Departamento de Microbiología

Facultad de Ciencias

TESIS DOCTORAL

Detection and characterization of mango malformation and its causal agent in Spain

(Detección y caracterización de la malformación del mango y su agente causal en España)

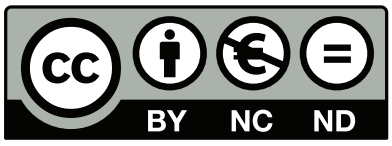
María Crespo Palomo

Málaga, Julio de 2014

Publicaciones y
Divulgación Científica

AUTOR: María Crespo Palomo

EDITA: Publicaciones y Divulgación Científica. Universidad de Málaga



Memoria presentada por

María Crespo Palomo

Para optar al grado de Doctor por la Universidad de Málaga

Detection and characterization of mango malformation and its causal agent in Spain

(Detección y caracterización de la malmormación del mango y su agente causal en España)

Directores:

Dr. Antonio de Vicente Moreno
Catedrático de Microbiología
Universidad de Málaga

Dr. Juan Antonio Torés Montosa
Investigador Científico del CSIC
Instituto de Hortofruticultura
Subtropical y Mediterránea la Mayora (IHSM- CSIC-UMA)

UNIVERSIDAD DE MÁLAGA
Departamento de Microbiología
Málaga 2014

EVALUATION COMMITTE

President

Dr. Rafael M. Jiménez Díaz

Departamento de Agronomía, Universidad de Córdoba;
Departamento de Protección de Cultivos, (IAS-CSIC)

Secretary

Dr. Alejandro Pérez García

Departamento de Microbiología, Universidad de Málaga

Vocals

Dr. Claude Alabouvette

UMR Microbiologie et Géochimie des Sols, Institut National De La Recherche Agronomique Institut (INRA), Dijon, Francia

Dra. Mª del Mar Jiménez Gascó

Department of Plant Pathology and Environmental Microbiology,
College of Agricultural Science, EE.UU

Dr. José García Jiménez

Departamento de Ecosistemas Agroforestales/Patología Vegetal
Universidad Politécnica de Valencia

Substitutes

Dr. Iñaki Hormaza Urroz

Instituto de Hortofruticultura
Subtropical y Mediterránea La Mayora, Consejo Superior de Investigaciones Científicas (CSIC)

Dr. Diego Romero Hinojosa

Departamento de Microbiología
Universidad de Málaga

Departamento de Microbiología

Facultad de Ciencias

Dr. D. JUAN JOSÉ BORREGO GARCÍA,

Director del Departamento de Microbiología de la Universidad de Málaga.

INFORMA: que Dña. María Crespo Palomo ha realizado en los laboratorios de este departamento la mayor parte del trabajo experimental conducente a la elaboración de la presente Memoria de Tesis Doctoral.

Y para que así conste, y tenga los efectos que correspondan, en cumplimiento de la legislación vigente, extiendo el presente informe en Málaga, a 9 de mayo de 2014

Fdo: D. Juan José Borrego García

Departamento de Microbiología

Facultad de Ciencias

Dr. D. ANTONIO DE VICENTE MORENO, Catedrático del Departamento de Microbiología de la Universidad de Málaga.

Dr. D. JUAN ANTONIO TORÉS MONTOSA, Investigador Científico del Instituto de Hortofruticultura Subtropical y Mediterránea La Mayora (IHSM-CSIC-UMA).

INFORMAN: que Dña. María Crespo Palomo ha realizado bajo nuestra dirección el trabajo experimental conducente a la elaboración de la presente Memoria de Tesis Doctoral.

Y para que así conste, y tenga los efectos que correspondan, en cumplimiento de la legislación vigente, extiendo el presente informe en Málaga, a 9 de mayo de 2014.

Fdo: Antonio de Vicente Moreno Fdo: Juan Antonio Torés Montosa

This present work was supported by the following funding sources:

- Ayudas CICE-Junta de Andalucía, Proyecto de Excelencia P07-AGR-02471. También cofinanciados por fondos FEDER (EU).

- Contrato 806/60.3219 con SAT-2803 TROPS y otros productores de mango.

Dª: María Crespo Palomo was supported by a collaboration grant with charges to the research project 806/60.3219, and also was supported by a grant from University of Málaga for a short stay in the Volcani Center, Bet Dagan, Israel.

A mi familia

Agradecimientos

Este trabajo no hubiera sido posible sin la colaboración, la ayuda y el apoyo prestados por muchas personas.

Gracias a Antonio de Vicente y a Juan Antonio Torés por su confianza, por su tesón y su buen hacer, y por la paciencia y cariño que han demostrado en todo momento; por enseñarme tanto y por no coartar demasiado mi "libre albedrío". Antonio, he aprendido mucho de ti, a no perder la perspectiva científica; me has enseñado a tener siempre presente que lo que buscamos es contestar las preguntas biológicas que nos planteamos, usando todas aquellas herramientas que sean necesarias, aprendiendo y sobre todo, disfrutando del camino. Por transmitirme cómo ser generosa en la Ciencia, algo que creo que es fundamental para avanzar en los proyectos y en las relaciones profesionales y personales. Gracias por apoyarme en los momentos en que me he derrumbado. Gracias a Juan Antonio por haberme hecho crecer como persona y haberme ayudado a empezar con buen pie en este arduo camino que es la investigación. Gracias por tu apoyo y el esfuerzo empleado en este trabajo; y gracias por las conversaciones, tu compañía y tu complicidad. Del mismo modo, quiero agradecer a Francisco Cazorla por su inestimable supervisión y ayuda en este trabajo, sus consejos y su apoyo incondicional. Os felicito a los tres, pues esta tesis es un trabajo de esfuerzos compartidos, y sin la visión global y particular de cada uno, no hubiésemos compuesto de la misma manera este trabajo del cual me siento tan orgullosa.

Gracias a todos los miembros del departamento de Microbiología, y en especial a Alejandro Pérez por haberme iniciado en la investigación como tutor, y por sus sugerencias y apoyo. A Carmen y a María por facilitar nuestro trabajo, tanto en el laboratorio como en la administración, gracias por la ayuda prestada, gracias por estar siempre disponibles. Gracias a Juan Carlos Codina, por estar siempre dispuesto a ayudar. Gracias a Cayo Ramos por sus propuestas y su interés durante los seminarios; y hago extensible este agradecimiento a todos los miembros de su grupo con los que he tenido la suerte de coincidir durante el desarrollo de éste trabajo.

Muchísimas gracias a todos mis compañeros, comenzando por David, que fue mi mentor en el laboratorio, gracias por tu ayuda en todo momento, por tu carisma y por ser tan buen maestro; y a Eva, que me supo aconsejar y enseñar cuando más perdida me encontraba, gracias por haber sido una luz en mi camino y por tu sincera amistad. Gracias a Claudia, Nacho, Carmen, Álvaro, Franches, Nuria, Guti, Eva, Irene, Conchita, Diego, Víctor, Jesús, Houda, Davinia, Laura, Joaquín. Gracias a todos por la ayuda prestada, por los buenos ratos y por haber compartido este tiempo conmigo. Gracias a Claudia por ser la alegría del laboratorio, a Nacho por su ayuda y sus consejos, a Carmen que es más "salá" que "to" las cosas, y a Álvaro, como no podía ser de otro modo, por sus abrazos. Gracias especiales a Nuria, por ser como es, a Irene por estar siempre dispuesta a ayudar sin pedir nada a cambio, y a Conchita por su buen hacer y su bondad, gracias a las tres por

vuestra amistad y complicidad. Gracias especiales también a Víctor por la ayuda bioinformática y por sus consejos. Gracias a todos por acompañarme en este camino.

Gracias a todos los que han colaborado desde la Estación Experimental "La Mayora", a Jose María Farré y Jorge González por vuestro apoyo y buena disposición, y especialmente a Jose María Hermoso y Emilio Guirado por la inestimable ayuda durante los muestreos y su excelente labor en la coordinación de los experimentos de inoculación llevados a cabo durante este trabajo. Muchas gracias a Jose Manuel Pulido por toda la ayuda prestada en el laboratorio, a Paco Ruiz por la ayuda prestada en el manejo del "chambao", y a Miguel Ruiz por cuidar tan bien de "mis claveles". Gracias a S.A.T TROPS y en especial a David Sarmiento, por facilitarnos el acceso a fincas comerciales, así como por su ayuda durante los muestreos, y a los dueños de las fincas por su colaboración. Quisiera hacer extensible este agradecimiento a otros miembros del sector como son Réyes Gutiérrez S.L. y Viveros Brokaw S.L. por el apoyo y la financiación concedida para la realización de este trabajo.

Many thanks to all of the members of the Volcani Center, Israel, for their warm hospitality and from whom I will always keep the most precious memories. Many thanks to Stanley Freeman for his dedication to this work, and for making the effort to make me feel like at home. Thanks for taking care of me, and for giving me the opportunity to be a part of your team. It was a fantastic experience, and it made me grow so much as a person. Many thanks

to Marcel Maymon, the "soul of the lab", who taught me how to manage in the laboratory and everything I needed to know to carry out my experiments with success. Thanks for your inestimable help, for making my life easy, and for the good times shared with you and your family out of the lab. And of course, thanks to the University of Málaga and to my Thesis directors for giving me the chance to do this short stay in Israel that was a work, but also a gift.

Gracias a todas las personas que se han cruzado en mi camino en estos cuatro años, tanto en congresos como en reuniones de grupo, gracias por vuestras aportaciones y por hacer de estas reuniones algo que recodaré con tanto cariño.

Gracias a Ana y a Manuel por existir, os quiero desde siempre, gracias por el apoyo, el ánimo y por quererme y aceptarme tal y como soy.

Gracias a mis amigas y almas gemelas, Elena, María y Penny por aparecer en mi vida cuando más os necesitaba.

Gracias a mi familia, a quien va dedicado este trabajo, sin ellos verdaderamente este trabajo no hubiese sido posible. A mi prima Nieves, gracias por el apoyo, por ser siempre tan positiva y por tus consejos tan acertados. A mi cuñado Jason McCarthy por echarme una mano en la elaboración del manuscrito en lengua inglesa. A mi hermana Elena, por el apoyo durante los tiempos feos y por la preocupación, aunque siempre le digo que no hay que preocuparse tanto. Gracias por ser mi hermana y por ayudarme con el niño y con cualquier cosa siempre que me ha hecho falta. A mi madre Elena, sin su ayuda esto hubiese sido imposible, gracias por tu apoyo y tu

cariño, y por cuidar de mi hijo tan bien durante mi ausencia, te queremos "abu". A Pipa, por su apoyo y su complicidad. Especialmente dedico este trabajo a mi padre, el Dr. D. Vicente Crespo, por sus conocimientos y por enseñarme a "ver" por un microscopio. Tu entusiasmo y dedicación a la micología son fuente de inspiración y admiración constantes. Gracias a vuestro apoyo he llegado hasta aquí, quiero deciros cuanto os quiero y os admiro, espero estéis orgullosos de mí, porque yo lo estoy de vosotros.

