20a	3.0 ± 0.0d	10.42 ± 0.49b	19.31± 0.21a
C. capsici	8	Falcate, with acute apex	22.83 ± 0.07a	3.02 ± 0.017c	8.17 ± 0.07c	18.78± 0.45a
C. capsici	9	Falcate, with acute apex	23.84 ± 0.21a	3.0 ± 0.0d	9.13 ± 0.56c	19.09± 0.37a

x Growth rate measured as lesion diameter in mm per day.
y Lesion diameter recorded 5 d.a.i.
z Mean values in the same column and followed by the same letter are not significantly different (P≤0.05) according to Tukey test.

2.4.3 ITS-5.8S sequence analysis

The ITS region, including the 5.8S gene of all isolates was successfully amplified and sequenced. The data set contained 450 characters of which 53 were parsimony informative. The BLAST similarity search confirmed the results obtained by the species-specific PCR analyses. Eleven reference sequences from other reports were downloaded from the NCBI database and used in this study: HQ896483, HQ845103, HQ264179, HM562711 and FJ972609 for *C. gloeosporioides*; HQ271458, GQ369594, HQ271468, DQ453990 and EF683602 for *C. capsici*; and GU227845 for *C. anthrisci*.

Phylogenetic analyses performed by UPGMA, Neighbor Joining and Maximum Likelihood methods produced similar topologies; one of the trees is shown in Fig. 2.4. Phylograms separated the *Colletotrichum* isolates into two distinct groups named A and B. Group A (100% bootstrap) includes all *C. gloeosporioides* isolates and group B (100% bootstrap) includes all *C. capsici* isolates. Group A was divided into four subgroups designated A1, A2, A3 and A4. Subgroup A1 included 59% of the *C. gloeosporioides* isolates, along with the reference sequences HQ896483, HQ264179 and HM562711. A2 included the CBS reference isolate *C. gloeosporioides* FJ972609, and isolates CGP14, CGP15 and CGP16, showing 99% identity.

Group B was divided into subgroups B1 and B2. Most of the isolates (57.14%) were included in subgroup B1. B2 included six isolates which were 99% identical to the reference isolates HQ271458, GQ369594, HQ271468, DQ453990 and the *C. capsici* sequence from the epitype strain EF683602. In some cases, isolates were separated according to geographical origin, for example group A3 contains isolates only from Quintana Roo state. Also, groups A2 and A4 are formed exclusively for isolates from Yucatan and Veracruz states respectively.

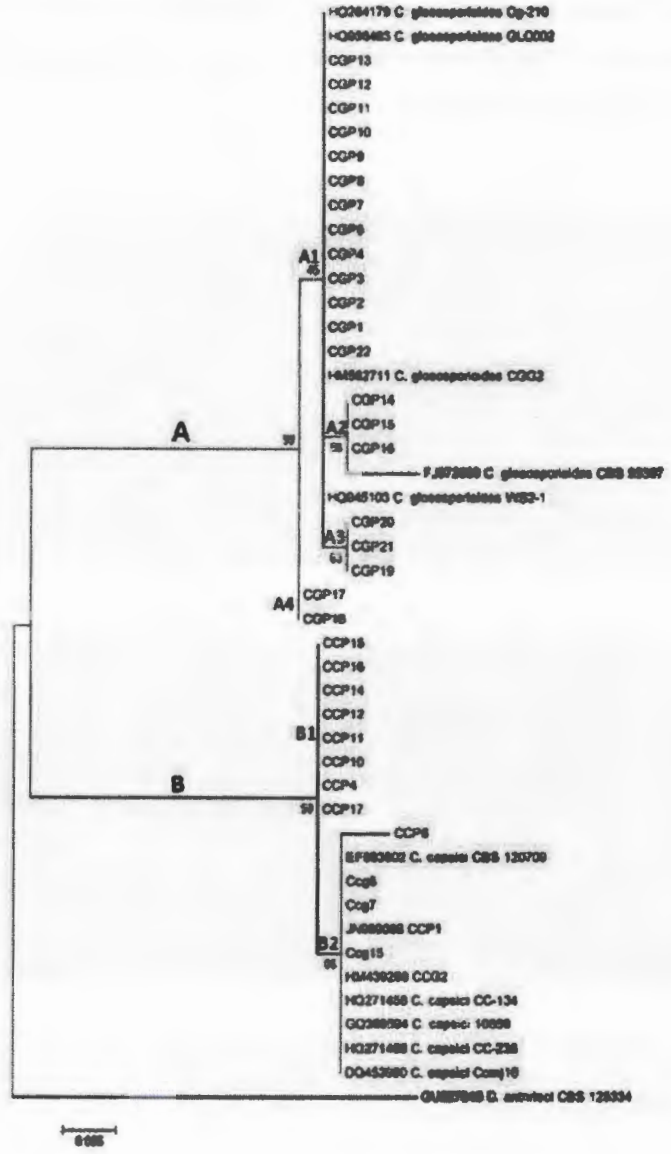

Figure 2.4 Maximum Likelihood (ML) tree based on the ITS data from collected isolates and published sequences. The tree was rooted with *Colletotrichum anthrisci* and Tamura-Nei model was used. Bootstrap test was done (1000 replicates) and values above 50% are shown on internal branches.

2.4.4 AP-PCR analysis

AP-PCR analysis was done to characterize 31 representative *Colletotrichum* isolates using 6 different primers. Gels showing diversity among representative isolates using the primer $(CAG)_5$ are presented (Fig.2. 5). The average number of polymorphic bands

per primer was 11 and ranged in size from 300 to 5000 bp. Information on banding patterns obtained from all the primers was used to determine genetic distance between isolates and to construct a dendrogram.

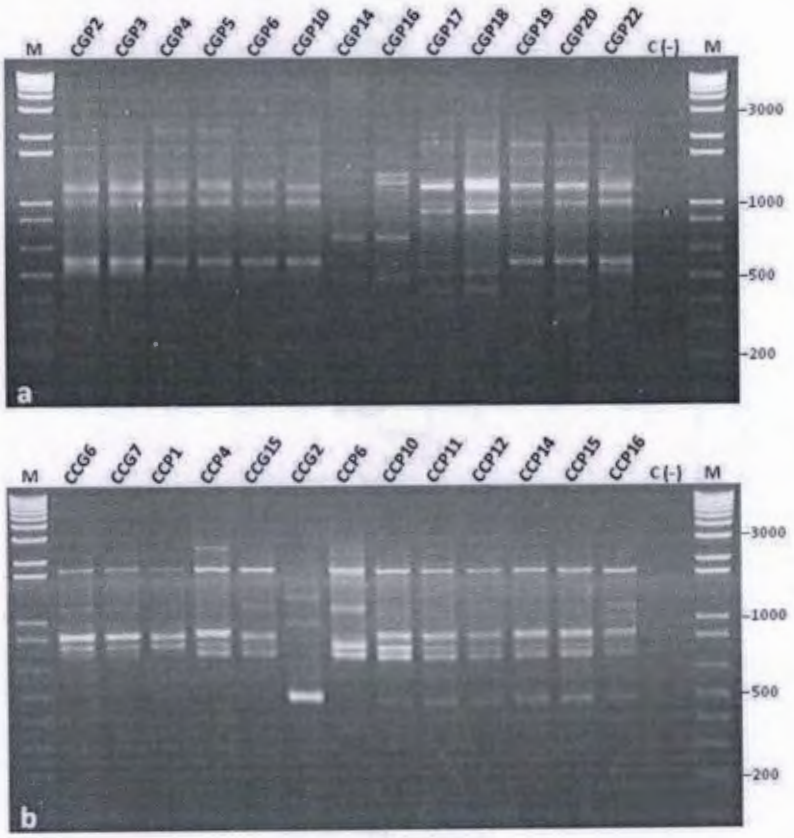

Figure 2.5 Band patterns of arbitrarily primed polymerase chain reaction amplified genomic DNA of representative (a) *Colletotrichum gloeosporioides* and (b) *C. capsici* isolates using primer (CAG)5. Lane M, DNA marker (1 kb DNA ladder); lane C, negative control.

According to this dendrogram, the *Colletotrichum* spp. we studied could be divided into 11 main groups (Fig. 2.6). Groups A1 to A6 had all the *C. gloeosporioides* isolates, while groups B1 to B5 contained the *C. capsici* isolates. The number of isolates within a cluster ranged from 2 to 5. However, isolate Ccg2 formed a separate group.

The clustering of the *C. gloeosporioides* isolates based on AP-PCR data showed a relationship with geographical distribution of isolates in well supported clusters. However, the clustering of the *C. capsici* isolates was not always associated with the

geographical localities from which the isolates were obtained as only isolates in groups B1 and B2 clustered according to their region, whilst groups B3 and B4 contained isolates from three different geographic regions in the same cluster.

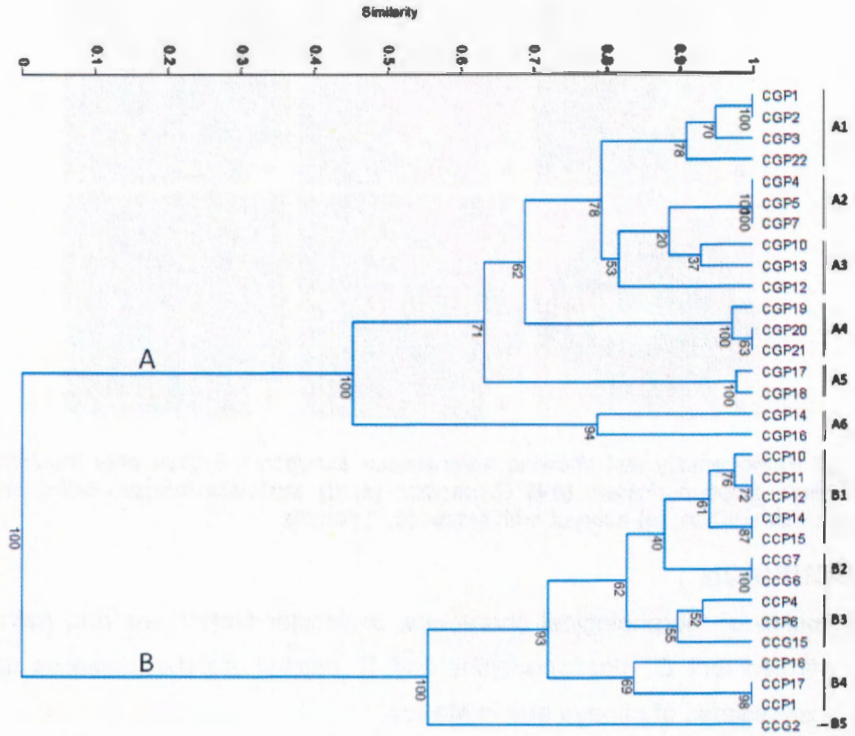

Figure 2.6 Unweighted Pair-Group Method with Arithmetic average dendrogram showing diversity and relationships among Colletotrichum isolates from papaya based on Arbitrarily Primed- PCR analyses using a combined dataset from primers (GAC)5, (GTG)5, (TCC)5, (CAG)5 and (GACAC)3. One thousand bootstrap test and Dice coefficient distance matrices were used.

2.4.5 Pathogenicity tests

Symptoms characteristics of anthracnose were observed at the inoculation site 5 d.a.i. All *C. gloeosporioides* and *C. capsici* isolates were capable of causing the infection, but there were significant differences between both species in mean lesion diameter (*P*= 0.001) (Table 2.3).

Two different types of lesions could be distinguished on the inoculated fruit, corresponding to each of the species tested (Fig. 2.7). Lesions produced by *C. gloeosporioides* were water soaked, sunken and contained orange conidial masses.

Lesions caused by *C. capsici* were brown to black, with grey spore masses and abundant acervuli arranged concentrically.

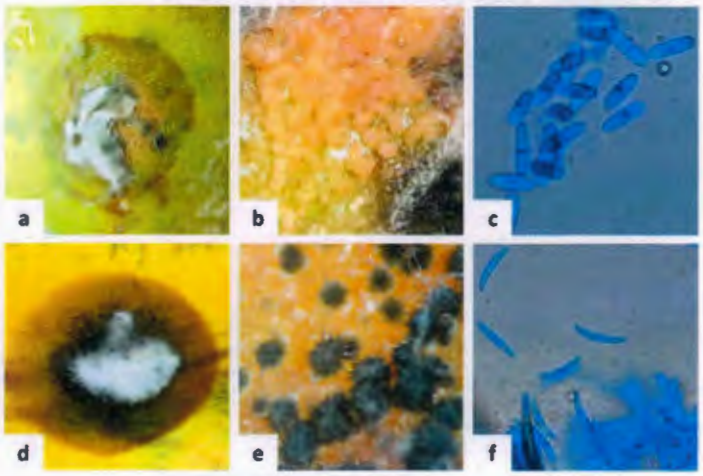

Figure 2.7 Pathogenicity test showing anthracnose symptoms 5 days after inoculation. **(a-c)** *Colletotrichum gloeosporioides*; **(d-f)** *C. capsici*; **(a, d)** artificially infected fruits; **(b)** conidial masses on fruit surface; **(e)** acervuli with setae; **(c, f)** conidia.

2.5 DISCUSSION

A combination of morphological characters, molecular techniques and pathogenicity tests confirmed that *C. gloeosporioides* and *C. capsici* are the causative agents for anthracnose disease of papaya fruit in Mexico.

C. gloeosporioides and *C. capsici* are both pathogens of many economically important hosts. *C. gloeosporioides* is a common pathogen on a variety of tropical crops such as mango, avocado and papaya (Silva-Rojas et al. 2011; Tapia-Tussell et al. 2008). On the other hand, *C. capsici* is best known as an aggressive pathogen in pepper (Shenoy et al. 2007) and has recently been associated with anthracnose in papaya (Tapia-Tussell et al. 2008) and physic nut (Torres-Calzada et al. 2011b). Consequently, there has been considerable interest in the development and utilization of PCR-based tools that allow the rapid and accurate diagnosis of these species. In our study, *C. gloeosporioides* isolates could be distinguished from *C. capsici* isolates by using species-specific primers. Previous reports indicated that *C. capsici*-specific primers were successfully used to detect the pathogen from different papaya parts such as fruit, petioles, leaves and flowers (Torres-Calzada et al. 2011a). Similarly, *C. gloeosporioides*-specific primers have been used for the detection of this species in other tropical crops like tamarillo, passiflora and mango (Afanador-Kafuri et al. 2003).

All these studies demonstrate the reliability of these primers for fast and accurate identification of pathogens, especially in cases where morphological identification is complicated (Whitelaw-Weckert et al. 2007).

The cultural characteristics we observed separated *C. gloeosporioides* into six different morphotypes. The isolates used in this study, belonging to groups 2, 3 and 6, were similar in appearance to those described by Photita et al. (2005) in reference to the groups they designated as 1, 2, and 3. Also, the *C. gloeosporioides* groups 4 and 6 fit the description of *C. gloeosporioides* group 4 and 2 characterized by Rampersad (2011) and Than et al. (2008), respectively. Cultural characteristics separated *C. capsici* isolates into three different morphotypes. The *C. capsici* group 3 described in this paper is consistent with the morphological characterization made by Shenoy et al. (2007). However, the appearances of morphotypes 1 and 2 have not been previously reported.

Within the *Colletotrichum* genus, analyses of ribosomal DNA have been extensively used for species delimitation (Mills et al. 1992). In the present study, ITS sequence analysis was reliable for interspecific separation among *Colletotrichum* spp. Moreover, this comparison was useful to elucidate the differences between the isolates even below the species level, separating *C. gloeosporioides* into groups A1 to A4 and *C. capsici* into groups B1 and B2. Interestingly, in some cases, geographical distribution was related to phylogeny, as shown in groups A2, A3 and A4, where the isolates within the groups belong to the same location. Johnston and Jones (1997) reported the presence of different groups based on rDNA sequence analyses among the *Colletotrichum* spp. isolates from fruit-rot in New Zealand. Their results discriminate among isolates of different morphological and cultural groups.

We also assessed the diversity of the *Colletotrichum* spp. populations using AP-PCR molecular markers. The clustering in the AP-PCR dendrogram for both analyzed species was associated with the geographic localities from which the isolates were obtained in all cases, except clusters B3 and B4, which contained isolates from different geographic regions. It is interesting to note that isolates within clusters A5, A6 and B1 also belong to the same morphological group. These findings indicate a link between genotype, geographical distribution, morphological characteristics and phylogeny. Genetic diversity of *C. gloeosporioides* and *C. capsici* has been described

previously for other hosts (Afanador-Kafuri et al. 2003; Than et al. 2008). Similarly to previous reports, higher variability was observed within the *C. gloeosporioides* populations where the genetic heterogeneity may be explained by the presence of a perfect stage (Freeman et al. 1998).

Pathogenicity tests with the *Colletotrichum* species isolated, showed that all were able to infect and cause symptoms in wounded papaya fruit, proving that both species were causal agents of anthracnose infection on papaya. These results provide evidence that, in anthracnose pathosystems, the same host is often infected by different *Colletotrichum* species as has been observed in crops such as avocado (Silva-Rojas and Avila-Quezada 2011) and pepper (Than et al. 2008). In this study, it was possible to isolate both *C. gloeosporioides* and *C. capsici* from fruit, flowers and leaves, which concurs with previous reports indicating that some *Colletotrichum* species are commonly found infecting not only fruit, but other parts of the plant (Howard et al. 1992). Further studies using different inoculation methods should be carried out to assess the cross-infection potential of these two species from papaya.

To our knowledge, this is the first study conducted to determine the genetic diversity of *Colletotrichum* species causing anthracnose in papaya. The high diversity observed in both species might be due to the different climate conditions found within our country. Also, the ability of both species to infect different hosts suggests the possibility of cross-infection among cultivars. In our region, papaya orchards are often located adjacent to other horticultural crops such as Habanero pepper (*Capsicum chinense*), facilitating dispersion of the pathogen from one crop to another and therefore representing a high-risk situation for disease control in the field. Determining the genetic diversity facilitates the identification of pathogen populations in crops in order to select appropriate control measures, as different species or even subgroups vary in their sensitivity to fungicides (Van Hemelrijck et al. 2010), thereby allowing producers to reduce the economic losses in crop production caused by this disease. Also this knowledge will be useful for breeding programs for genetic resistance to anthracnose in papaya, considering that the obtention of an anthracnose resistant papaya variety is the best control measure.

2.6 ACKNOWLEDGEMENTS

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CAPÍTULO III. GENETIC DIVERSITY AND LACK OF HOST SPECIFICITY OF *Colletotrichum truncatum* ISOLATES FROM DIFFERENT HOSTS[1]

3.1 ABSTRACT

Colletotrichum truncatum (syn. *C. capsici*), has been identified as the causative agent of anthracnose in different hosts. The aim of this study was to determinate if *C. truncatum* populations infecting papaya, pepper and physic nut in the southeast region of Mexico are genetically and pathogenically different in order to improve more effective disease management strategies. A total of 113 *truncatum* isolates, collected from 18 fields in five producer states, were subjected to phenotypic and genotypic characterization and pathogenicity testing. *C. truncatum* isolates were divided into six different morphological groups, based on colony appearance. This extensive phenotypic variability of the pathogen populations is revealed for the first time. The ISSR markers used were efficient in separating the isolates, all of which were grouped according to their host or geographical origin, but not according to their aggressiveness. The pathogenicity tests revealed that all isolates could infect the three hosts and produce typical anthracnose symptoms, indicating the lack of host specificity of this species and therefore its pathogenic potential on other plants. This has important implications for the genetic improvement of the hosts, in the search for anthracnose resistance, indicating that plant breeders should seek a broad genetic basis of resistance.

3.2 INTRODUCTION

The genus *Colletotrichum* comprises several species considered as major plant pathogens worldwide, mainly in tropical and temperate regions. They cause significant damage in various economically important crops, particularly during the fruiting stage. The disease is commonly known as anthracnose and symptoms of infection are characterized by sunken necrotic tissue with concentric rings of acervuli (3).

Numerous cases have been reported in which several *Colletotrichum* species are associated with a single host, but it is also common to find that a single species infects multiple hosts (11). This is the case of *Colletotrichum truncatum* (syn. *C. capsici,* 6) which has been identified as the causative agent of anthracnose in different hosts (10),

[1] **Torres-Calzada C.**, E. Huchin-Poot, I. Higuera-Ciapara, R. Tapia-Tussell y D. Perez-Brito (2014). Genetic diversity and lack of host specificity of *Colletotrichum truncatum* isolates from different hosts. Phytopathology. Submitted September 15th, 2014. Manuscript ID: PHYTO-09-14-0246-R

such as, pepper (*Capsicum* spp.), papaya (*Carica papaya*) and physic nut (*Jatropha curcas*), all of which have been severely infected by this pathogen in Mexico (23).

In Mexico, the production areas of these crops are concentrated mainly in the south-southeast, and the constant presence of the pathogen is to be expected given the high humidity caused by greater rainfall in this area, in comparison with the north of the country. Consequently, losses in the field and postharvest are considerable, with a marked effect on the economy of the country since Mexico is the largest exporter of papaya to the U.S. and one of the leading producers worldwide of pepper (9).

Effectiveness in the control of the disease relies on chemical use, and information on the distribution of pathotypes is critical for the deployment of more accurate control strategies such as host resistance breeding. To date, three pathotypes of *C. truncatum* have been described (14); however, information is restricted to the presence of the species in only one host (pepper).

Genetic variation within populations of pathogens may be created by the presence of alternate hosts, as well as by mutation or migration of individuals, leading to an increase in the virulence of the pathogen (4). Phenotypic and DNA-based markers have been used to measure the variability among individuals. ISSR markers have been successfully used to infer the genetic diversity of different *Colletotrichum* spp. (12, 15).