Y por último gracias a mi hijo Seth por ser un niño tan magnífico, por apoyarme y cuidarme, por quererme tanto y por ser luz que alumbra mi existencia.

INDEX

INDEX

RESUMEN

RESUMEN

El mango (*Mangifera indica* L.) es un árbol originario de la región indobirmana, laderas del Himalaya y Sri Lanka, donde aún existen poblaciones silvestres y ha sido cultivado desde la antigüedad en la India como atestiguan las sagradas escrituras hindúes, los libros de los Vedas, redactadas entre el 1500 y el 1000 a. C. (Galán-Saúco, 2009). La dispersión del mango fue muy rápida por el subcontinente de la India y el archipiélago malayo con la apertura del comercio entre Asia y Europa. El mango llegó a África Oriental por viajeros árabes y fenicios, y los españoles lo introdujeron desde Filipinas a los puertos comerciales del continente americano hacia el siglo XVII. Los portugueses llevaron el mango al sur de África en el siglo XVI y a Brasil en 1700 (Galán-Saúco, 2009). Hacia la segunda mitad del siglo XVIII aproximadamente, el mango fue introducido en la Península desde las Islas Canarias. A finales del siglo pasado, el cultivo del mango comenzó a extenderse comercialmente por Andalucía oriental, fundamentalmente en las costas de Málaga y Granada. En España, su tasa de plantación está siendo relativamente rápida debido a la oportunidad comercial de este fruto en Europa (Ferrer, 1992).

La distribución del cultivo del mango es amplia, abarcando países tropicales y subtropicales tanto del hemisferio Norte como del hemisferio Sur. Los principales países productores a nivel mundial son India, China, Indonesia, y Méjico. En España las principales variedades cultivadas son Osteen, Keitt, Kent y

Tommy Atkins apareciendo otras variedades en menor medida. En la península Ibérica, las primeras fincas comerciales de mango se inician alrededor del año 1985 y se localizan principalmente en la zona de la Axarquía (Málaga). En la actualidad se estima que existen 3.500 ha plantadas. En los últimos años, el ritmo de plantación ha sido de 350-400 ha por año. Datos de ASAJA (Asociación agraria de jóvenes agricultores) en 2010 revelaron que en la zona de la Axarquía ese año se produjeron más de 11000 toneladas de mango con una facturación de 13 millones de euros (Gutiérrez-Barranquero, 2012). Desde el punto de vista económico y comercial, este cultivo podría enfrentarse a graves problemas derivados del monocultivo de la principal variedad, Osteen; que constituye el 80% de la superficie total de cultivo (Díaz-Robledo & Hermoso, 2009). Actualmente, la situación de este cultivo está cambiando, y se está apostando en mayor medida por la diversificación de las variedades cultivadas.

El mango es la especie de mayor importancia agronómica de la familia de las Anacardiáceas tanto por su amplia distribución geográfica como por el valor económico de su fruto (Galán-Saúco, 2009). La propagación se suele realizar mediante varios métodos de injerto, desde el tradicional sistema de injerto por aproximación utilizado en la India desde tiempo inmemorial hasta diversos tipos de injerto de yema o púa.

Las enfermedades o plagas que lo afectan se encuentran determinadas por las condiciones de cultivo, su localización geográfica, y la idiosincrasia del cultivar. Entre las plagas más

importantes que atacan al mango se encuentran diversas especies de moscas de la fruta, cochinillas, trips y ácaros. Resaltar la importancia de la mosca de la fruta del Mediterráneo, *Ceratitis capitata* Wied. por su amplia distribución; y el taladrador de la semilla, encarnado por el coleóptero *Sternochetus (=Cryptorhynchus) mangiferae* (Fabricius), una plaga importante del mango cuya presencia limita la propagación de plantas en los viveros y cuyo control resulta complicado (Galán-Saúco, 1999). Una de las enfermedades más destructivas y difundidas es sin duda la antracnosis producida por *Colletotrichum gloesporioides* (Penz.) Penz. & Sacc., que ataca a hojas, inflorescencias y frutos. Se manifiesta con mayor intensidad en climas húmedos. Otra enfermedad de difusión mundial es el oídio, *Oidium mangiferae* Berthet, hongo que causa graves problemas si no se controla a tiempo, principalmente en la fase de floración y el cuajado del fruto. El oídio se ve favorecido por un ambiente seco y bajas temperaturas nocturnas (Torés, 1997).

La necrosis apical del mango (NAM) es una enfermedad de difusión más restringida, que afecta a yemas, hojas, tallos e inflorescencias, llegando a ocasionar importantes pérdidas económicas en plantaciones, principalmente del área mediterránea (España, Portugal, Italia e Israel), o zonas con clima parecido (Australia) (Cazorla *et al*., 1998; Golzar & Cother, 2008). Los síntomas incluyen la necrosis de las yemas vegetativas y florales, pudiendo impedirse el desarrollo de las mismas como consecuencia de la necrosis. La bacteria causante de la

enfermedad *Pseudomonas syringae* pv. syringae (Cazorla *et al*., 1998), se ve favorecida en períodos fríos y húmedos, jugando la lluvia o el rocío un importante papel en la diseminación del inóculo entre distintas partes del árbol y a árboles adyacentes. En nuestro grupo de investigación, también se ha descrito por primera vez la implicación de cepas patógenas de *Pantoea agglomerans* como agente causal de la NAM en las Islas Canarias (Gutiérrez-Barranquero *et al*., 2012a). El control de esta enfermedad en el campo es muy complejo, debido a la cantidad de factores que pueden estar involucrados en el desarrollo de los síntomas. Un tratamiento efectivo para esta enfermedad ha sido la aplicación de compuestos basados en el cobre, en particular el caldo bordelés (Cazorla *et al*., 2006); sin embargo, debido a los problemas de toxicidad y contaminación que estos productos pueden generar en el suelo, se desarrolló en nuestro grupo de investigación una alternativa más respetuosa con el medio ambiente en forma de gel de silicio, y que en la actualidad se utiliza ampliamente para controlar la NAM en fincas del sur de España (Gutiérrez-Barranquero *et al*., 2012b). Otra enfermedad bacteriana relevante es la producida por *Xanthomonas campestris* pv. mangiferaeindicae, denominada mancha negra bacteriana o mancha angular, por la aparición de manchas húmedas en hojas y fruto que ennegrecen rápidamente, así como chancros negros en tallos y tronco (Galán-Saúco, 2009). Hasta la fecha, esta enfermedad no ha sido detectada en el área mediterránea.

1.1 La malformación del mango

La malformación es una de las enfermedades más importantes del mango en el mundo y causa importantes pérdidas económicas. La malformación fue observada por primera vez en la India en 1891 (Marasas *et al*., 2006) y en la actualidad se ha descrito en la mayoría de los países productores del mundo tales como Egipto, Sudáfrica, Sudán, Israel, Malasia, Omán, China, Pakistán, Bangladesh, Brasil, Méjico y EE.UU., entre otros (Marasas *et al*., 2006; Youssef *et al*., 2007; Kavas *et al*., 2008; Zhan *et al*., 2010; Kumar *et al*., 2011), y en el presente trabajo se ha descrito en España (Crespo *et al*., 2012). La malformación afecta tanto a brotes vegetativos como a brotes florales, dándose la primera sobre todo en plantas jóvenes y en plantas de vivero, especialmente cuando éstas se cultivan bajo árboles afectados, lo cual es una práctica común en Oriente Medio (Ploetz *et al.,* 2001; Youssef *et. al*., 2007). En la malformación vegetativa la pérdida de dominancia apical conduce a que las yemas vegetativas axilares o apicales produzcan brotes deformes que muestran reducción de los entrenudos y de la lámina foliar (Kumar *et al*., 1993). La enfermedad limita el crecimiento de plantas jóvenes y puede conducir a la muerte de la planta en el caso de que todas las yemas se encuentren afectadas. El desarrollo de la malformación vegetativa en la mayor parte de las ramas en un árbol, conduce a una reducción considerable de la floración o a la ausencia total de ésta (Kumar & Beniwal, 1992). Generalmente, una rama que muestra síntomas de malformación vegetativa también produce

inflorescencias malformadas (Singh *et al.,* 1961). Además, la enfermedad debilita seriamente los plantones utilizados como portainjerto y compromete la seguridad del movimiento nacional e internacional de germoplasma (Ploetz, 2001). En cuanto a los síntomas de la malformación floral, los más característicos y comunes, las inflorescencias presentan una reducción en la longitud del eje primario y secundario, los cuales son más gruesos que los ejes normales, además pueden ser muy ramificados, presentando un aspecto de racimo. Las panículas malformadas producen un número de flores hasta tres veces superior al de las panículas normales, con una proporción mayor de flores masculinas que hermafroditas, y además pudiendo alcanzar éstas hasta el doble de su tamaño normal (Singh *et al.,* 1961). Las inflorescencias afectadas, generalmente no producen fruto y cuando lo hacen los pierden prematuramente, lo que conlleva importantes pérdidas económicas. Además, las inflorescencias afectadas si no son retiradas constituyen una importante fuente de inóculo secundario y contribuyen a la dispersión de la enfermedad entre árboles cercanos.

Esta enfermedad ha sido atribuida a varias especies de hongos del género *Fusarium*. *Fusarium mangiferae* y *Fusarium sterilihyphosum* fueron descritas en 2002 como especies asociadas con la malformación del mango, y en la actualidad se encuentran, una o ambas especies, en muchas zonas productoras del mundo, tanto del hemisferio Norte como del hemisferio Sur (Britz *et al*., 2002; Marasas *et al*., 2006; Youssef *et al*., 2007; Kvas *et al*., 2008;

Lima *et al*., 2009b; Kumar *et al*., 2011), incluyendo el sur de España, como se describe en este trabajo (Crespo *et al*., 2012). *Fusarium proliferatum*, también ha sido descrita como causante de la malformación floral en Egipto, sur de China y Malasia (Haggag *et al*., 2009; Zhang *et al*., 2010; Nor *et al*., 2013). En Méjico, *Fusarium mexicanum* ha sido descrito como el principal agente causal de la enfermedad (Otero-Colina, 2010). Hasta la fecha, la última nueva especie descrita asociada a esta enfermedad ha sido *Fusarium tupiense* en Brasil y Senegal (Lima *et al*., 2012; Senghor *et al*., 2012), especie que también se ha detectado en el Sur de España en el presente trabajo.