Knowledge of the variability and pathogenicity in the *C. truncatum* populations in Mexico could lead to the development of more effective control measures for the disease. Thus, the aim of this study was to investigate the pathogenicity and genetic diversity in *C. truncatum* populations infecting papaya, pepper and physic nut in the southeast region of Mexico. Isolates were analyzed by ISSR markers. Phenotypic characters, i.e. colony morphology, lesion size and growth rate were also included.

3.3 MATERIALS AND METHODS

3.3.1 Collection of *C. truncatum* isolates

Samples of papaya, pepper and physic nut with anthracnose symptoms were collected during different growing seasons from 2008 to 2013. In total, 108 *C. truncatum* isolates were collected from 18 fields in five producer states: Quintana Roo, (ROO), Yucatan,

(YUC); Campeche, (CAM); Chiapas, (CHS) and Tabasco, (TAB) (Table 1). Identity of the isolates was confirmed by molecular analysis (22).

Table 3.1 Description of *Colletotrichum truncatum* isolates used in this study.

Name	Location	Collection date	Host	Tissue
CCG6	Morelos, ROO	2009	*Carica papaya*	Fruit
CCG7	Morelos, ROO	2009	*C. papaya*	Fruit
CCG15	Valladolid, YUC	2008	*C. papaya*	Leaf
CCP1	Champoton, CAM	2009	*C. papaya*	Fruit
CCP4	Champoton, CAM	2009	*C. papaya*	Fruit
CCP6	Morelos, ROO	2009	*C. papaya*	Fruit
CCP10	Morelos, ROO	2011	*C. papaya*	Fruit
CCP11	Morelos, ROO	2011	*C. papaya*	Fruit
CCP12	Morelos, ROO	2011	*C. papaya*	Fruit
CCP14	Morelos, ROO	2011	*C. papaya*	Fruit
CCP15	Morelos, ROO	2011	*C. papaya*	Fruit
CCP16	Morelos, ROO	2011	*C. papaya*	Fruit
CCP17	Morelos, ROO	2011	*C. papaya*	Fruit
CCP20	Hopelchen, CAM	2012	*C. papaya*	Fruit
CCP21	Tizimin, YUC	2012	*C. papaya*	Fruit
CCP22	Tizimin, YUC	2012	*C. papaya*	Petiole
CCP23	Tizimin, YUC	2012	*C. papaya*	Fruit
CCP24	Tizimin, YUC	2012	*C. papaya*	Fruit
CCP25	Tizimin, YUC	2012	*C. papaya*	Fruit
CCP26	Tizimin, YUC	2012	*C. papaya*	Fruit
CCP27	Tizimin, YUC	2012	*C. papaya*	Petiole
CCP28	Tizimin, YUC	2012	*C. papaya*	Petiole
CCP29	Balankan, TAB	2012	*C. papaya*	Fruit
CCP30	Balankan, TAB	2012	*C. papaya*	Fruit
CCP31	Balankan, TAB	2012	*C. papaya*	Fruit
CCP32	Balankan, TAB	2012	*C. papaya*	Fruit
CCP33	Balankan, TAB	2012	*C. papaya*	Fruit
CCP34	Balankan, TAB	2012	*C. papaya*	Fruit
CCP35	Balankan, TAB	2012	*C. papaya*	Fruit
CCP36	Balankan, TAB	2012	*C. papaya*	Fruit

CCP37	Balankan, TAB	2012	*C. papaya*	Fruit
CCP38	Balankan, TAB	2012	*C. papaya*	Fruit
CCP39	Arriaga, CHS	2013	*C. papaya*	Fruit
CCP40	Arriaga, CHS	2013	*C. papaya*	Fruit
CCP41	Arriaga, CHS	2013	*C. papaya*	Fruit
CCP42	Arriaga, CHS	2013	*C. papaya*	Fruit
CCP43	Arriaga, CHS	2013	*C. papaya*	Fruit
CCP44	Arriaga, CHS	2013	*C. papaya*	Fruit
CCP45	Arriaga, CHS	2013	*C. papaya*	Fruit
CCG9	Peto, YUC	2008	*Capsicum annuum*	Fruit
CCG12	Tekax, YUC	2008	*C. annuum*	Fruit
CCG13	Tekax, YUC	2008	*C. annuum*	Fruit
CCC1	Balankan, TAB	2012	*C. chinense*	Stem
CCC2	Balankan, TAB	2012	*C. annuum*	Fruit
CCC3	Balankan, TAB	2012	*C. annuum*	Fruit
CCC4	Balankan, TAB	2012	*C. annuum*	Fruit
CCC6	Balankan, TAB	2012	*C. annuum*	Fruit
CCC7	Balankan, TAB	2012	*C. annuum*	Fruit
CCC11	Muna, YUC	2012	*C. annuum*	Fruit
CCC13	Muna, YUC	2012	*C. annuum*	Fruit
CCC17	Balankan, TAB	2012	*C. chinense*	Stem
CCC18	Balankan, TAB	2012	*C. annuum*	Fruit
CCC19	Balankan, TAB	2012	*C. chinense*	Stem
CCC20	Balankan, TAB	2012	*C. chinense*	Stem
CCC21	Balankan, TAB	2012	*C. chinense*	Fruit
CCC22	Balankan, TAB	2012	*C. chinense*	Fruit
CCC23	Calkini, CAM	2012	*C. annuum*	Fruit
CCC25	Calkini, CAM	2012	*C. annuum*	Fruit
CCC28	Calkini, CAM	2012	*C. annuum*	Fruit
CCC29	Calkini, CAM	2012	*C. annuum*	Fruit
CCC30	Calkini, CAM	2012	*C. annuum*	Fruit
CCC31	Chiapa de Corzo, CHS	2012	*C. chinense*	Fruit
CCC32	Chiapa de Corzo, CHS	2012	*C. chinense*	Fruit
CCC33	Chiapa de Corzo, CHS	2012	*C. chinense*	Fruit
CCC35	Chiapa de Corzo, CHS	2012	*C. chinense*	Fruit
CCC36	Chiapa de Corzo, CHS	2012	*C. chinense*	Stem

CCC38	Chiapa de Corzo, CHS	2012	*C. chinense*	Stem
CCC40	Chiapa de Corzo, CHS	2012	*C. chinense*	Stem
CCC41	Chiapa de Corzo, CHS	2012	*C. chinense*	Stem
CCC42	Chiapa de Corzo, CHS	2012	*C. chinense*	Fruit
CCC43	Conkal, YUC	2012	*C. annuum*	Fruit
CCC44	Conkal, YUC	2012	*C. annuum*	Fruit
CCC45	Conkal, YUC	2012	*C. annuum*	Fruit
CCC46	Hopelchen, CAM	2012	*C. chinense*	Fruit
CCC47	Hopelchen, CAM	2012	*C. chinense*	Fruit
CCC48	Hopelchen, CAM	2012	*C. chinense*	Fruit
CCC49	Hopelchen, CAM	2012	*C. chinense*	Fruit
CCC50	Tekax, YUC	2012	*C. chinense*	Flower
CCC51	Tekax, YUC	2012	*C. chinense*	Flower
CCC52	Tekax, YUC	2012	*C. chinense*	Flower
CCC53	Tekax, YUC	2012	*C. chinense*	Flower
CCC54	Morelos, ROO	2012	*C. chinense*	Fruit
CCC55	Morelos, ROO	2012	*C. chinense*	Fruit
CCC60	Solidaridad, ROO	2013	*C. chinense*	Fruit
CCC61	Solidaridad, ROO	2013	*C. chinense*	Fruit
CCC62	Cardenas, ROO	2013	*C. chinense*	Fruit
CCC63	Cardenas, ROO	2013	*C. chinense*	Fruit
CCC64	Cardenas, ROO	2013	*C. chinense*	Fruit
CCC65	Cardenas, ROO	2013	*C. chinense*	Fruit
CCG5	Muna, YUC	2009	*Jatropha curcas*	Leaf
CCG14	Uman, YUC	2008	*J. curcas*	Leaf
CCJ2	Muna, YUC	2012	*J. curcas*	Leaf
CCJ3	Muna, YUC	2012	*J. curcas*	Leaf
CCJ4	Muna, YUC	2012	*J. curcas*	Leaf
CCJ5	Muna, YUC	2012	*J. curcas*	Leaf
CCJ8	Muna, YUC	2009	*J. curcas*	Leaf
CCJ10	Tekax, YUC	2012	*J. curcas*	Leaf
CCJ11	Tekax, YUC	2012	*J. curcas*	Leaf
CCJ12	Tekax, YUC	2012	*J. curcas*	Leaf
CCJ13	Merida, YUC	2012	*J. curcas*	Leaf
CCJ14	Merida, YUC	2012	*J. curcas*	Leaf
CCJ16	Merida, YUC	2012	*J. curcas*	Leaf

CCJ17	Opichen, YUC	2012	*J. curcas*	Leaf
CCJ18	Opichen, YUC	2012	*J. curcas*	Leaf
CCJ19	Opichen, YUC	2012	*J. curcas*	Leaf
CCJ20	Chiapa de Corzo, CHS	2013	*J. curcas*	Leaf
CCJ21	Chiapa de Corzo, CHS	2013	*J. curcas*	Leaf
CCJ22	Chiapa de Corzo, CHS	2013	*J. curcas*	Leaf

3.3.2 Phenotypic characterization

C. truncatum isolates were placed on PDA at 25 °C for five days. Mycelial plugs were punched from sporulating areas near the growing edge of the plate and placed onto PDA dishes. The plates were incubated at 25 °C for seven days and colony diameter was recorded daily. Growth rate was calculated as the 7-day average of mean daily growth (mm per day). The color and shape of the cultures were recorded visually. Three cultures of each isolate were evaluated and the experiment was conducted twice. To study the conidial morphology of each isolate, a conidial suspension was prepared by flooding the cultures with sterile distilled water (SDW). The suspension was placed on microscope slides and stained with lactophenol/ cotton blue. The length and width of 50 conidia per isolate were measured.

3.3.3 Pathogenicity tests

Twenty isolates (CCP4, CCP6, CCP27, CCP31, CCP43, CCG9, CCG12, CCC3, CCC18, CCC30, CCC33, CCC38, CCC43, CCC60, CCG14, CCJ2, CCJ11, CCJ13, CCJ19 and CCJ20) from all morphological groups and locations were selected for the pathogenicity tests. Conidial suspension was prepared in SDW by harvesting acervulli from 7-day old cultures and the concentration was adjusted to $1x10^6$ spores ml^{-1}.

Differences in the pathogenicity of the isolates were tested with a cross-inoculation assay, using pepper, papaya and physic nut. Leaves of greenhouse-grown physic nut plants were injected with 20 µL of conidial suspension. After inoculation, plants were placed in a growth chamber at 25±1 °C and 16-h light period. Disease-free mature fruits of pepper (var. Habanero) and papaya (var. Maradol) were inoculated with 20 µL of conidial suspension as previously described (23). After 7 days, the fruits/leaves were evaluated by measuring the area of the lesion. Six fruits/leaves were tested per isolate and experiments were conducted twice. Results were presented as the mean size of

lesion and were analyzed by ANOVA using the statistical software R v. 2.14.0 (R Core Team). Means were separated using Tukey´s multiple range test.

3.3.4 Genotypic analysis

DNA was extracted using the method described by Tapia-Tussell *et al.* (2006) and diluted to a concentration of 20 ng/µL until use. The same subgroup of 20 isolates was tested by ISSR markers. Five 15-bp primers derived from the repeated sequences $(GTG)_5$, $(CAG)_5$, $(GAC)_5$, $(TCC)_5$, and $(GACAC)_3$ were selected from previous reports (15) and used for genotypic characterization.

PCR reaction was carried out in 25 µL reaction volumes containing 50 ng of DNA, 1× PCR buffer (Invitrogen), 0.25 mM of each dNTP (Invitrogen), 2 mM $MgCl_2$, 0.8 µM of each primer and 1 U *Taq* polymerase (Invitrogen). DNA amplification was performed in a GeneAmp 9700 DNA Thermal Cycler (Perkin-Elmer), and consisted of an initial denaturing step at 95 °C for 5 min, followed by 40 cycles including: denaturing at 95 °C for 40 s, annealing for 1 min at either 42 °C for $(GACAC)_3$ and $(TCC)_5$, or 50 °C for $(GTG)_5$ and $(GAC)_5$, or 60 °C for $(CAG)_5$, extension for 1 min at 72 °C; and a final extension step of 5 min at 72 °C. PCR products were separated on 1.5% agarose gels stained with ethidium bromide.

3.3.5 Data analysis

Amplification products were scored computationally using the Gel Analyzer 2010 software (http://www.gelanalyzer.com). The presence or absence of bands was recorded in a binary matrix used to calculate genetic similarities between the isolates, based on the Dice similarity coefficient (7), and cluster analysis was performed by the Unweighted Pair Group with Arithmetic averaging. A Principal Coordinate Analysis was conducted to elucidate the genetic relationship among the tested isolates using the software PAST (13).

3.4 RESULTS

C. truncatum isolates were divided into six different morphological groups, based on colony appearance. A summary of the characteristics for each group is shown in Table 3.2, Figure 3.1.

Table 3.2. Summary of morphological characteristics of each *Colletotrichum truncatum* group identified.

Morphological Group	% isolates	Colony appearance
1	20.3	Light salmon to pale grey colour, with conidial masses in centre.
2	2.7	White to light salmon colony, with abundant mycelium and conidial masses produced in concentric rings.
3	39.0	Pale grey to black colour, with cottony mycelium and conidial masses in concentric rings.
4	17.7	White to pale grey thin mycelium with abundant cream to grey conidial masses in centre.
5	5.3	Olive colony colour with cottony mycelium and no visible conidial mass.
6	15.0	Salmon coloured colony with slight mycelium and cream conidial masses produced in concentric rings.

Most of the isolates belonged to morphotype 3, which had grey to black colonies, with cream-like conidial masses distributed concentrically around the plate. Isolates belonging to morphotype 2 had cottony, light salmon mycelia and were the least abundant. Both morphological groups 1 and 6 had similar colored colonies, but the conidial masses were differently distributed. Isolates grouped in morphotype 4 had white, thin mycelia with abundant cream-colored conidial masses arranged in the center of the plate. Isolates located in group 5, were collected only from pepper and papaya, and had olive-colored, cottony mycelia, with no apparent conidial mass in the plate.

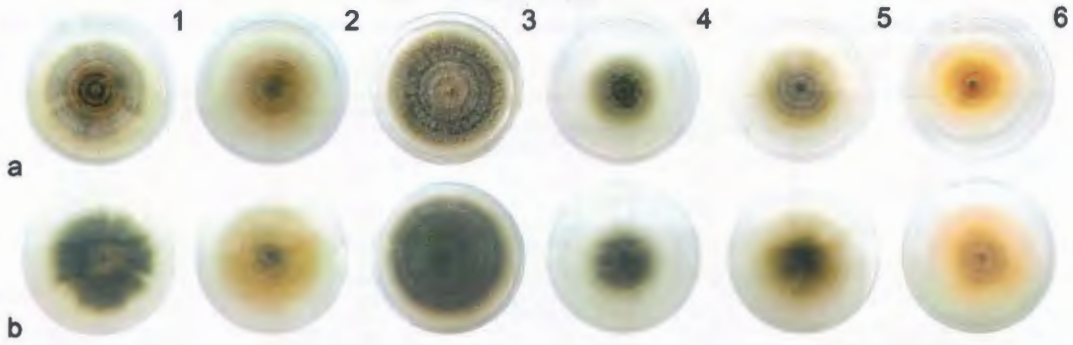

Figure 3.1. Morphotypes of *Colletotrichum truncatum*, a) upper colony surface and b) lower colony surface of isolates in groups 1-6.

The average length and width of the conidia ranged from 21.36 to 23.12 µm and 2.84 to 3.00 µm, respectively (Table 3.3). The isolates obtained from physic nut were significantly thinner than the other isolates ($P<0.05$). With respect to growth rate, little variation was found between the isolates, with average growth rates of 8.61 to 8.95 mm day^{-1}.

Table 3.3. Conidial dimensions and growth rate of the *Colletotrichum truncatum* isolates according to their host.

Host	Conidia length (mm) Mean ± SD	Conidia width (mm) Mean ± SD	Growth rate (mm) Mean ± SD
C. papaya	23.12±0.23a	3.00±0.03a	8.61±0.26a
Capsicum spp.	22.14±0.50a	2.89±0.11b	8.64±0.39a
J. curcas	21.36±0.41a	2.84±0.05b	8.95±0.30a

All isolates were able to infect fruits and leaves of the three hosts tested and produced visible spore masses under laboratory conditions, showing no host specificity. Pathogenic variability was also evaluated according to differences in the scored lesion sizes (Table 3.4).

Table 3.4. Pathogenicity of *C. truncatum* isolates on detached pepper and papaya fruits and on attached physic nut leaves.

Host	Isolate	Lesion diameter (mm) Mean ± SD		
		Papaya	Pepper	Physic nut
Carica papaya	CCP4	21.89±0.4d	6.10± 0.41def	3.99±0.39hij
C. papaya	CCP6	21.14±1.57de	6.09±0.29def	10.61±0.62a
C. papaya	CCP27	18.2±1.45fgh	6.76±0.98d	4.49±0.49ghi
C. papaya	CCP31	19.91±1.86ef	5.84±0.66def	4.17±0.66hij
C. papaya	CCP43	15.07±0.47j	5.73±1.14def	4.17±0.66hij
Capsicum annuum	CCG9	12.72±0.37k	5.46±0.36def	3.92±0.72hij
C. annuum	CCG12	26.2±1.67b	27.48±0.58a	8.22±0.29b
C. annuum	CCC3	17.26±0.26ghi	4.30±0.30fg	5.79±0.22efg
C. annuum	CCC18	22.45±0.96d	6.52±0.90de	7.09± 0.55bcde
C. annuum	CCC30	18.31±0.33fgh	5.49±0.24def	7.46±1.02bc
Capsicum chinense	CCC33	24.42±1.48c	5.84±0.24def	2.97± 0.31j
C. chinense	CCC38	18.94±1.83fg	4.66±0.40defg	3.50±0.18ij
C. annuum	CCC43	17.69±0.48gh	15.38±0.90b	7.33±0.36bcd
C. chinense	CCC60	22.17±2.76d	9.25±1.52c	5.87±0.62efg
Jatropha curcas	CCG14	27.04±2.27ab	3.02± 0.29g	5.04±0.16fgh
J. curcas	CCJ2	16.76±0.61hij	3.01±0.18g	5.13±0.07fgh
J. curcas	CCJ11	22.79±0.86cd	5.98±0.40def	6.56±0.56cde
J. curcas	CCJ13	15.59±0.33ij	4.18±0.25fg	4.08±0.46hij
J. curcas	CCJ19	11.18±0.61k	4.47±0.23efg	6.01± 0.24def
J. curcas	CCJ20	28.11±0.21a	13.94±2.71b	10.53±1.21a

Fifteen of the isolates exhibited similar aggressiveness and, in some cases, lesions were not significantly different from one other, comparing within the same host inoculated. Isolates CCJ20 and CCG12 were highly aggressive, causing the largest lesions on the three inoculation assays. In contrast, isolates CCG9, CCJ19 and CCC38 produced the smallest symptoms on both fruits and leaves (Figure 3.2).

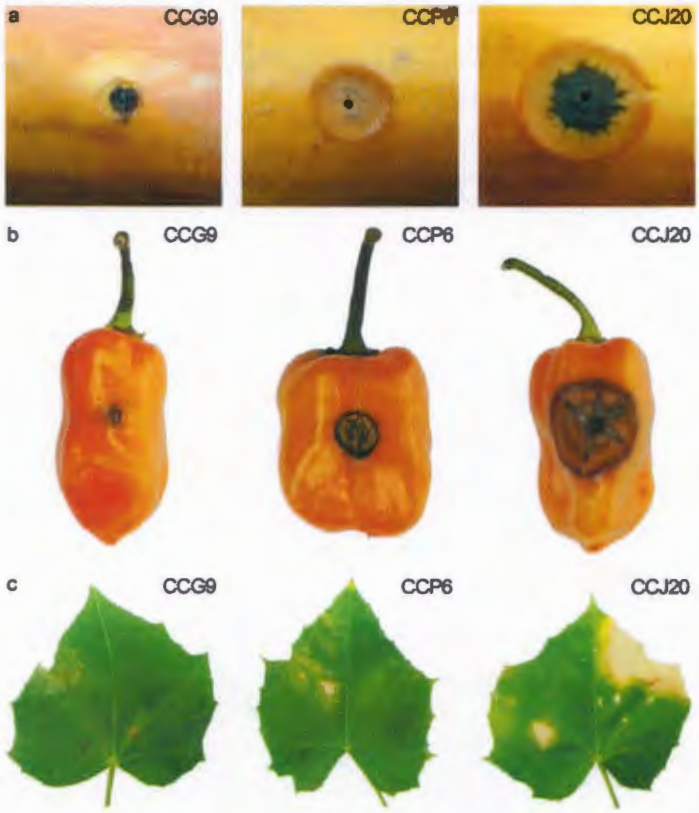

Figure 3.2. Cross inoculation of a) papaya fruits, b) pepper fruits and c) physic nut leaves with representative isolates. CCP6, isolated from *Carica papaya*; CCG9, isolated from *Capsicum annuum* and CCJ20, isolated from *Jatropha curcas*.

ISSR analysis was conducted on 20 isolates representing the different sampling sites and morphotypes. The five primer combinations used generated a total of 67 bands, of which 60 (89.55%) were polymorphic. The highest percentage of polymorphic loci (100%) was generated with the $(CAG)_5$ and $(TCC)_5$ primers, and the lowest proportion of polymorphic loci (76.4%) was found with the $(GAC)_5$ primer. The genetic fingerprint obtained for each isolate was different, although some isolates (e.g. CCJ11 and CCJ13) had very similar band patterns.