Estas especies se pueden diferenciar y caracterizar mediante el empleo de herramientas genéticas y moleculares que incluyen la determinación del grupo de compatibilidad vegetativa (VCG); polimorfismos de longitud de fragmentos de DNA amplificados (AFLPs) (Lima *et al*., 2009b) y polimorfismos de fragmentos de DNA amplificados al azar (RAPD-PCR), basados en la reacción en cadena de la polimerasa (PCR); la secuenciación parcial de genes "housekeeping" (O´Donnell *et al*., 1998, 2000; Steenkamp *et al*., 2000; Marasas *et al*., 2006; Lima *et al*., 2009b; Crespo *et al*., 2014), y la determinación del tipo sexual (mating-type) (Britz *et al*., 2000). Con el propósito de poder realizar un diagnóstico rápido y específico de algunas de las especies asociadas a la malformación, mediante PCR, Zheng y Ploetz (2002) desarrollaron una pareja de cebadores, 1-3F/R, que amplifican un fragmento específico de 608 pb incluido en las ITS de *F. mangiferae*, que permite el diagnóstico

de esta especie. Un segundo par de cebadores, 61-2F/R, diseñados originalmente para el diagnóstico de *Fusarium subglutinans* aislado de maíz (Möller *et al*, 1999), que no amplifican el DNA de *F. mangiferae*, pero sí amplifican cuando los protocolos de amplificación se adecúan, un fragmento de 445 pb a partir de aislados de *F. sterilihyphosum* y *F. mexicanum* (Zheng y Ploetz, 2002; Rodriguez-Alvarado *et al*., 2007).

Como se ha mencionado anteriormente, es posible caracterizar la diversidad poblacional de estas especies mediante el empleo de varias técnicas genéticas y moleculares. Conocer la diversidad de una población fúngica desconocida, en nuestro caso las especies de *Fusarium* patógenas de mango en España, puede resultar muy relevante para comprender aspectos sobre la introducción y la dispersión del patógeno en un área determinada, y por lo tanto desarrollar medidas de control más eficaces.

La epidemiología de la malformación del mango no está aún clara, el patógeno parece transmitirse principalmente en el proceso de injerto y a través de material de vivero infectado, aunque restos de inflorescencias con malformación actúan como fuente de inóculo secundario. Los microconidios constituyen propágulos infecciosos ya que son el principal tipo de esporas producidas de manera profusa por el hongo en las panículas afectadas (los macroconidios son menos frecuentes). La dispersión dentro de las plantaciones es lenta debido a la rápida muerte de los conidios del hongo en el suelo, no obstante parece

verse favorecida por determinados artrópodos que pueden actuar como vector del hongo como es el caso del ácaro *Aceria mangiferae* (Gamliel-Atinsky *et al*., 2009a). Aunque numerosos aspectos del patrón de colonización del hongo y de su ciclo de vida están aún por dilucidar, parece claro que las heridas facilitan la infección y el desarrollo posterior de la enfermedad (Gamliel-Atinsky *et al*., 2009c). Su control es muy difícil y tras la infección se produce un avance progresivo de la enfermedad hacia brotes sanos, desde brotes afectados e inflorescencias marchitas. La dispersión a pequeña escala es evidente en los viveros (Prakash & Srivastava, 1987), pero a mayores distancias la diseminación probablemente tiene lugar a través de material propagativo (Lima *et al*., 2009a). El empleo de diversos fungicidas se ha mostrado muy poco eficaz, y hasta el momento el método más adecuado es la eliminación manual de los brotes con síntomas y su destrucción, y el control eficaz del empleo de material propagativo sano (Cazorla *et al*., 2009; Freeman *et al*., 2014).

1.2 Detección y aislamiento de especies de *Fusarium* causantes de malformación del mango en la Axarquía (España)

Nuestro objetivo en este trabajo ha sido confirmar la presencia de la malformación del mango en el Sur de España y conocer su distribución geográfica; identificar las especies de *Fusarium* asociadas a esta enfermedad en la región de la Axarquía y confirmar su patogenicidad. Analizar la diversidad de las poblaciones de *Fusarium* spp., y determinar las relaciones

filogenéticas entre estos aislados y otros *Fusarium* spp. asociados con la malformación del mango a nivel mundial.

Hasta 2006, los árboles de mango en España se consideraban libres de malformación, aunque desde hacía algunos años se venían observando de forma esporádica árboles con síntomas sospechosos de malformación en plantaciones de mango del sur de la península. La aparición de los primeros síntomas en la costa andaluza es difícil de precisar, pero es en la primavera de 2006 cuando se realizaron las primeras tomas de muestras en plantaciones comerciales de la Axarquía y fue entonces cuando se planteó la necesidad de abordar este trabajo, que tenía como finalidad confirmar la presencia de esta enfermedad en los cultivos de mango en la provincia de Málaga, concretamente en la Axarquía, y evaluar su incidencia y distribución espacial en la zona de estudio; así como determinar cuál o cuáles de sus posibles agentes causales estaban presentes en la Axarquía, la variabilidad de sus poblaciones, y proponer medidas preventivas de vigilancia y control de esta enfermedad. Para ello, en este estudio se llevaron a cabo prospecciones sistemáticas durante los años 2009-2012 en diferentes términos municipales de la Axarquía: Algarrobo, Almáchar, Benamargosa, Benamocarra, Cajiz, Cútar, Frigiliana y varias localidades de Vélez-Málaga, y coincidiendo con los meses de floración del mango, es decir, desde abril hasta julio aproximadamente, cuando los síntomas se hacen más evidentes. Se obtuvieron muestras en un total de 43 fincas con síntomas aparentes de malformación del mango; en 36

de las cuales se aisló *Fusarium*, y en trece de ellas se llevó a cabo un seguimiento durante varios años con el fin de evaluar la dispersión del patógeno, y la eficacia de las labores de saneamiento y control.

1.3 Identificación del agente/s causal/es de la malformación del mango en España

Como resultado de estos muestreos, se obtuvo una colección de 127 aislados monospóricos de *Fusarium* spp. procedentes de muestras de tejidos de mango infectados, de 10 municipios diferentes de la Axarquía. A esta colección se añadieron los siete aislados previamente obtenidos en 2006.

Para conocer la identidad del agente causal de la malformación del mango en el sur de España, se emplearon dos estrategias metodológicas complementarias, el estudio de las características micromorfológicas del hongo a microscopía óptica, y el empleo de técnicas de biología molecular basadas en el empleo de cebadores específicos de distintas especies de *Fusarium* y la reacción en cadena de la polimerasa (PCR). El examen detallado a microscopio óptico de aislados monospóricos cultivados en agar patata dextrosa (PDA) y agar hoja de clavel fresca (FCLA) permitió diferenciar dos grupos principales de aislados. Así, 40 aislados mostraron características morfológicas que concordaban con las descritas para *F. mangiferae*, es decir, micelio aéreo de coloración salmón-púrpura, conidióforos en forma de mono y polifiálides, macroconidios con tres a cinco

septos, y abundantes microconidios en falsas cabezas; esporodoquios de color anaranjado en la superficie del agar y ausencia de clamidosporas. Esta identificación presuntiva fue comprobada mediante PCR, con el empleo del par de cebadores específicos 1-3F/R descritos para esta especie, descartándose los aislados UMAF F0602, UMAF F1043 y UMAF F1063, que aunque mostraban características fenotípicas muy similares a *F. mangiferae*, no amplificaron con el par de cebadores descritos para esta especie. Por otra parte, el aislado UMAF F0923 cuya morfología no coincidía con la descrita para *F. mangiferae* sí mostró amplificación con los cebadores específicos para esta especie, aunque con el análisis posterior de caracterización adicional (VCG, patogenicidad, análisis filogenético y el tipo de compatibilidad sexual), fue diagnosticado como *F. mangiferae* atípico al tratarse de un aislado con algunas características diferentes y no patogénico.

El grupo mayoritario de aislados (90) mostraba algunas características morfológicas diferentes a las descritas para *F. mangiferae,* presentando así mismo mono y polifiálides, microconidios dispuestos en falsas cabezas y ausencia de clamidosporos, pero los macroconidios eran más cortos y anchos que aquellos producidos por los aislados de *F. mangiferae*, y además no se obtuvo amplificación con el par de cebadores específicos de *F. mangiferae* con ninguno de ellos. Sin embargo, sí se obtuvo amplificación con el par de cebadores 61-2F/R en estos 90 aislados y en los tres que morfológicamente eran más

parecidos a *F. mangiferae* pero no fueron diagnosticados como tal (UMAF F0602, UMAF F1043 y UMAF F1063). Finalmente otros tres aislados (UMAF F0927, UMAF F0928 y UMAF F1062) mostraron morfologías no concordantes con ninguna de las descritas para las especies de *Fusarium* asociadas a la malformación del mango y no se obtuvo amplificación con ninguna de las dos parejas de cebadores. En total, se analizaron 134 aislados de *Fusarium* spp. que fueron identificados en dos especies principales, 38 aislados diagnosticados como *F. mangiferae* aunque entre éstos, el aislado UMAF F0923 presentaba características morfológicas atípicas y no era patogénico; 93 se diagnosticaron como *F. tupiense,* ya que aunque en la fase inicial del trabajo no se obtuvo una identificación concluyente, sí lo fueron mediante las técnicas genéticas y moleculares adicionales empleadas en esta tesis. Finalmente, otros tres aislados de *Fusarium* sp. no incluidos en ninguna de estas dos especies, dos de los cuales en análisis filogenéticos posteriores quedaban agrupados en un clúster próximos a *Fusarium phyllophilum* denominándose en este trabajo como *F. phyllophilum*- like.

1.4 Confirmación de la patogenicidad de los aislados de *Fusarium* de la Axarquía

Llegados a este punto, era necesaria también la comprobación de la patogenicidad de dichos aislados mediante ensayos de inoculación de árboles sanos de mango, y en

particular de los aislados no identificados de manera concluyente a nivel de especie. Las inoculaciones se llevaron a cabo en condiciones controladas en tres ensayos independientes que se iniciaron en marzo y noviembre de 2010 y noviembre de 2011. Como resultado de éstos ensayos de patogenicidad se comprobó de manera inequívoca la implicación en la producción de síntomas claros de malformación floral y/o vegetativa en árboles de mango, de siete aislados representativos de *F. mangiferae*, mientras que el aislado atípico UMAF F0923 resultó no patogénico. También se confirmó la implicación de los 12 aislados representativos de *F. tupiense* y del aislado de *Fusarium phyllophilum*-like (UMAF F0927) ensayados en la inducción de síntomas de la enfermedad, confirmándose así el papel de todos ellos como agentes causales de la misma en la zona de la Axarquía. El aislado de *Fusarium* sp. UMAF F1062 resultó no patogénico.

Se comprobó que la totalidad de los aislados recuperados eran idénticos morfológicamente a aquellos inoculados, además el fragmento específico de 608 pb o de 445 pb se amplificó por PCR en todos los aislados con la excepción del aislado *F. phyllophilum*-like (UMAF F0927) como cabía esperar.

1.5 Estudio de la diversidad de las poblaciones de *Fusarium* patógenas de mango en la Axarquía

El hecho de que se hayan aislado tres especies diferentes de *Fusarium* patógenas de mango en la Axarquía, indica claramente

que la diversidad de las poblaciones de este hongo causantes de la malformación del mango en la Axarquía es elevada; por ello, nos planteamos profundizar en el estudio de la diversidad genética intraespecífica de las mismas, con la hipótesis de que la diversidad podía ser aún mayor, lo que pondría además de manifiesto la introducción de inóculo primario incluso en más ocasiones. Este estudio de la diversidad de las poblaciones del hongo se abordó empleando varios métodos experimentales. En primer lugar, se analizaron genotípicamente la práctica totalidad de los aislados (131) mediante las técnicas de apPCR y RAPD-PCR. El análisis de diversidad por las mencionadas técnicas se basa en la realización de reacciones de PCR utilizando cebadores arbitrarios, que permiten detectar variabilidad genética entre diferentes especies, así como intraespecífica. En este estudio de diversidad se han empleado cinco cebadores $(GACAC)_3$, $(GACA)_4$, $(CAG)_5$, OPF-08 y OPF-13, y con todos ellos se han observado claras diferencias entre los perfiles de bandas de otras especies de *Fusarium* implicadas en la malformación del mango a nivel mundial, *F. sterilihyphosum*, *F. mexicanum*, *F. tupiense*. Los aislados de *F. mangiferae* encontradas en la Axarquía mostraron un perfil similar al del aislado de *F. mangiferae* de referencia MRC7560. En el caso de los aislados de *F. mangiferae* incluyendo 3 aislados de referencia de *F. mangiferae* aislados de Israel, Egipto y EE.UU., los cebadores $(GACA)_4$, y OPF-13 permitieron además detectar diferencias intraespecíficas. Como resultado pudimos diferenciar genotípicamente dos poblaciones diferentes de *F.*

mangiferae, el genotipo 1 formado por la mayor parte de los aislados, y el genotipo 2 constituido por solo 4 aislados de una misma finca, aunque aislados en años diferentes, y también por los aislados de Israel y Egipto (MRC7560 y EM50B). Sin embargo, los 92 aislados de *F. tupiense* de la Axarquía ensayados, con la única excepción del aislado UMAF F1182, mostraron un perfil de bandas homogéneo entre todos ellos con los cinco cebadores empleados, e idéntico a un aislado procedente de Brasil y perteneciente al VCG I usado como referencia (*F. tupiense* NRRL 53995). Respecto a los dos aislados de *F. phyllophilum*-like (UMAF F0927 y UMAF F0928) procedentes ambos de una misma finca, mostraron un perfil homogéneo entre sí y diferente de todos los demás aislados y cepas de referencia ensayados.

Para profundizar aún más en este análisis de la diversidad genética de las poblaciones de *Fusarium* en la Axarquía se determinaron los grupos de compatibilidad vegetativa (VCG) entre aislados representativos de las tres especies. Los VCG en hongos se determinan para conocer la diversidad en las poblaciones fúngicas y son de enorme utilidad en estudios de epidemiología. Su determinación se basa en la compatibilidad miceliar (anastomosis de hifas) entre aislados diferentes del mismo VCG, lo que permite clasificar los aislados en distintos VCG (Puhalla, 1985). Cuando dos aislados complementan entre sí se dice que pertenecen al mismo VCG, y por lo tanto son genéticamente muy próximos. Para la clasificación de nuestros aislados de *F. mangiferae* se enfrentaron 33 aislados

representativos (incluyendo el aislado UMAF F0923, no patogénico), entre sí y frente a una colección de aislados de *F. mangiferae* de referencia de los 6 VCG descritos por Zheng y Ploetz, (2002), y un aislado procedente de Israel, así como dos aislados de *F. sterilihyphosum* y dos aislados de *F. mexicanum* procedentes de diferentes colecciones. De igual forma se determinaron los VCG de 41 aislados representativos de *F. tupiense* junto a aislados de referencia de las especies antes mencionadas y una colección de 6 aislados de *F. tupiense* de referencia, uno por cada VCG descrito para esta especie (Lima *et al*., 2009a), y los dos aislados de *F. phyllophilum*-like. Con este experimento se ha conseguido confirmar que los aislados de la malformación del mango en la Axarquía se corresponden, en base a sus características genotípicas diferenciadas, con tres grupos homogéneos, como cabía esperar de especies diferentes. No obstante, se observa que los aislados de *F. mangiferae* se distribuyen a su vez en tres grupos de compatibilidad vegetativa, VCG 7 constituido por la mayoría de los aislados correspondientes al genotipo 1, y VCG 8 formado por los cuatro aislados procedentes de una misma finca y con genotipo 2, y un tercer VCG agrupando 4 aislados de la Axarquía también de genotipo 1, entre ellos el aislado UMAF F0923 con características morfológicas atípicas, y dos aislados de Sudáfrica en el VCG 5 descrito por Zheng y Ploetz, 2002; sugiriendo que esta especie se ha introducido en España al menos en tres ocasiones (Crespo *et al*., 2014). Además, se puso de manifiesto que los aislados de *F.*

mangiferae de la Axarquía no se complementan con ninguno de los aislados de *F. mangiferae* procedentes de Egipto, EE.UU. e Israel, utilizados en el experimento, indicando que aunque son la misma especie, tienen un genotipo y origen diferenciados. En cuanto a los aislados de las otras dos especies, todos los aislados de *F. tupiense* ensayados mostraron pertenecer a un mismo VCG al igual que mostraron un genotipo homogéneo, sugiriendo que la población de *F. tupiense* en la Axarquía posiblemente sea una población clonal. Estos aislados han resultado pertenecer a uno de los VCG descritos para *F. tupiense* en Brasil (VCG I), lo que sugiere que ambas poblaciones podrían tener un origen común, y que la introducción en España probablemente se haya realizado a través de material infectado procedente de países donde está presente como Brasil o Senegal. Los dos aislados de *F. phyllophilum*-like como cabía esperar constituyeron un VCG separado de los demás.

Para completar este estudio de diversidad de las poblaciones de *Fusarium* en la Axarquía, también se determinó el tipo de compatibilidad sexual (mating-type) que presenta cada uno de los aislados españoles. Este análisis resulta de crucial importancia a la hora de realizar cruzamientos entre aislados. El locus MAT determina el tipo sexual de los hongos y los genes MAT tienen el potencial de delimitar las fronteras entre especies (Yun *et al*., 2000) y su utilidad ha sido demostrada en análisis filogenéticos (Leslie & Klein, 1996). Los resultados obtenidos tras analizar 132 aislados de *Fusarium* procedentes de muestras de mango de la Axarquía

indican que la mayoría de los mismos (117 aislados) son del tipo *MAT*-2, confirmando así que la reproducción de las especies de *Fusarium* detectadas en la Axarquía ocurre casi exclusivamente de forma asexual. No obstante, se han encontrado algunas excepciones, dos aislados del genotipo 1 de *F. mangiferae* (UMAF F12123 y UMAF F12126) son del tipo *MAT*-1, así como un único aislado de *F. tupiense* (UMAF F1168), lo que implica la evidencia de un nuevo elemento de diversidad en las poblaciones de la Axarquía. Por otra parte, con doce aislados no fue posible determinar el tipo de compatibilidad sexual.

Con el objeto de profundizar en el diagnóstico de los aislados implicados en la malformación del mango en la Axarquía, así como para confirmar la identificación de los aislados de *F. tupiense* de la Axarquía se llevó a cabo también la secuenciación parcial de varios genes "housekeeping" de los mismos. La secuenciación de uno o más de estos genes conservados se suele realizar para asignar especie o para obtener información sobre las relaciones filogenéticas entre los aislados (Leslie & Summerell, 2006). Este análisis filogenético se realizó en dos ensayos independientes incluyendo un número representativo de aislados de la Axarquía, (29). En nuestro laboratorio se secuenciaron y compararon bioinformáticamente las secuencias parciales de los genes factor de elongación 1-alfa (TEF) y β-tubulina, de aislados representativos seleccionados teniendo en cuenta la máxima diversidad posible, 9 de *F. mangiferae* incluyendo el aislado UMAF F0923, 17 de *F. tupiense* y

2 *F. phyllophilum*-like, con secuencias equivalentes de otras cepas de diferentes especies incluidas en el complejo *Gibberella fujikuroi* (GFSC) y disponibles en el banco de genes del NCBI (GenBank) y en la base de datos de *Fusarium* (*Fusarium*-ID). El árbol filogenético resultante agrupó los aislados de *F. mangiferae* de la Axarquía con los otros aislados de *F. mangiferae* procedentes de Israel e India en el denominado clado asiático. Los aislados de *F. tupiense* españoles agruparon con los aislados de *F. tupiense* de Brasil en el clado americano, y los dos aislados de *F. phyllophilum*-like agruparon en un clúster próximo a *Fusarium udum* en el clado africano. Un segundo estudio filogenético más en profundidad se llevó a cabo mediante la secuenciación parcial de siete genes "housekeeping": factor de elongación 1-alfa (TEF), β-tubulina, calmodulina, histona H3, nuclear ribosomal intergenic spacer region (IGS rDNA), y dos subunidades de la RNA polimerasa (RPB y RPB2), en colaboración con el Bacterial and Foodborne Pathogen and Mycology Research Unit, U.S. Department of Agriculture-Agricultural Research Service (IL, EE.UU.). El análisis filogenético se llevó a cabo con varios aislados representativos españoles, dos aislados de *F. mangiferae*, cuatro de *F. tupiense* y uno de *F. phyllophilum*-like, y se incluyeron secuencias de varias especies de *Fusarium* implicadas en la malformación del mango junto con otras especies pertenecientes al GFSC. Los resultados obtenidos fueron similares, aunque, el aislado de *F. phyllophilum*-like agrupó también en el clado africano pero esta vez más

próximo a *F. phyllophilum* que a *F. udum*; por esto en este trabajo se han denominado como *F. phyllophilum*-like.