Principal Coordinate Analysis (PCO) showed a high variability, revealing the intraspecies division among the *C. truncatum* isolates. The first two coordinates scored 43.95% of the total variation (27.58% and 16.37%, respectively). The scatter plot is an indicator of the variance of the data and clearly separated the isolates into different

groups. Although there is no clear population structure within these groups, isolates from the same host clustered together (Figure 3.3). Also, isolates CCP4 and CCP6 clustered together and separated from the rest.

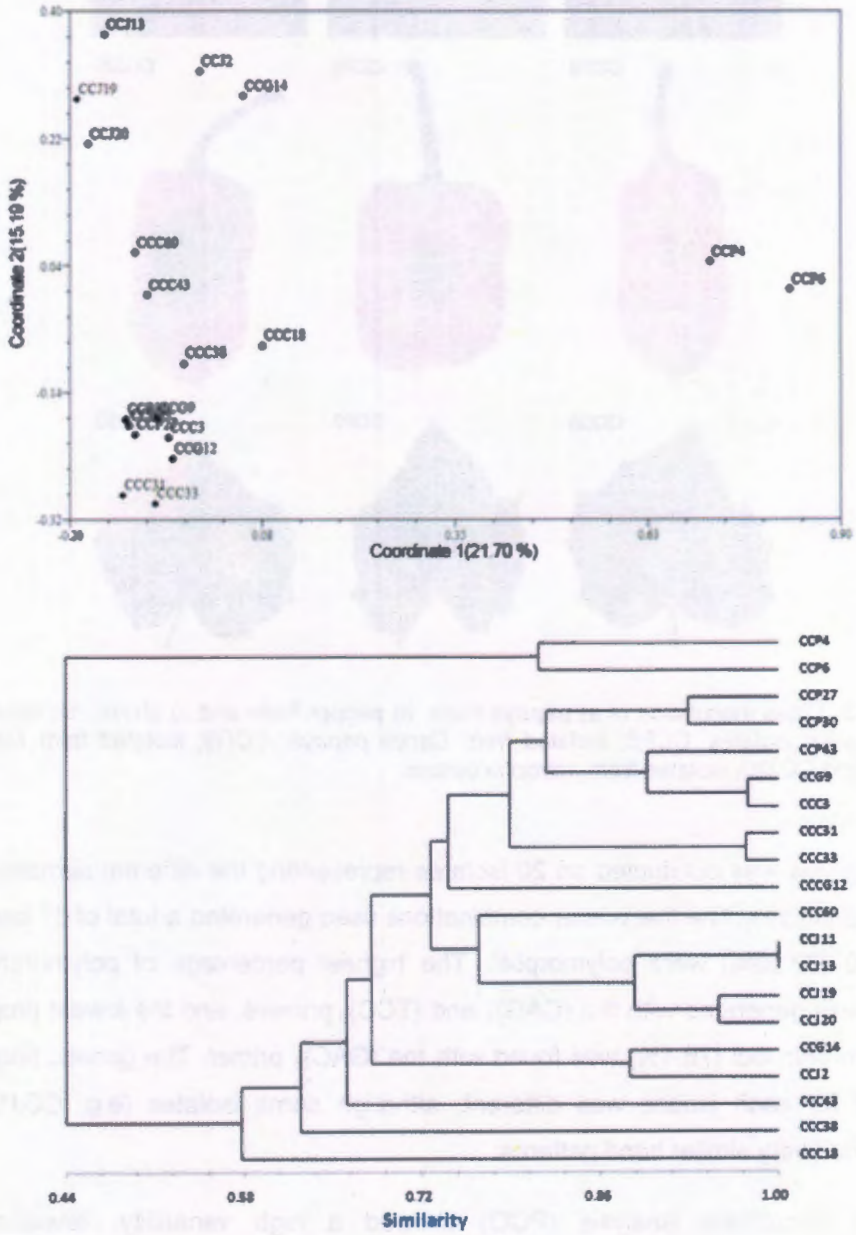

Figure 3.3. Principal Coordinate Analysis of the *C. truncatum* isolates and dendrogram generated with UPGMA method based on ISSR distance matrix.

Dice similarity indices varied from 0.31 to 0.97. The widest range was obtained among the papaya isolates (0.39 to 0.93), followed by the pepper isolates (0.44 to 0.95); the physic nut isolates were the most closely related, with Dice similarity coefficients ranging from 0.70 to 0.97.

The dendrogram based on UPGMA and similarity indices grouped the isolates into six different groups, with groups 3, 4 and 5 consisting of only one isolate (CCC43, CCC38 and CCC18, respectively), all from pepper. Group 1 had the largest number of isolates (13), and was divided into two subgroups. Subgroup 1A consisted of 7 isolates obtained from papaya and pepper collected from three of the five states. Subgroup 1B consisted of isolates collected from the other two states and, with the exception of isolate CCC60, all of them were collected from physic nut. Groups 2 and 6 were also formed with isolates from the same host (group 2 with isolates from physic nut, and group 6 from papaya), confirming the close relationship among the isolates from the same host.

3.5 DISCUSSION

C. truncatum isolates from the south-southeast region of Mexico were studied to assess their genetic variability and pathogenicity. Isolates were collected from papaya, pepper and physic nut in five geographic locations and were characterized according to their morphology and pathogenicity; ISSR fingerprinting was also performed.

The fungal isolates investigated here have extensive variability in morphological characteristics. C. *truncatum* isolates with the characteristics of morphotype 3 were the most abundant, which is consistent with a previous study of our group conducted on papaya (23), and, surprisingly, it is the only morphotype reported on pepper (17, 20). In the present work, the isolates were classified into six morphological groups. Group 5 was the only morphotype absent in the physic nut isolates. Nevertheless, *C. truncatum* isolates belonging to group 5 from pepper and papaya were able to infect physic nut leaves in the cross-inoculation assay, showing no restrictions for the pathogen in the infection process regarding the host. Several studies have reported the morphological diversity within the species of the genus (1, 11). Morphological descriptions for the *C. gloeosporioides* and *C. acutatum* species have been widely reported (18, 21). However, to our knowledge, this is the first study to reveal the extensive phenotypic variability in the *C. truncatum* populations.

The results of the pathogenicity tests revealed that all isolates could infect the three hosts and produce typical anthracnose symptoms, indicating their pathogenic potential on other plants. In the present study, *C. truncatum* isolates caused diseased symptoms on the papaya, pepper and physic nut cross-infection assay with variable intensity, suggesting the possible existence of more than one race or biotype, as has been indicated in previous reports for the *C. acutatum* species (16). In general, the most aggressive isolates were obtained from pepper and physic nut. Specifically, of the 20 isolates tested, CCJ20 and CCG12 were highly aggressive on the fruits and leaves of the three different hosts.

In the Yucatan Peninsula, physic nut has been used for decades as a living fence, and only recently (around 2010) has it begun to be used as a commercial crop, thus leading to the introduction of diverse cultivars from different origins, such as Asia, Central America and Brazil, which may have influenced the pathogen population by favoring the presence of the more aggressive genotypes in this host. In contrast, pepper has long been known as the main host for this fungal pathogen, which has promoted the appearance of several genotypes of this fungus.

C. truncatum isolates CCG9 and CCC38 were the least aggressive. Surprisingly, isolates CCP6, CCC18 and CCG14 were more pathogenic on alternative hosts. Similarly, it has been found that *C. acutatum* and *C. gloeosporioides* isolates did cause anthracnose symptoms on different plants both by artificial cross-infection and under field conditions in Brazil and Australia (2, 8, 24), indicating a lack of host specificity. This can favors the migration of the pathogen from one host to another, even more if these hosts are planted in surrounding areas as it occurs in Mexico.

The ISSR markers used were efficient in separating the isolates in this study. The resultant dendrogram showed high levels of genetic variation among isolates, all of which were grouped according to their host or geographical origin, but not according to their pathogenicity.

According to the PCO analysis, the populations under study did not have a defined structure. However, most of the isolates that clustered together were collected from the same host, which is similar to the results observed in the dendrogram. Previous reports have proved the effectiveness of the molecular markers in describing the genetic variations in different pathogen populations (4, 5). The results obtained herein suggest

the need to further investigate the phylogeny of this fungus with the help of multiloci analyses.

The fact that *C. truncatum* isolates were able to infect all hosts under study, has important implications for the genetic improvement of the hosts, in the search for anthracnose resistance, indicating that plant breeders must seek a broad genetic basis of resistance so that cultivars obtained from any of the three plant species, can have an adequate level of tolerance to the large number of possible genotypes of the fungus with pathogenic characteristics.

3.6 ACKNOWLEDGEMENTS

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CAPÍTULO IV. SENSITIVITY OF *Colletotrichum truncatum* FROM PAPAYA, PEPPER AND PHYSIC NUT TO FOUR FUNGICIDES AND MOLECULAR CHARACTERIZATION OF BENZIMIDAZOLE-RESISTANT ISOLATES[1]

4.1 ABSTRACT

Anthracnose, caused by *Colletotrichum truncatum* (syn. *C. capsici*) has become a common disease of tropical crops, affecting severely the quantity and quality of fruits and seeds, and therefore reducing their market value. For years, chemical control has been used extensively for the management of this disease. However, appearance of isolates resistant to the most commonly employed fungicides is increasingly widespread. Twenty *C. truncatum* isolates from pepper, papaya and physic nut were tested *in vitro* against four fungicides in order to determine their sensitivity. All of the isolates evaluated were resistant to azoxystrobin and thiabendazole, and susceptible to the cyprodinil + fludioxonil mix. To determine the molecular mechanism conferring QoI and benzimidazole resistance, the *cytochrome b* gene and the TUB-2 gene were characterized, revealing a glutamic acid to alanine substitution at position 198 of the TUB-2 gene in six of the twenty isolates tested. This work confirms the emergence of benzimidazole-based fungicides resistant mutants in the *C. truncatum* populations and highlights the need of monitoring fungicide sensitivity as an essential activity for the development of effective control schemes.

4.2 INTRODUCTION

Anthracnose, caused by the species of the genus *Colletotrichum*, is one of the most common diseases of plants (Bailey & Jeger, 1992). The losses caused by this disease occur mainly as a direct reduction in the quality and quantity of the harvested product. Usually, the fungus infects more than one part of the plant, causing the prevalence of the disease throughout the growing season (Waller, 1992). In Mexico, *Colletotrichum truncatum* (syn. *C. capsici,* Damm *et al.*, 2009) has been identified as the causative agent of anthracnose in different hosts, such as pepper (*Capsicum* spp.), papaya (*Carica papaya*) and physic nut (*Jatropha curcas*), affecting severely the quantity and quality of the fruits and seeds, and reducing their market value.