Con el objeto de determinar la fertilidad de los aislados de *Fusarium* de la Axarquía con tipos de compatibilidad sexual opuesta, y comprobar si los aislados de *F. tupiense* españoles podían cruzarse con aislados de Brasil, se realizaron diferentes ensayos de cruzamiento sexual. Por un lado entre aislados españoles de *F. mangiferae*, y por otro lado entre aislados de *F. tupiense,* incluyendo en este caso dos aislados de referencia de Brasil. La mayoría de los ascomicetes, incluyendo *Fusarium* son generalmente haploides y se pueden propagar de forma vegetativa o reproducirse de manera sexual. Con frecuencia, solo se conoce la forma asexuada del hongo (anamorfo), y la identificación del hongo se hace en base a las características de éste. La forma sexuada o perfecta (teleomorfo) es en muchos casos desconocida. Todas las especies de *Fusarium* estudiadas son heterotálicas, y un aislado puede servir como macho o como hembra, pero no puede fertilizarse a sí mismo por que se requieren diferentes tipos de compatibilidad sexual (*MAT*-1 y *MAT*-2) para que el cruzamiento tenga éxito. La capacidad de dos aislados para producir el teleomorfo constituye una prueba adicional y concluyente de que ambos aislados pertenecen a la misma especie (Leslie & Summerell, 2006). El estado perfecto de *F. mangiferae* aún no ha sido descrito por lo que no se dispone de aislados hembra fértiles de referencia para realizar los cruzamientos; sin embargo, en el caso de *F. tupiense* sí se dispone

de estos aislados, ya que el estado perfecto de este hongo fue descrito por Lima *et al*., (2012). De esta forma, dos aislados brasileños de *F. tupiense* hembra fértiles (CLM1000 y CLM1843) se incluyeron en ensayos de cruzamiento con el ánimo de confirmar también por esta vía, la identidad de los aislados de *F. tupiense* de la Axarquía. A pesar de las reiteradas repeticiones experimentales, en ningún caso se consiguió obtener el estado perfecto en los cruzamientos realizados entre aislados de *F. mangiferae* de la Axarquía, ni en el caso de los cruzamientos entre aislados de *F. tupiense* de Brasil y España. Este resultado puede deberse a la baja fertilidad de los aislados de campo y/o a condiciones experimentales inadecuadas.

A modo de resumen de los diferentes estudios sobre el diagnóstico y la diversidad de las poblaciones de *Fusarium* patógenas de mango en la Axarquía, podemos resaltar algunos aspectos. La población de *Fusarium* patógenos de mango en la Axarquía entre 2009 y 2012, consta de al menos tres especies diferentes: *F. mangiferae*, *F. tupiense* y *F. phyllophilum*-like, esta tercera especie restringida a una única finca y nunca antes descrita como patógena de mango. En cuanto a las dos especies mayoritarias, en el caso de *F. mangiferae* se han diferenciado claramente tres genotipos en base a los diferentes análisis de diversidad realizados, uno mayoritario y otros dos asociados a fincas concretas. Por el contrario, en el caso de *F. tupiense* aparentemente se trata de una población clonal homogénea, cuyo genotipo ha resultado similar a uno de los descritos para *F.*

tupiense en Brasil. Si bien esta introducción pudo haber llegado procedente de Brasil también pudo haberlo hecho procedente de Senegal donde recientemente se ha detectado esta misma especie (Senghor *et al*., 2012).

Desde el punto de vista epidemiológico nuestros estudios indican de forma concluyente que al menos se han producido cinco entradas diferentes de inóculo primario del patógeno en España, en todos los casos anteriores a 2009. Es evidente que el control sobre la entrada de material vegetal infectado no fue el adecuado, y se ha mostrado claramente insuficiente, como evidencian estos resultados, explicando de esta manera la reiterada llegada de esta enfermedad a España por diferentes vías. Ello nos hace llamar la atención sobre lo imprescindible de extremar el control sobre la importación de material vegetal, para evitar la entrada de nuevos inóculos. De las cinco entradas mencionadas, tres de ellas han quedado muy restringidas, aparentemente a una sola finca, o muy pocas, es el caso de *F. phyllophilum*-like y de *F. mangiferae* genotipo 2 y genotipo 1-VCG 5, con la circunstancia positiva adicional de que en los dos primeros casos en dichas fincas en 2012 ya no se han observado síntomas tras la aplicación reiterada de las medidas de saneamiento recomendadas. Por el contrario, los otros dos casos, *F. mangiferae* genotipo 1 (VCG 7) y *F. tupiense* han sufrido una importante diseminación por toda la región, que mientras en el primer caso ya parece haberse estabilizado, en el segundo caso parece continuar en expansión, siendo actualmente la especie

predominante en la Axarquía. Asimismo, estos resultados también sugieren que una forma principal de diseminación de esta enfermedad en la región es el uso de material vegetal de propagación infectado, siendo por ello también muy importante extremar el cuidado en la selección de la planta madre de dónde se obtienen las púas de injerto, para evitar emplear plantas infectadas y asintomáticas y que con ello prosiga la diseminación de la enfermedad.

Es evidente que para que una enfermedad que no estaba presente en una zona de cultivo aparezca en la misma es necesaria la introducción del patógeno mediante un inóculo primario. Esta introducción de inóculo primario, dado el aislamiento geográfico de la costa andaluza respecto a otras áreas de cultivo, se ha debido con mucha probabilidad a la importación de material infectado, y como se acaba de exponer, en base a los resultados de diversidad genética, este hecho se ha producido aparentemente en al menos cinco ocasiones. Una vez que el patógeno está presente en la zona de cultivo la transmisión de la enfermedad se facilita por medio de la dispersión del inóculo secundario por vías alternativas.

Por tanto, podemos señalar tres aspectos críticos para controlar la difusión de la enfermedad en la Axarquía y que es imprescindible considerar:

- *Entrada de inóculo primario*: Es fundamental evitar la llegada de nuevo material infectado, y en este sentido solo cabe extremar las

medidas de control y cuarentena sobre la importación de material vegetal, para así disminuir todo lo posible el riesgo de nuevas entradas de patógeno.

- *Dispersión a través de material de injerto infectado*: Esta es posiblemente la vía principal y más peligrosa de dispersión de inóculo secundario de esta enfermedad en la región en estos momentos. Si la vareta está infectada, el árbol producido lo estará también. Por ello, nunca deben utilizarse varetas (púas) para injertar obtenidas en fincas con síntomas o sospecha de esta enfermedad, ni siquiera aunque estos no sean evidentes, y se deben extremar las medidas fitosanitarias en la producción de árboles de mango en viveros.

En este trabajo se han realizado algunos ensayos dirigidos a la puesta a punto de métodos de termoterapia para la obtención de material de vivero libre del patógeno. No obstante, en los ensayos preliminares con varios aislados de *Fusarium*, la aplicación de tratamientos a 40-45 ºC durante hasta 3 horas no se han mostrado suficientemente eficaces para la inactivación de suspensiones de esporas del hongo, y el empleo de temperaturas superiores compromete la viabilidad de las púas.

- *Saneamiento de las fincas infectadas*: Otra vía importante de diseminación de la enfermedad, es la dispersión de esporas desde árboles afectados a otros árboles en la propia finca o fincas colindantes, especialmente en la época de la floración. Ya que los

tratamientos químicos no se han mostrado eficaces (Freeman *et al.*, 2014), la mejor manera de combatir esta vía es la realización de podas de saneamiento de las fincas afectadas. Para evitar la dispersión del patógeno una vez se ha detectado su presencia en la finca, se recomienda realizar labores de saneamiento consistentes en cortar las ramas afectadas por debajo de la madera de dos o tres años de edad depositándolas sobre un plástico donde quedarán expuestas al sol durante los meses de verano, alcanzando temperaturas elevadas (Cazorla *et al*., 2009). De igual forma se aconseja la desinfección de las herramientas utilizadas en las tareas de saneamiento. Durante marzo y abril, cuando se produce el crecimiento de las panículas florales con síntomas, el nivel de esporas es aún bajo, y es el momento más adecuado para realizar la primera poda de ramos afectados. En fincas bastante infectadas se recomienda realizar un mínimo de 3-4 pases de poda en el periodo marzo-agosto, eliminando cualquier foco de infección visible en inflorescencias o brotes vegetativos. Estas recomendaciones han dado resultados satisfactorios en Israel y Sudáfrica, disminuyendo progresivamente año tras año el nivel de infección, pero exigen que se realicen de una forma constante y exhaustiva hasta conseguir la erradicación. En nuestra región, se han obtenido resultados satisfactorios cuando esta práctica se ha aplicado de forma adecuada e intensa, aunque del total de fincas afectadas en que se ha realizado seguimiento (13), únicamente se ha observado erradicación la enfermedad en dos de ellas.

Para concluir, tras las prospecciones realizadas en distintas fincas con síntomas de malformación de mango en la Axarquía, se ha confirmado la presencia de esta enfermedad en la región; alcanzando un considerable grado de dispersión, así, se ha confirmado y aislado el agente causal en 35 fincas repartidas en varios términos municipales: Algarrobo, Almáchar, Benamargosa, Benamocarra, Cútar y en varias localidades de Vélez-Málaga. Asimismo se ha observado la infección sobre la mayoría de cultivares, en particular en la principales variedades cultivadas en la Axarquía: Osteen, Keitt, Kent y Tommy Atkins.

En relación con la epidemiología de la enfermedad se ha puesto de manifiesto una elevada diversidad genética en las poblaciones de *Fusarium*, hongo causante de la enfermedad. Así se han identificado aislados de tres especies diferentes: *F. mangiferae* (descrito como el principal agente causal en el hemisferio Norte), *F. tupiense*, y dos aislados de *Fusarium* próximos a *F. phyllophilum*, pero también patógenos aunque no corresponden a ninguna especie antes descrita como agente causal de la MMD. Así mismo se han detectado tres genotipos diferentes entre los aislados de la especie *F. mangiferae*. Todos ellos ya presentes en 2009 en la zona, lo que indica que al menos se han producido cinco entradas diferentes de inóculo primario antes de 2009, no habiéndose detectado nuevos genotipos a partir de ese año. Asimismo los dos genotipos mayoritarios en la zona (*F. mangiferae* genotipo 1 y *F. tupiense*) ya fueron aislados en 2006.

En cuanto a la distribución de las dos especies mayoritarias, mientras en 2009 se observaba un predominio de *F. mangiferae*, en años posteriores la especie predominante ha sido *F. tupiense*. Esta especie se está expandiendo en la Axarquía de una forma aparentemente más eficaz que *F. mangiferae*. Así en 20 de las 22 fincas infectadas, muestreadas por primera vez entre 2010 y 2012, el patógeno aislado ha sido *F. tupiense*. Aunque su procedencia es desconocida, el hecho de que la población (homogénea) de aislados de esta especie encontrados en la Axarquía sean prácticamente idénticos a uno de los genotipos descritos en Brasil sugiere que tuvo lugar al menos una introducción del patógeno y que éste se ha ido dispersando probablemente a través de material de injerto contaminado.

En buena medida, parece observarse que las fincas afectadas que han sido sometidas a un manejo adecuado y exhaustivo han reducido la afectación, resta insistir en la necesidad de extremar las medidas de control y cuarentena sobre la importación de material vegetal para disminuir todo lo posible el riesgo de nuevas entradas de patógeno.

SUMMARY

SUMMARY

Mango (*Mangifera indica* L.), is one of the most important fruit crops in the world. Native to Southern Asia, mango has been introduced in tropical and subtropical areas worldwide. In Europe, this crop is found mainly in Southern Spain and Portugal. The mango belongs to the *Anacardiaceae* family, order *Sapindales*, genus *Mangifera*, consisting of approximately 69 species, from which only mango and another three species are used in agriculture (Samson, 1986; Litz, 1994).

The largest global producers are India, China, Indonesia and Mexico. In Spain this crop was introduced at the end of the last century via the Canary Islands, and currently is located mainly in the coastal areas of Málaga and Granada. Its planting rate is relatively fast mostly due to its commercial opportunities in Europe (Ferrer, 1992). The main cultivars in commercial orchards in Spain are Osteen, Keitt, Kent and Tommy Atkins, with other cultivars appearing to a lesser extent. The commercial mango orchards in Southern Spain are primarily located in the Axarquía region, (Málaga province), started in 1985, and substantially increasing in the number of hectares cultivated in recent years (350-400 ha per year). From a commercial point of view, in the Axarquía region this crop could suffer severe problems derived from monoculture, with Osteen cultivar representing 80% of the total area cultivated (Díaz-Robledo & Hermoso, 2009). Currently, this scene is changing and cultivar diversification is increasing.

Data from ASAJA (Agrarian association of young farmers) in 2010 reported more than 11000 tons of mango produced in the Axarquía with a turnover of 13 million euros (Gutiérrez-Barranquero, 2012).

The mango crop may suffer several pathologies both biotic and abiotic in origin. Among the most important pests affecting this crop are several species of fruit fly, trips and mites. Highlighting the importance of the Mediterranean fruit fly *Ceratitis capitata* Wied. for its wide distribution; and the seed borer beetle *Sternochetus* (=*Cryptorhynchus*) *mangiferae* (Fabricius), an important pest that limits plant production in nurseries and is difficult to control (Galán-Saúco, 1999). Anthracnose disease (*Colletotrichum gloesporoides*) (Penz.) Penz. & Sacc. is one of the most destructive and widespread diseases affecting leaves, panicles and fruits. Another important fungal disease is the powdery mildew disease (*Oidium mangiferae* Berthet), causing severe damage if not treated, especially during flowering and fruit set periods (Torés, 1997).

One of the most important diseases affecting mango crops in the Mediterranean area (Spain, Portugal, Italy and Israel) and other countries with similar weather conditions (Australia) (Cazorla *et al*., 1998; Golzar & Cother, 2008) is the bacterial apical necrosis (BAN) elicited by *Pseudomonas syringae* pv. syringae. BAN disease is characterized by rapidly expanding necrotic lesions on buds and leaves. Stem and panicles can also be affected. The bacterium is favoured by cool, humid weather

conditions, rainfall being an important vehicle for disseminating primary inoculum among nearby trees. A new etiology of the BAN associated to this crop in the Canary Islands (Spain), was described by this group for the first time, caused by pathogenic strains of *Pantoea agglomerans* (Gutiérrez-Barranquero *et al*., 2012a). The control of this disease in the field is very complex, due to the amount of factors that may be involved in the development of certain symptoms. Copper based compound Bordeaux mixture, have been proved to control BAN disease effectively (Cazorla *et al*., 2006); nevertheless, its toxicity can lead to contamination problems in soil. Silicon gel, a more environmental friendly treatment against the disease was developed by this group as an alternative to Bordeaux mixture. The efficacy of Silicon gel has been demonstrated and nowadays is been commercially used to control BAN disease in commercial orchards in Spain (Gutiérrez-Barranquero *et al*., 2012b). A second relevant bacterial disease is the black spot (French, 1989) caused by *Xanthomonas campestris* pv. mangiferaeindicae, affecting mostly aerial parts of the mango, including fruits. Leaf and fruit symptoms are most common, but twig and branch cankers are found when the infection is severe (Gagnevin & Pruvost, 2001). Until now, this disease has not been observed in the Mediterranean area.

1.1 Mango malformation disease

Mango Malformation Disease (MMD), currently the most important disease of mango worldwide, is of growing concern because it is widespread and destructive and because control is not well understood. Malformation is well known in India where it was first detected in 1891 (Kumar *et al*., 1993; Marasas *et al*., 2006), and has also been confirmed in most of the mango-growing countries: Egypt, South Africa, Sudan, Israel, Malaysia, Oman, China, Pakistan, Bangladesh, Brazil, Mexico and USA, among others (Marasas *et al*., 2006; Youssef *et al*., 2007; Kavas *et al*., 2008; Zhan *et al*., 2010; Kumar *et al*., 2011), and in the present work is described in Spain (Crespo *et al*., 2012). Malformed inflorescences in a tree generally do not bear fruit, and in the cases they do, lose them prematurely; thus causing losses in yield. Yield loss from the disease can reach 83% (Kumar *et al*., 1993).

There are two stages of MMD: vegetative and inflorescence malformations. Vegetative malformation (VM), first described in 1953 by Nirvan (Kumar *et al*., 1993) usually occurs in young seedlings particularly in nurseries, especially when seedlings are grown beneath affected trees, which is a common practice in the Middle East (Ploetz *et al*., 2001; Yuossef *et al*., 2007). Vegetative malformation also appears in mature trees. Typical symptoms in seedlings are loss of apical dominance and swelling of vegetative buds in the leaf axil or at the tip. Apical and axillary buds

produce misshapen shoots with shorten internodes and dwarf leaves that are brittle and recurved towards the supporting stem. Shoots may not expand fully, resulting in a bunched appearance: the so called bunchy-top symptom of the disease. Young nursery plants remain stunted and die young if all buds on a plant are affected. Development of VM on most branches in a tree leads to considerably reduced or no flowering (Kumar & Beniwal, 1992). Generally, a branch showing VM produces malformed inflorescences (Singh *et al*., 1961). Furthermore, the disease seriously debilitates seedlings used as rootstock and complicates the safe national and international movement of germplasm (Ploetz, 2001).

Inflorescence malformation occurs in mature trees at flowering. This form of malformation results in an enlargement of the inflorescence, increased panicle growth and the abortion of fruit production (Kumar *et al.,* 1993), thus it is a more important problem than vegetative malformation. Primary or secondary axes on affected panicles are often shortened, thickened and greatly branched. Malformed panicles produce up to three times the normal number of flowers, ranging from half to two times normal size, and have an increased proportion of male vs. perfect flowers (Singh *et al*., 1961). Malformed panicles may also produce dwarfed and distorted leaves.

Since it was first detected in India more than a century ago, MMD has spread and, as mention before, currently is present in most of the mango growing countries of four continents (Marasas

et al., 2006; Youssef *et al*., 2007; Kavas *et al*., 2008; Lima *et al*., 2009a; Zhan *et al*., 2010), and in this work in Spain (Crespo *et al*., 2012). Several *Fusarium* species have been associated with this disease. *Fusarium mangiferae*, the most prevalent species in the Northern hemisphere, have been reported to be the causal agent of MMD in at least South Africa, Egypt, Israel, Oman and United States (Britz *et al*., 2002; Youssef *et al*., 2007; Kvas *et al*., 2008) and in this work in Spain (Crespo *et al*., 2012, 2014). Nevertheless, in the Southern hemisphere other *Fusarium* species associated with this disease are more prevalent; *Fusarium sterilihyphosum* in South Africa and Brazil (Steemkamp *et al*., 2000; Britz *et al*., 2002; Lima *et al*., 2009b), and recently, *Fusarium tupiense* in Brazil and Senegal (Lima *et al*., 2012; Senghor *et al*., 2012), and also it is described in Spain in this study. Likewise, *Fusarium mexicanum* have been recovered from infected mango tissue in Mexico (Otero-Colina *et al*., 2010), and *Fusarium proliferatum* was reported from China, Egypt and Malaysia (Haggag *et al*., 2009; Zhan *et al*., 2010; Nor *et al*., 2012)

All of the species associated with MMD belongs to the *Gibberella fujikuroi* species complex and have similar morphological characters (Leslie & Summerell, 2006; Kvas *et al*., 2009; Lima *et al*., 2012). PCR primer pairs have been used to diagnose some of the taxa above. The 1-3F/R primer pair (Zheng & Ploetz, 2002), that amplifies a 608 pb DNA fragment for *F. mangiferae* have been used for diagnostic purposes of this species (Youssef *et al*., 2007; Crespo *et al*., 2012). Another pair, 61-2F/R, originally developed to diagnose *Fusarium subglutinans* from

maize (Möller *et al*., 1999), reported to amplify a 445 pb fragment from *F. sterilihyphosum* and *F. mexicanum* (Rodriguez-Alvarado *et al*., 2008).

The population diversity of these species can be differentiated and characterized using several genetic and molecular tools, among them vegetative compatibility groups (VCGs), amplified length polymorphisms (AFLPs) (Lima *et al*., 2009b), random amplified polymorphic DNA (RAPD), multilocus DNA sequence data (O´Donnell *et al*., 1998a, 2000; Steenkamp *et al*., 2000; Marasas *et al*., 2006; Lima *et al*., 2009b; Crespo *et al*., 2014) and identification of mating-type idiomorphs based on polymerase chain reaction (PCR) assays (Britz *et al*., 2000). Determining the variability of an unknown *Fusarium* population, in our case *Fusarium* spp. causing MMD in Spain, can be very useful to know more about the introduction and dispersion of the pathogen in a restricted area, and subsequently, develop more effective control strategies. To determine the genetic diversity of the *Fusarium* spp. Spanish isolates pathogenic on mango, various methodological approximations were taken into account. Arbitrary PCR (ap-PCR), RAPD and VCG analysis has been shown to be a useful technique for examining genetic diversity among populations of plant pathogenic fungi (Zheng & Ploetz, 2002). Genetic diversity was previously determined among *F. mangiferae* isolates from different origins. Zheng & Ploetz (2002) identified six different VCGs for *F. mangiferae*, and found additional heterogeneity for RAPD bands within some of the *F.*

mangiferae VCGs. In 2009, Lima *et al*., described 6 VCGs for *F. tupiense* in Brazil and found correlation between AFLP and VCG analysis (Lima *et al*., 2009a).