Effective control of the disease includes a set of chemical, cultural and biological practices. For the chemical control, several fungicides have been used for many years in

[1] **Torres-Calzada C.**, R. Tapia-Tussell, I. Higuera-Ciapara, R. Martin-Mex, A. Nexticapan-Garcez y D. Perez-Brito (2015). Sensitivity of *Colletotrichum truncatum* from papaya, pepper and physic nut to four fungicides and molecular characterization of benzimidazole-resistant isolates. Plant Disease. Accepted January 10, 2015. Manuscript ID: PDIS-11-14-1183-RE

the field, mainly fungicides based on benzimidazole. However, it is increasingly common to find isolates that are less sensitive or even resistant to these fungicides, as it has been seen in populations of *C. gloeosporioides* (Astua *et al.*, 1994; Solano & Arauz, 1995). The benzimidazole fungicides act by inhibition of nuclear division, binding to the b-tubulin molecule and disrupting microtubule-based processes (Brennan *et al.*, 2007). Resistance to benzimidazole fungicides is conferred by a single-point mutation in the b-tubulin gene and to date, different levels of resistance has been reported in several fungal pathogens (Chung *et al.*, 2006; Wong *et al.*, 2008). This resistance was attributed to missense point mutation in DNA resulting in amino acid exchanges in the β-tubulin protein (Ma & Michailides, 2005). In fungi, molecular analysis revealed four amino acid substitutions that led to the development of resistance to benzimidazole. The changes from glutamic acid (E) to alanine (A), glycine (G) or lysine (K) at position 198, or from phenylalanine (F) to tyrosin (Y) at position 200 cause the emergence of resistant isolates. Previous studies proposed that the resistance-conferring mutations allowed the formation of hydrogen bonds closing off the binding pocket and thus decreasing the binding affinity (Brennan *et al.*, 2007).

The strobilurines (QoI) are a different group of fungicides that have become one of the most important agricultural products used for the control of several diseases, including anthracnose (Avila-Adame *et al.*, 2003). These fungicides inhibit mitochondrial respiration by binding to the Qo center of cytochrome b and thus, blocking the transfer of electrons between the cytochrome b and the cytochrome c1, halting the production of ATP (Fernandez-Ortuño *et al.*, 2012). Several QoI resistant isolates have been described and for most of them, the major mechanism of resistance is the amino acid substitution of glycine with alanine at position 143 (G143A) of the cytochrome b protein (Farman *et al.*, 2001; Gisi *et al.*, 2002). The mutations F129L and G137R have been reported as mechanisms for QoI tolerance in *Alternaria solani* and *Pyrenophora teres*, but these changes are considered of minor importance because they lead to lower resistance compared with the mutation G143A (Stammler *et al.*, 2013).

For the effective control of anthracnose disease, monitoring of fungicide resistance is crucial. There are no previous reports exploring the resistance in the *C. truncatum* populations in Mexico. Thus, the objectives of this study were to (i) determine the sensitivity of *C. truncatum* isolates obtained from various host species (papaya, pepper and physic nut) to five different fungicides: benzimidazole, azoxystrobin, mancozeb,

cyprodinil and fludioxonil using in vitro bioassays, (ii) analyze the sequence of the *cytochrome b* gene and (iii) characterize benomyl-resistant genotypes based on amino acid sequence comparisons of the TUB-2 gene.

4.3 MATERIALS AND METHODS

4.3.1 Isolate collection and molecular characterization

Colletotrichum truncatum isolates were collected from lesions on leaves, fruits and seeds from diseased plants of papaya (*Carica papaya* L.), pepper (*Capsicum* spp.) and physic nut (*Jatropha curcas* L.) sampled in five different states in Mexico. Pure cultures were obtained by single spore isolation and maintained on PDA plates at 25°C. The identity of the isolates was determined by molecular identification using species-specific primers (Torres-Calzada *et al.*, 2011).

4.3.2 Selection of fungicides

Five chemicals were selected based on their current use to control the disease in Mexico. All fungicides tested were used as commercial formulations and were obtained from local suppliers, with one of them having two active ingredients (Table 4.2). Observing a more accurate response of the pathogen would be achieved by using the commercial formulations instead of the active ingredient (a. i.). Tecto and Dithane were prepared in dimethyl sulfoxide (DMSO) as stock solutions of 10 000 µg/ml. Amistar was diluted in methanol as a stock solution of 1000 µg/ml. The final concentration of DMSO and methanol were below 0.1%. Switch was prepared at a concentration of 100 µg/ml in sterile distilled water (SDW).

Table 4.1 Fungicides used in the *C. truncatum* assays. Based on Fungicide Resistance Action Committee website and fungicide labels.

Trade name	Manufacturer	FRAC group	Active Ingredient	Maximum rate	Chemical group	Mode of Action/ Target site
Tecto	Syngenta Crop Protection	1	60% Thiabendazole	2.0 kg/Ha	Benzimidazoles	B1: b-tubulin assembly in mitosis
Dithane	BASF, Corporation	M13	80% Mancozeb	2.5 kg/Ha	Dithiocarbamates	Multi-site contact activity
Amistar	Syngenta Crop Protection	11	50% Azoxystrobin	500 mg/Ha	Methoxy-acrylates	C3: complex III cytochrome bc1 at Qol site
Switch	Syngenta Crop Protection	9	37.5% Cyprodinil	1.0 kg/Ha	Anilino-pyrimidines	D1: methionine biosynthesis
		12	25% Fludioxonil		Phenylpyrroles	E2: MAP/ Histidine- kinase in osmotic signal transduction

4.3.3 *In vitro* assessment of fungicide sensitivity

Sensitivity of the *Colletotrichum* isolates to the different fungicides was determinate using mycelia growth assays. Each isolate was plated onto PDA at 25°C. 5-mm plugs were punched from actively sporulating areas near the growing edge of a 5-day-old culture of these isolates. Mycelia plugs were placed onto PDA dishes amended with 0, 0.01, 0.1, 1 and 10 µg/ml a. i. of cyprodinil + fludioxonil, and 0, 0.1, 1, 10 and 100 µg/ml a.i. of mancozeb, and 0, 10, 100, 1000 and 10 000 µg/ml a.i. of azoxystrobin or thiabendazole. For the azoxystrobin sensitivity assay, an additional test was performed. Salicylhydroxamic acid (SHAM) at a final concentration of 100 µg/ml was added to the culture media instead of the fungicide to observe any effect of the alternative oxidase on in vitro growth. The plates were incubated at 25°C for five days. Control plates consisted of a 5-mm diameter plug of the solates inoculated on nonamended PDA. The diameter of each colony was used to calculate the percent relative growth (%RG) compared with the control, as well as the percent of the relative growth inhibition (%RGI = 100-%RG). The 50% effective dose (ED_{50}) value was determined by plotting the % RG on each fungicide-amended medium compared with growth on the control versus the log concentration of the fungicide treatment, and then calculating the regression line through the linear portion of the dose-response curve (Wong *et al.*, 2008).

4.3.4 Analysis of *cytochrome b* gene

The total genomic DNA was extracted according to the method described by Tapia-Tussell *et al.* (2006) and diluted to a final concentration of 50 ng/µl. The pair of primers P1 and P2 described by Avila-Adame *et al.* (2003) was used to amplify a fragment of the *cyt b* gene. The PCR reaction mixture and program were carried out according to previous reports with the following parameters: 94°C for 7 min, followed by 30 cycles including: 30 s at 94°C, annealing for 30 s at 55°C, 1 min at 72°C; and a final extension step of 72°C for 7 min. PCR products were separated by electrophoresis on ethidium-bromide stained 1.5% agarose gels.

4.3.5 Analysis of *β-tubulin* gene

Resistance or sensitivity among the isolates to the benzimidazole-based fungicide was evaluated. The *β-tubulin* gene, including the aminoacid codons 198 and 200 was amplified using the TUB2-1 and TUB2-1 primer pair. The PCR reaction mixture and reaction program followed were those reported by Wong *et al.* (2008). The PCR amplifications were carried out using a (termociclador) and consisted of an initial denaturing step at 95°C for 1 min, followed by 30 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C, and a final extension step of 1 min at 72°C. PCR products were separated by electrophoresis in 1.5% (w/v) agarose gels and visualized by ethidium-bromide staining.

4.4 RESULTS

In total, 134 isolates were collected from the three different hosts, mainly from *Capsicum* spp. Twenty representative isolates were selected for this study: five from papaya, nine from pepper and six from physic nut (Table 4.2).

Table 4.2 *C. truncatum* isolates selected for the *in vitro* sensitivity assay

Isolate	Host	Origin	Collection year
CCP4	*Carica papaya*	Champoton, CAM	2009
CCP6	*C. papaya*	Morelos, ROO	2009
CCP27	*C. papaya*	Tizimin, YUC	2012
CCP31	*C. papaya*	Balankan, TAB	2012
CCP43	*C. papaya*	Arriaga, CHS	2013
CCG9	*Capsicum annuum*	Peto, YUC	2008
CCG12	*C. annuum*	Tekax, YUC	2008
CCC3	*C. annuum*	Balankan, TAB	2012
CCC18	*C. annuum*	Balankan, TAB	2012
CCC30	*C. annuum*	Calkiní, CAM	2012
CCC33	*Capsicum chinense*	Chiapa de Corzo, CHS	2012
CCC38	*C. chinense*	Chiapa de Corzo, CHS	2012
CCC43	*C. annuum*	Conkal, YUC	2012
CCC60	*C. chinense*	Solidaridad, ROO	2013
CCG14	*Jatropha curcas*	Uman, YUC	2008
CCJ2	*J. curcas*	Muna, YUC	2012
CCJ11	*J. curcas*	Tekax, YUC	2012
CCJ13	*J. curcas*	Merida, YUC	2012
CCJ19	*J. curcas*	Opichen, YUC	2012
CCJ20	*J. curcas*	Chiapa de Corzo, CHS	2013

4.4.1 Response tu fungicides

Differences in sensitivities to the fungicides were observed for all isolates (Table 4.3). Isolates with ED_{50} values <1 µg/ml were considered as susceptible (S), whereas isolates with ED_{50} values ranged from 1 to 100 µg/ml were grouped as intermediate resistant (IR). Finally, isolates with ED_{50} values greater than 100 µg/ml were highly resistant (HR).

For the 20 isolates selected, the calculated ED_{50} for the benzimidazole experiment ranged from 6.6 to 2673.9 µg/ml. All isolates resulted to be resistant to this fungicide and there was no effective dose response to the concentrations tested. Similar results were obtained with the isolates evaluated against azoxystrobin.

The ED_{50} values for this fungicide ranged from 37.2 to 6318.7. With mancozeb, 70% of the isolates were IR showing ED_{50} values between 1.2 and 36.5 µg/ml, and only six isolates were susceptible, with ED_{50} values between 0.5 and 0.9 µg/ml. No HR isolates were found with this fungicide. Results from the experiment using cyprodinil + fludioxonil showed that all isolates were susceptible to this fungicide, with the ED_{50} values ranged from 0.008 to 0.37 µg/ml.

Generally, isolates CCC38, CCC43, CCC60 and CCG14 were inherently more sensitive to the four fungicides and exhibited the lowest ED_{50} values in the experiments. In all cases, they were either S or IR, with the exception of CCG14 when was evaluated with amistar. Interestingly, almost all isolates showed the same level of sensitivity between benzimidazole and azoxystrobin.

4.4.2 Importance of fungicide class in *C. truncatum* resistance

Results from the sensitivity bioassay showed that isolates of *C. truncatum* collected from physic nut were more sensitive to benzimidazole than isolates from papaya and pepper. Eighty percent of isolates from papaya were highly resistant and similar levels of resistance were found in the pepper isolates. The highest levels of resistance were observed to the QoI fungicide, with 100% of isolates from papaya were highly resistant, as well as 67% of isolates from pepper and 50% of isolates form physics nut.