Microconidia are infected propagules since they are the primary spores that are produced by the fungus (macroconidia are less common) and form profusely on dead malformed tissues. Propagation of the disease within an orchard is limited, due to the reduced survival of conidia on the soil surface or when buried, nevertheless can be favoured by some arthropods as the mango mite, *Aceria mangiferae*. Presumably, mites feeding on buds facilitates infection, although it does not appear to play a significant role in disseminating the pathogen among trees (Gamliel-Atinsky *et al*., 2009a and c). The pathogen is spread by grafting, and also in infected nursery stocks. Spread on a smaller scale is clearly evident in nurseries (Prakash & Srivastava, 1987). Thus, dissemination across large distances is most likely to occur via propagation material (Lima *et al*., 2009a). The best way to avoid problems with the disease is to establish new plantings with pathogen-free nursery stock. Scion material should never be taken from an affected orchard, and affected plants that are observed in the nursery should be removed and destroyed. Once the disease is found in an orchard, cultural management has been most effective (Freeman *et al*., 2014).

1.2 Detection and isolation of *Fusarium* strains causing MMD in the Axarquía region (Spain)

Our goal in this thesis was to confirm the presence of MMD in Southern Spain and know its distribution; to identify the *Fusarium* species associated with this disease in the Axarquía region and to confirm their pathogenicity. Analyze the *Fusarium* spp. population diversity, and determine the relationships amongst these strains and other *Fusarium* spp. associated with MMD worldwide.

Symptoms of MMD were first observed in the Axarquía region in the year 2006 where few samples of symptomatic trees were collected and *Fusarium* sp. isolated without further identification. To confirm the presence of MMD in the Axarquía region, in this work surveys were conducted during four consecutive years (2009-2012) in different districts of the Axarquía region: Algarrobo, Almáchar, Benamargosa, Benamocarra, Cútar, Frigiliana and Vélez-Málaga. From 36 of these orchards *Fusarium* sp. was isolated from vegetative shoots and floral tissue of symptomatic mango trees and 13 of these orchards were supervised for incidence of the disease during several consecutive years. As result of these surveys, a total of 127 single-spore isolates of different *Fusarium* sp. strains were obtained from mango infected tissues, from 10 different districts of the Axarquía region. The seven isolates, previously obtained in 2006, were added to this collection. Therefore, in this study we

confirm the presence of MMD in the Axarquía region (Spain), showing a relevant dispersion and affecting the majority of mango cultivars, in particular the most prevalent in the Axarquía region: Osteen, Keitt, Kent and Tommy Atkins.

1.3 Identification of the causal agents of MMD in Spain

In order to assess the identity of *Fusarium* strains isolated from mango malformed tissue, morphological and molecular analysis were carried out. Forty of these isolates possessed dark purple-to-salmon-coloured mycelium when grown on potato dextrose agar (PDA) medium, and on fresh carnation leaf agar (FCLA) medium, mycelium contained aerial conidiophores possessing three- to five-celled macroconidia and abundant microconidia in false heads from mono- and polyphialides; while cream-orange-colored sporodochia were produced on the surface of the medium, typical for *F. mangiferae.* Thirty-eight isolates were also diagnosed as *F. mangiferae* by a specific PCR assay with the primer pair 1-3F/R that amplified a 608-bp DNA fragment; the majority of these isolates also showing typical morphology of this species with the exception of isolate UMAF F0923 that amplified with F1-3F/R primer pair, but showed morphological characteristics distinct from *F. mangiferae*, and particularly it was non-pathogenic on mango inoculations assays. The isolates UMAF F1043 y UMAF F1063 that showed morphological

characteristics similar to *F. mangiferae*, and isolate UMAF F0602 did not amplified with the specific primers for this species.

On the other hand, 90 isolates presented microconidia in false heads from mono- and polyphialides and the absence of chlamidospores, but macroconidia were shorter and wider compared to those produced by *F. mangiferae* isolates with the exception of isolate UMAF F0602 that presented cultural degeneration in the way of lacking aerial mycelium and subsequently, its morphology was difficult to assess. All of these 93 isolates (including UMAF F0602, UMAF F1043 y UMAF F1063) together with *F. sterilihyphosum*, *F. mexicanum* and *F. tupiense* reference strains amplified a 445 pb fragment with 61-2F/R primer pair; subsequently, these Spanish isolates were identified as *F. tupiense*, based on additional molecular techniques described further in this thesis. Three other isolates (UMAF F0927, UMAF F0928 and UMAF F1062) with atypical morphological characteristics (different of those described for *Fusarium* species associated with MMD) were initially identified morphologically as *Fusarium* spp. and additionally they did not amplified with any of the tested primers, thus, the species level in this isolates was not conclusively determined. Two of this isolates (UMAF F0927 and UMAF F0928) in a phylogenetic analysis carried out further described on this thesis were clustered close to *Fusarium phyllophilum*, and so on they will be referred as *F. phyllophilum*-like in the present work.

1.4 Pathogenicity assays

At this point was necessary to confirm the role of the *Fusarium* spp. isolated from mango in Southern Spain as causal agents of the disease. Therefore, inoculation assays on mango trees were carried out with some Spanish representative isolates: 8 *F. mangiferae* (including isolate UMAF F0923), 12 *F. tupiense*, 1 *F. phyllophilum*-like and 1 *Fusarium* sp. as well as with a *F. mangiferae* control strain (MRC7560) from Israel. These experiments were conducted in three independent assays, in two different years (March and November 2010; and November 2011). Seven Spanish isolates of *F. mangiferae,* twelve *F. tupiense* isolates and the *F. phyllophilum*-like isolate (UMAF F0927), as well as the control strain inoculated on mango trees, induced typical symptoms of MMD. The exceptions were *F. mangiferae* isolate UMAF F0923 (diagnosed as *F. mangiferae* by PCR) and *Fusarium* sp. isolate UMAF F1062. The totality of the isolates newly recovered from artificially induced symptoms were identical morphologically to those inoculated, and the specific DNA fragment (608 pb and 445pb) was also amplified with PCR in all of them with the exception of *F. phyllophilum*-like isolate UMAF F0927, as expected.

1.5 Population diversity of *Fusarium* spp. Spanish isolates pathogenic on mango

With the aim of elucidate epidemiological aspects and design more efficient control strategies, *F. mangiferae, F. tupiense,*

and *F. phyllophilum*-like Spanish isolates were included in a population study. Population diversity among *Fusarium* spp. isolates associated with MMD in Spain was determined by ap-PCR, RAPD-PCR, vegetative compatibility groups (VCGs), mating type and phylogenetic analyses. The ap-PCR and RAPD-PCR analysis was performed with five different primers $(GACAC)_3$, $(GACA)_4$, $(CAG)_5$, OPF-08 y OPF-13, using DNA extracted from 131 *Fusarium* Spanish isolates, and seven representative isolates from different *Fusarium* species causing MMD worldwide as reference: *F. mangiferae* from Israel, Egypt and Florida (USA), *F. mexicanum*, *Fusarium pseudocircinatum, F. sterilihyphosum* and *F. tupiense*. As result, clear differences in the banding pattern were observed among the *Fusarium* Spanish isolates, differentiating clearly the three different species. Regarding *F. mangiferae* Spanish isolates, intraspecific diversity was also detected with primers $(GACA)_4$ and OPF-13, resulting in the detection of two different genotypic patterns among the Spanish isolates, genotype 1 comprising the majority of the isolates, and genotype 2 grouping four isolates collected in the same orchard but in different years, and the reference isolates MRC7560 and EM50B from Israel and Egypt.

These two genotypes partially corresponded with the three VCGs detected among the 33 *F. mangiferae* Spanish studied isolates; VCG 7 grouping the majority of isolates (also determined as genotype 1), VCG 8 grouping the four isolates of genotype 2, and a third VCG grouping four Spanish isolates from the same

orchard (UMAF F0923, UMAF F12125, UMAF 12126 and UMAF 12127, and also of genotype 1) with isolates X3875-2 and X3875-5 from South Africa in VCG 5, previously described by Zheng & Ploetz (2002). None of the 33 representative Spanish isolates complemented with the tested *F. mangiferae* reference isolates from Egypt, USA and Israel; nevertheless, *F. mangiferae* isolate MRC7560 from Israel was included with *F. mangiferae* isolates EM50B and EM43C from Egypt in VCG 2, according to Zheng & Ploetz (2002). Although these isolates showed close genetic similarity according to the ap- PCR markers with the Spanish isolates in VCG 8 (genotype 2), they were located in a distinct VCG (VCG 2). Similarly, isolates UMAF F12125, UMAF F12126 and UMAF F12127 shared a similar ap-PCR profile with the 30 *F. mangiferae* Spanish isolates of VCG 7 (genotype 1), but were located in a different VCG, VCG 5. These results suggest that the Spanish population of *F. mangiferae* is different from the populations of *F. mangiferae* tested from Egypt, USA and Israel (Crespo *et al*., 2014). In the case of *F. tupiense* Spanish isolates, with the exception of isolate UMAF F1182, all isolates showed an identical banding pattern among them with all of the primers tested. This profile resulted also identical to one of the *F. tupiense* isolates from Brazil (CLM386) located in VCG I according to Lima *et al*., (2009a). All 41 representative Spanish *F. tupiense* tested isolates were grouped in the same VCG together with *F. tupiense* isolate CLM386 (VCG I). *Fusarium phyllophilum*-like Spanish isolates UMAF F0927 and UMAF F0928, were located on a single

VCG (named as VCG D), and shared an unique and identical band profile with all the primers tested.

Furthermore, we identified mating-type idiomorphs (*MAT*-1 or *MAT*-2) for *Fusarium* Spanish isolates. Among the *F. mangiferae* Spanish isolates, only two were identified as *MAT*-1 whereas the majority of isolates appeared as *MAT*- 2. On the other hand, the majority of *F. tupiense* isolates were identified as *MAT*-2 with the unique exception of isolate UMAF F1168 identified as *MAT*-1. These results confirm the population variability, and also suggest that probably in field these populations reproduce only vegetatively.

Concerning the unsolved identity of the *Fusarium sp.* Spanish isolates pathogenic on mango, as well to confirm the identification of *F. tupiense* Spanish isolates, a phylogenetic analysis with several housekeeping genes was carried out in two independent assays. Firstly in our lab, the translation elongation factor 1-α (TEF) and β-tubulin DNA sequences of some representative Spanish isolates: 10 *F. mangiferae*, 17 *F. tupiense* and 2 *F. phyllophilum*-like comprising the maximum possible diversity, were compared with similar sequences from strains of other species in the *G. fujikuroi* species complex already available in GenBank. The resulted phylogenetic tree grouped *F. mangiferae* Spanish isolates in the Asian Clade with other *F. mangiferae* reference isolates from Israel and India. *F. tupiense* Spanish isolates grouped with *F. tupiense* isolates from Brazil in the American Clade, and the two pathogenic *Fusarium phyllophilum*-

like Spanish isolates were grouped together in a cluster in the African Clade, closer to *Fusarium udum*.