In general, isolates exhibit intermediate levels of resistance against mancozeb, which is considerate as a midrange fungicide. Isolates from physic nut were the most resistant to this fungicide (83%). Forty percent of the isolates from papaya and 34% of the isolates from pepper resulted susceptible to mancozeb.

The lowest levels of fungicide resistance were obtained with cyprodinil + fludioxonil, showing very low growth in all isolates compared with the controls.

Table 4.3 Phenotypic response and half maximal effective concentration (ED_{50}) values (µg mL^{-1}) obtained for *C. truncatum* isolates with four different fungicides.

Isolate	Fungicide sensitivity (ED_{50} µg mL^{-1})			
	Tecto	Amistar	Dithane	Switch
CCP4	IR (81.6)	HR (947.3)	IR (3.6)	S (0.045)
CCP6	HR (2673.9)	HR (325.3)	S (0.6)	S (0.37)
CCP27	HR (689.8)	HR (5980.0)	IR (36.5)	S (0.008)
CCP31	HR (463.5)	HR (1145.9)	IR (2.2)	S (0.008)
CCP43	HR (305.3)	HR (6318.7)	S (0.8)	S (0.019)
CCG9	HR (910.7)	HR (3335.1)	IR (5.0)	S (0.027)
CCG12	HR (750.4)	HR (701.3)	S (0.7)	S (0.009)
CCC3	HR (920.6)	HR (163.3)	IR (4.5)	S (0.009)
CCC18	HR (1728.1)	HR (6252.9)	IR (4.6)	S (0.008)
CCC30	HR (700)	HR (141.3)	IR (1.2)	S (0.121)
CCC33	HR (923)	HR (563.2)	IR (8.0)	S (0.009)
CCC38	IR (20.4)	IR (77.2)	S (0.8)	S (0.008)
CCC43	IR (6.6)	IR (92.7)	S (0.9)	S (0.008)
CCC60	IR (7.0)	IR (83.3)	IR (7.4)	S (0.047)
CCG14	IR (7.6)	HR (238.5)	S (0.5)	S (0.064)
CCJ2	HR (1909.1)	HR (143.2)	IR (1.4)	S (0.008)
CCJ11	IR (7.7)	IR (51.2)	IR (5.8)	S (0.009)
CCJ13	IR (37.7)	IR (37.7)	IR (1.8)	S (0.125)
CCJ19	HR (611.8)	HR (611.8)	IR (2.7)	S (0.094)
CCJ20	IR (83.0)	IR (83.0)	IR (23.3)	S (0.007)

HR, highly resistant
IR, intermediate resistant
S, susceptible

4.4.3 Analysis of *cytochrome b* gene

PCR products using primer pair P1 and P2 corresponding to amino acids 82 to 273 were sequenced for all isolates. All twenty isolates exhibit identical sequences, conserving the GGT at codon 143 of *cyt b*. The G143A amino acid exchange responsible for the resistant phenotype was not detected in the isolates, neihter other exchanges at different positions throughout the sequence.

4.4.4 Molecular identification of benzimidazole-resistant isolates

Nucleotide sequences from the amplification of the TUB-2 gene of the twenty isolates were obtained. The deduced amino acid sequences corresponding to residues 152 to 303 contained the residues at positions 198, 200 and 240 typically associated with benzimidazole resistance. The mutation at the translated sequences of the isolates are shown (Table 4.4), 75% of the isolates classified as highly resistant showed a point mutation from GAG to GCG at codon 198 (E198A) and six of the seven intermediate resistant isolates retained the E at position 198. However, no mutations were detected at the sequences corresponding with codons 200 (F) and 240 (L).

Table 4.4 Mutation and deduced amino acid substitutions in the partial sequence of the *b-tubulin* gene in *C. truncatum* isolates with different *in vitro* responses to benzimidazole.

Isolate	Response	% Relative growth	Sequence in codon	
			198	200
CCP4	IR	41.11	GCG	TTC
CCP6	HR	71.46	GCG	TTC
CCP27	HR	57.91	GCG	TTC
CCP31	HR	56.37	GCG	TTC
CCP43	HR	52.57	GCG	TTC
CCG9	HR	76.22	GCG	TTC
CCG12	HR	63.25	GCG	TTC
CCC3	HR	61.81	GCG	TTC
CCC18	HR	88.93	GCG	TTC
CCC30	HR	55.04	GCG	TTC
CCC33	HR	63.27	GAG	TTC
CCC38	IR	40.51	GAG	TTC
CCC43	IR	17.92	GAG	TTC
CCC60	IR	24.96	GAG	TTC
CCG14	IR	26.59	GAG	TTC
CCJ2	HR	67.42	GAG	TTC
CCJ11	IR	30.63	GAG	TTC
CCJ13	IR	23.11	GAG	TTC
CCJ19	HR	54.46	GAG	TTC
CCJ20	IR	46.12	GAG	TTC

Sequences of six of the isolates revealed base substitutions that led to four different codon changes: S153F for CCP4 and CCP6, D155N for CCG9 and CCP27, G158E for CCC18 and G158A for CCJ20. These codon changes need further evaluation in seeking for their relation in the benzimidazole-resistant phenotype of the isolates carrying them (Fig. 4.1).

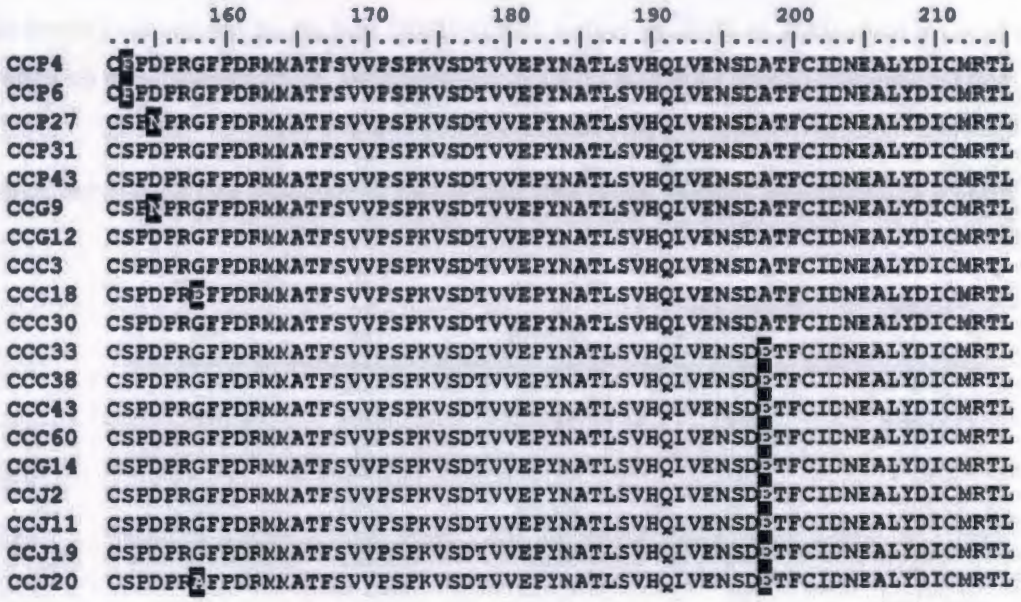

Fig. 4.1 Amino acid sequences spanning residues 152 to 251 of the β-tubulin protein for 20 *Colletotrichum truncatum* isolates. The amino acids exchanged are highlighted.

4.5 DISCUSSION

In the present study, isolates from various hosts were evaluated for their sensitivities to different fungicides with diverse modes of action. The molecular mechanisms conferring resistance to benzimidazoles and to the QoI fungicide azoxystrobin were also investigated.

According to our results, isolates were classified as sensitive, intermediate resistant, or highly resistant to four commercial fungicides in a mycelia growth assay and the relative growth of these isolates at the discriminatory doses selected were consistent to those reported previously (Wong *et al.*, 2008). The results strongly suggest the development of resistance to QoI and benzimidazole-fungicides in populations of *C. truncatum*.

All isolates showed a similar high resistance to both fungicides, higher than when tested with other products. Additionally, we observed a high frequency (70%) of resistance against dithane in every host and location sampled. According to the results, site location did not influence the level of resistance or sensitivity, making apparent that the isolates are distinguished based on their host. This may be due to the intensive production of pepper and papaya, with constant use of fungicides during the growing season, then exposing these isolates to an increased contact with this chemicals, compared with isolates from physic nut, which until a couple of years was used only as a living fence and intensive cultivation is recent, and therefore with lower chemical applications.

Results from this research showed that all isolates were sensitive to the fludioxonil + cyprodinil fungicide mix (Switch; Syngenta Crop), suggesting that fungicide mixtures provide an adequate control of the pathogen, and also reduce the risk of fungicide resistance development. Further testing is needed in order o determine whether one of the two active ingredients is the most active in the mix, or that there is some synergy between them.

Quinone outside inhibitors (QoIs) are another site-specific fungicides, which inhibit mitochondrial respiration at the ubiquinol oxidation center site of the cytochrome bc1 enzyme complex. Since their introduction to the market, QoIs have been extensively applied for the control of most fungal diseases. Azoxystrobin, the first QoI fungicide was launched for agricultural pest management in 1996, and now is registered for its use on 84 different crops in 72 countries, making it the most used fungicide around the world (Bartlett *et al.*, 2002). However, to date the development of resistance among many phytopathogenic fungi have been reported (Banno *et al.*, 2009; Yin *et al.*, 2012). A point mutation at codon 143 in the *cyt b* gene giving rise to a substitution from Glycine to Alanine (G143A) is considered to confer resistance to QoIs, and various fungal pathogens carrying the mutation have been reported (Heaney *et al.*, 2000; Sierotzki *et al.*, 2007). Other reports described F129L and G137R point mutations at cyt b gene also confer resistance to these fungicides, but in lower levels (Farman, 2001; Olaya *et al.*, 2003).

In this study, azoxystrobin caused practically null reduction in mycelia growth in all tested isolates, regardless of location or host origin and according to the ED50 values obtained in the assay, 70% of the isolates were classified as highly resistant. Analysis of the *cyt b*

gene revealed no amino acid substitutions at positions related to QoI resistance, suggesting that other mechanisms might be involved in the resistant phenotype. Previous reports had described an alternative respiration pathway sustained by an alternative oxidase (Bohr *et al.*, 1999; Miguez *et al.*, 2004; Wood *et al.*, 2003), as well as the expression of a gene coding an efflux transporter involved in preventing the accumulation of toxic concentrations of fungicides inside the cells (Andrade *et al.*, 2000; De Waard *et al.*, 2006., Roohparvar *et al.*, 2007). Therefore, we hypothesize that a combination of these mechanisms could be involved in maintaining the resistance of the isolates under study.

The phenotypic response of benzimidazole resistance in *C. truncatum* isolates was supported with the results of the molecular analysis of the tubuline gene. Site-specific mutations in the TUB2 gene have been identified for resistance in several fungal species (Downing *et al.*, 2000) and previous reports determined that mutations leading to the amino acid substitutions E198A, E198K and F200Y, are involved in conferring resistance to benzimidazole-based fungicides (Jung *et al.*, 1990; Peres *et al.*, 2004; Yarden & Katan, 1993). In this study, the amino acid substitution E198A was observed in the protein sequence of *C. truncatum* isolates and to our knowledge, this is the first report of the mutation in this species. We found that 75% of the fungal isolates with the amino acid substitution at position 198 were considered as highly resistant, with ED50 values higher than 300 µg/ml. The E198K and F200Y amino acid changes were not observed among the isolates collected in this work. However, some amino acid substitutions have been noted at other positions different to those usually reported with benzimidazole resistance but whether these changes are involved in the resistant phenotype, or are due to normal allelic variation needs further verification.

Monitoring of fungicide sensitivity is an essential activity for the development of effective control schemes. The results described here exhibit the rapid emergence of benzimidazole-based and QoI fungicides resistant mutants in the *C. truncatum* populations. Continuous monitoring programs, either with bioassays, or with PCR methods detecting mutations in the target genes is essential to evaluate changes in the EC values leading to the emergence of resistant phenotypes and also allows us to understand the population dynamics of the pathogen according to different chemical control programs in order to design adequate and effective management strategies for anthracnose.

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CAPÍTULO V. DISCUSIÓN GENERAL Y CONCLUSIONES

5.1 DISCUSIÓN GENERAL

El objetivo principal de este trabajo fue caracterizar una colección de cepas de *Colletotrichum truncatum* (syn. *C. capsici*, Damm *et al.*, 2009) aisladas de *Carica papaya*, *Capsicum* spp. y *Jatropha curcas* en cinco estados diferentes del sur-sureste de México. Se colectaron en total 108 aislamientos, los cuales provinieron de diferentes órganos de las plantas, principalmente de los frutos, aunque también se colectaron de hojas, peciolos, tallos y flores. Del total de la colecta, 39 fueron aislados de *Carica papaya*, 19 de *Capsicum annuum*, 31 de *C. chinense* y 19 de *Jatropha curcas*. Los síntomas de la enfermedad observados en campo coincidieron con los reportados por Sutton (1992) y consistieron en lesiones húmedas, ligeramente hundidas, circulares, de color café oscuro, con la formación de anillos concéntricos de acérvulos y masas de conidios de color crema o grises.

La caracterización morfológica de estos aislados puso de manifiesto la gran variabilidad fenotípica de la especie. Los aislamientos crecidos *in vitro* fueron separados por su morfología en seis grupos distintos. De acuerdo con Shenoy *et al.* (2007), la cepa tipo de *C. capsici,* descrita por Sydow, tiene las siguientes características: micelio aéreo blanco a gris, oscureciéndose con el paso del tiempo, con el reverso color gris, y acérvulos color café oscuro, con setas oscuras y el morfotipo 3, descrito en este trabajo, coincide con este reporte. Al igual que Shenoy, Rampersad (2011) reportó un solo morfotipo de *C. capsici,* el cual fue hallado en *C. papaya*, y coincide con las características del morfotipo 5 descritas aquí. Sin embargo, los otros morfotipos no han sido reportados previamente en ningún otro trabajo.

Con respecto a las características microscópicas, los seis grupos tuvieron la misma morfología. Los conidios fueron unicelulares, hialinos, falcados, con ápices agudos; en todos se observó la formación de acérvulos circulares, con setas abundantes y rígidas lo que coincide con lo reportado anteriormente para esta especie (Shenoy *et al.*, 2007).

El análisis de la huella genética utilizando marcadores moleculares sirvió para complementar la caracterización de los aislamientos bajo estudio. La información de los patrones de bandas obtenidos con los iniciadores ISSR, se utilizó para calcular la distancia genética entre ellos y para la construcción de un dendrograma, el cual

mostró altos niveles de variabilidad genética entre los aislamientos, agrupándolos de acuerdo a su hospedero de origen o a su lugar de procedencia. Por otro lado, no se encontró relación entre los genotipos y sus morfotipos, ya que, en algunos casos, aislamientos de un mismo genotipo presentaron morfotipos diferentes (Manuel *et al.*, 2009). El conocimiento de la diversidad genética y morfológica del patógeno, así como de la posible relación entre ellas, resulta indispensable para determinar si las diferencias a nivel genético o morfológico están relacionadas con la capacidad del patógeno para infectar a un amplio rango de hospederos, o en caso contrario, para restringir su patogenicidad (Freeman *et al.*, 1998).

El análisis de coordenadas principales (PCO) reveló que las poblaciones bajo estudio no tienen una estructura definida. Sin embargo, la mayoría de los aislamientos se agruparon nuevamente de acuerdo a su hospedero de origen, tal como se observó en el dendrograma.

Dentro del género *Colletotrichum*, existen muchos trabajos enfocados en el conocimiento de la diversidad genética. Se ha visto que especies como *C. acutatum* y *C. gloeosporioides* muestran mayor heterogeneidad genética, explicada, tal vez, por la presencia de un teleomorfo (Freeman *et al.*, 1998). Van Hemelrijck *et al.* (2010) reportan que las poblaciones de *C. acutatum* aisladas de fresa en Bélgica, son genéticamente muy diferentes, a pesar de que estudios previos realizados en Francia, Israel, Bulgaria y España (Garrido *et al.* 2009; Jelev *et al.*, 2008; Urena-Padilla *et al.*, 2002), demostraron que no existía gran diversidad genética en esta especie, y que una posible explicación a esto, es que el material vegetal del cual se obtuvieron los aislamientos de *C. acutatum*, provenía de diferentes lugares, lo cual corrobora nuevamente la influencia del lugar de procedencia en la variabilidad de los patógenos.

Con base en los resultados obtenidos en la caracterización molecular, y observando los resultados de las colectas y la posterior caracterización morfológica, se seleccionaron veinte aislamientos, provenientes de diferentes localidades, que además procedían de los tres hospederos bajo estudio, y presentaban las características de los diferentes grupos morfológicos. Los aislamientos seleccionados fueron inoculados en los tres hospederos de origen, con el fin de observar las diferencias en severidad que pudieran existir entre ellos. Los resultados de este trabajo demostraron que el fenómeno de infección cruzada ocurre entre los aislamientos de *C. truncatum*. Los

aislamientos CCG12 y CCJ20 fueron más agresivos, en comparación con los otros aislamientos inoculados. Por el contrario, las lesiones más pequeñas se obtuvieron al inocular los aislamientos CCG9 y CCC38. El tamaño de las lesiones no estuvo relacionado con el hospedero inoculado, indicando que entre los aislamientos no existe especificidad hacia el hospedero de origen, en contraste con lo reportado previamente por Kim *et al.* (2009), lo cual sugiere la adaptación del patógeno a hospederos alternos, desarrollando su capacidad para infectar a diferentes cultivos y favoreciendo por lo tanto, su migración de un hospedero a otro, principalmente si los cultivos se encuentran localizados en áreas cercanas, tal como sucede con las plantaciones de papaya y chile en México (Tapia-Tussell *et al.*, 2008).

La respuesta fenotípica de los aislamientos hacia las pruebas de sensibilidad a fungicidas, puso de manifiesto la resistencia del patógeno hacia los fungicidas Tecto (thiabendazole) y Amistar (azoxistrobin), que son los productos más utilizados para el control de las enfermedades en campo. Esta respuesta de resistencia *in vitro* hacia el thiabendazole, fue complementada con los resultados del análisis molecular del gen de tubulina. Se detectó una mutación puntual en el codón que codifica al aminoácido 198. El cambio de un ácido glutámico por una alanina, ha sido reportado previamente como el responsable de la resistencia en otras especies de hongos (Peres *et al.*, 2004; Yarden & Katan, 1993).

Por otra parte, todos los aislamientos fueron sensibles al fungicida Switch, que es una mezcla que contiene dos ingredientes activos. Esto demuestra que, tal como sugiere Fairchild *et al.* (2013), el empleo de fungicidas en mezcla provee un control más adecuado del patógeno y reduce el riesgo de la aparición de resistencia.

Los resultados de este trabajo ponen de manifiesto la rápida aparición de resistencia hacia los fungicidas empleados en campo. Por lo que, es necesario establecer programas de monitoreo continuo, que nos permitan detectar la aparición de fenotipos resistentes, con el fin de aplicar programas de manejo que resulten efectivos en el control de la enfermedad.

5.2 CONCLUSIONES

- *Colletotrichum truncatum* aislado de *Carica papaya, Capsicum* spp, y *Jatropha curcas* mostró una gran variabilidad fenotípica.
- Las características morfológicas *C. truncatum* permitieron establecer seis morfotipos, de los cuales, el morfotipo tres es el más abundante y coincide con el reportado para esta especie.
- Los conidios de los aislados obtenidos de *C. papaya* fueron significativamente más grandes que aquellos provenientes de *Capsicum spp.* y *Jatropha curcas.*
- Las huellas genéticas obtenidas de las cepas de *C. truncatum*, mostraron una relación entre los aislamientos y el hospedero del cual se aislaron
- Todos los aislamientos colectados son capaces de infectar a papaya, chile y Jatropha, sin importar su hospedero de origen.
- Las cepas de *C. truncatum* aisladas de chile fueron los que generaron mayores tamaños de lesión en todos los hospederos inoculados.
- Todos los aislamientos de *C. truncatum* evaluados *in vitro* fueron resistentes a los fungicidas Tecto y Amistar
- La resistencia de los aislamientos hacia el fungicida Tecto está dada por una mutación puntual en el gen que codifica la proteína β-tubulina.

5.3 PERSPECTIVAS

Colletotrichum truncatum (syn. *C. capsici*) es un patógeno muy versátil. En este trabajo ha quedado de manifiesto su gran variabilidad genética y morfológica, así como su alta capacidad infectiva.

Los resultados obtenidos del análisis de la diversidad genética mediante marcadores ISSR facilitaron la identificación de las poblaciones del patógeno presentes en los cultivos bajo estudio. Sin embargo, considerando la variabilidad encontrada, los resultados sugieren la necesidad de profundizar en el estudio del patógeno, mediante el empleo de otras técnicas moleculares, tales como los análisis filogenéticos multilocus, u otras técnicas de marcadores moleculares que nos permitan obtener mayor información sobre el genoma del hongo.

Por otra parte, los ensayos de sensibilidad *in vitro* de *C. truncatum* permitieron determinar la efectividad de los productos químicos empleados en campo para controlar al patógeno, lo cual es un punto crítico en el cuidado de los cultivos

afectados. A pesar de haberse demostrado la efectividad de los productos en el laboratorio, es necesario evaluarlos *in vivo* para garantizar su efectividad en campo.

Se ha visto que la resistencia de los hongos a los fungicidas puede estar dada por mutaciones en el genoma. En este trabajo se identificaron, además de las mutaciones previamente reportadas para conferir resistencia a bencimidazoles, otras mutaciones sitio específicas en posiciones diferentes, por lo que se requiere analizar si estos cambios están involucrados en la respuesta fenotípica del patógeno hacia el fungicida.

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