A second and deeper phylogenetic analysis with DNA sequence data from portions of seven genes, TEF, β-tubulin, calmodulin, histone H3, nuclear ribosomal intergenic spacer region (IGS rDNA), and the RNA polymerase subunits RPB1 and RPB2 was carried out in the Bacterial and Foodborne Pathogen and Mycology Research Unit, U.S. Department of Agriculture-Agricultural Research Service (IL, USA). This phylogenetic analysis was carried out with seven representative Spanish isolates: two *F. mangiferae,* four *F. tupiense* and one *F. phyllophilum*-like, and including sequence data of several MMD-associated *Fusarium* spp. and other members of the *Gibberella fujikuroi* species complex. Similar results were obtained, nevertheless *F. phyllophilum*-like isolate (UMAF F0927) was grouped on a separate cluster in the African Clade as well, but closer to *Fusarium phyllophilum* than to *F. udum.* The two *F. phyllophilum*-like Spanish isolates are not conclusively identified, nevertheless they appear closer to *Fusarium phyllophilum*, and for this on this study they are referred as *F. phyllophilum*-like.

In this study, we also tested the fertility of *F. mangiferae* and *F. tupiense* Spanish isolates in cross-fertility assays. Most ascomycetes, including *Fusarium* are usually vegetatively haploid and can propagate vegetatively or reproduce sexually. Typically, only the anamorph or the asexual stage of the fungus is found in the field and in culture, and the fungus is identified on this basis;

the teleomorph or sexual stage (perfect stage) is unknown in many cases. All the *Fusarium* species are monoecious (an individual produces both male a female sexual structures), with an idealized strain in a heterothallic species described as "self-sterile" hermaphrodite. This idealized strain can serve as male and as a female, but cannot fertilize itself because different mating types are required for a sexual cross to be successful. For a sexual cross to occur, both strains have to be in the same biological species and carry different *MAT* allele. In general, male-fertile/female-sterile strains are relatively common under field conditions. High fertile female *F. mangiferae* tester strains are not available for using in crossing protocols as the teleomorph of this species have not been found; on the other hand, the teleomorph of *F. tupiense* have been previously described (Lima *et al*., 2012) and two female/male fertile tester strains were included on this study. In our experiments, any of the *F. mangiferae* tested isolates produced perithecia, possibly due to a reduced female fertility of the field isolates, or to unsuitable cross conditions. In any of the crosses of *F. tupiense* Spanish isolates with the tester strains of *F. tupiense* from Brazil we found the formation of perithecia. This most likely was as result of inappropriate cross conditions, impossible to solve after several unsuccessful attempts.

In conclusion, in this study, we confirm the presence of MMD in the Axarquía region in Spain, showing a relevant dispersion and affecting the majority of mango cultivars. In spite of control measurements carried out in a greater or lesser extent,

the disease was very difficult to control, and only in one orchard was totally eradicated. These results suggest that cleaning labors of affected panicles and shoots should be done with a higher frequency and intensity. With all the data obtained in this work, MMD in the Axarquía region is caused by three different *Fusarium* species: *F. mangiferae*, *F. tupiense* and a newly described *Fusarium* sp. and pathogenic on mango which is closer to *F. phyllophilum*. The diversity studies carried out in these *Fusarium* species pathogenic on mango suggest that *F. mangiferae* isolates represent a population different to the populations tested found in Egypt, Israel and Florida (USA). In the case of *F. tupiense*, the Spanish isolates represent a clonal population identical to one of the populations described in Brazil. Finally, two *Fusarium* sp. isolates close to *F. phyllophilum* were found associated with the disease.

Spatial distribution of the *Fusarium* genotypes in the Axarquía region also strengthens the hypothesis that in this area spread of the pathogen is most likely via propagation material; thus, stronger sanitary measurements should be consider involving movement of propagation plant material into the country to avoid new introductions of primary inocula, and through the nurseries stocks to prevent the spread of the pathogen in the region.

PREFACE

1. PREFACE

1.1. Mango crop

Mango (*Mangifera indica* L.), is a tree originated from Southeastern Asia (Birmania, Himalayan foothills, and Sri Lanka) where wild populations can still be found, and where mango had been cultivated since ancient times as testified by the Vedas, the sacred scriptures of Hinduism dated to 1500-1000 BCE (Galán-Saúco, 2009). The spread of this crop was fairly fast through the Indian subcontinent and Malaysia with the increase of commerce between Asia and Europe. Introduced in Eastern Africa by Arab and Phoenician travelers, the Spaniards introduced to the American continent from Philippines in the XVI century and the Portuguese to Brazil in 1700 (Galán-Saúco, 2009). In Spain this crop was introduced at the end of the last century via the Canary Islands, and is currently located mainly in the coastal areas of Málaga and Granada.

The mango belongs to the *Anacardiaceae* family, order *Sapindales*, genus *Mangifera*, consisting of approximately 69 species, from which only mango and another three species are used in agriculture (Samson, 1986; Litz, 1994). Generally, mango varieties are divided into two groups; the Indian, monoembrionic and more aromatic, from which most commercial cultivars are derived; and the Indochina (East India and Philippines),

polyembrionic and less aromatic, used as rootstock. In nurseries, propagation is usually done by several grafting techniques, from the traditional approach method used in India since time immemorial, to several methods of grafting using scions.

The mango crop is relatively more resistant to wind than to cool (Ibar, 1986), with an optimal temperature of 26-27ºC. This crop may develop well at temperatures between 20ºC and 25ºC, reaching the tree a dormancy period when temperatures drop below 15ºC (Samson, 1986). The largest global producers are India, China, Indonesia and Mexico. In Spain, the planting rate of mango is relatively fast mainly due to its commercial opportunities in Europe (Ferrer, 1992). The main cultivars in commercial orchards in Spain are Osteen, Keitt, Kent and Tommy Atkins, with other cultivars appearing to a lesser extent. The commercial mango orchards in Southern Spain are mainly located in the Axarquía region, (Málaga province), started in 1985, and substantially increasing in the number of hectares cultivated in recent years (350-400 ha per year). From a commercial point of view, in the Axarquía region this crop could suffer severe problems derived from monoculture, with Osteen cultivar representing 80% of the total area cultivated (Díaz-Robledo & Hermoso, 2009). Currently, this scene is changing and cultivar diversification is increasing. Data from ASAJA (Agrarian Association of Young Farmers) in 2010 reported more than 11000 tons of mango produced in the Axarquía with a turnover of 13 million euros (Gutiérrez-Barranquero, 2012).

The plagues and diseases that affect mango crop are determined by the crop conditions, its geographical location and the idiosyncrasy of the cultivar. Among the most important pests affecting and limiting this crop are several species of fruit fly, trips and mites. Highlighting the importance of the Mediterranean fruit fly *Ceratitis capitata* Wied. for its wide distribution; and the seed borer beetle *Sternochetus* (=*Cryptorhynchus*) *mangiferae* (Fabricius), an important pest that limits plant production in nurseries and is difficult to control (Galán-Saúco, 1999). Anthracnose disease (*Colletotrichum gloesporoides*) (Penz.) Penz. & Sacc. is one of the most destructive and widespread diseases affecting leaves, panicles and fruits. Another important fungal disease is the powdery mildew disease (*Oidium mangiferae* Berthet), causing severe damage if not treated, especially during flowering and fruit set periods (Torés, 1997).

The main disease affecting mango crops in the Mediterranean area (Spain, Portugal, Italy and Israel) and other countries with similar weather conditions (Australia) is the bacterial apical necrosis (BAN) elicited by *Pseudomonas syringae* pv. syringae (Cazorla *et al*., 1998; Golzar & Cother, 2008). BAN disease is characterized by rapidly expanding necrotic lesions on buds and leaves. Stem and panicles can also be affected. The bacterium is favored by cool, humid weather conditions, rainfall being an important vehicle for disseminating primary inoculum among nearby trees (Cazorla *et al*., 1998). A new aetiology of the BAN associated to this crop in the Canary Islands (Spain), was

also described by this group for the first time, caused by pathogenic strains of *Pantoea agglomerans* (Gutiérrez-Barranquero *et al*., 2012a). The control of this disease in the field is very complex, due to the amount of factors that may be involved in the development of certain symptoms. Copper based compound Bordeaux mixture, have been proved to control BAN disease effectively (Cazorla *et al*., 2006); nevertheless, its toxicity can lead to contamination problems in soil. Silicon gel, a more environmental friendly treatment against the disease was developed by this group as an alternative to Bordeaux mixture. The efficacy of Silicon gel has been demonstrated and nowadays is being commercially used to control BAN disease in commercial orchards in Spain (Gutiérrez-Barranquero *et al*., 2012b). A second relevant bacterial disease worldwide is the black spot (French, 1989) caused by *Xanthomonas campestris* pv. mangiferaeindicae, affecting mostly aerial parts of the mango, including fruits. Leaf and fruit symptoms are most common, but twig and branch cankers are found when the infection is severe (Gagnevin & Pruvost, 2001). Until now, this disease has not been observed in the Mediterranean area.

1.2. Mango malformation disease

Mango Malformation Disease (MMD), currently the most important disease of mango worldwide, is of growing concern because it is widespread and destructive and because control is not well understood. Malformation is well known in India where it was first detected in 1891 (Kumar *et al*., 1993; Marasas *et al*., 2006), and has also been confirmed in most of the mango-growing countries: Egypt, South Africa, Sudan, Israel, Malaysia, Oman, China, Pakistan, Bangladesh, Brazil, Mexico and USA, among others (Marasas *et al*., 2006; Youssef *et al*., 2007; Kvas *et al*., 2008; Zhan *et al*., 2010; Kumar *et al*., 2011), and in this work also described in Spain (Crespo *et al*., 2012). Malformed inflorescences in a tree generally do not bear fruit, and in the cases that fruit has already started to grow, lose them prematurely; thus causing losses in yield. Yield loss of the disease can reach 83% (Kumar *et al*., 1993).

1.2.1. Symptoms

There are two stages of MMD: vegetative and inflorescence malformations. Vegetative malformation (VM), first described in 1953 by Nirvan (Kumar *et al*., 1993) usually occurs in young seedlings particularly in nurseries, especially when seedlings are grown beneath affected trees, which is a common practice in the Middle East (Ploetz *et al*., 2001; Yuossef *et al*., 2007). Vegetative

malformation also appears in mature trees. Typical symptoms in seedlings are loss of apical dominance and swelling of vegetative buds in the leaf axil or at the tip. Apical and axillary buds produce misshapen shoots with shorten internodes and dwarf leaves that are brittle and recurved towards the supporting stem. Shoots may not expand fully, resulting in a bunched appearance: the so called bunchy-top symptom of the disease (Figure 1). Young nursery plants remain stunted and die young if all buds on a plant are affected. Development of VM on most branches in a tree leads to considerably reduced or no flowering (Kumar & Beniwal, 1992). Generally, a branch showing VM produces malformed inflorescences (Singh *et al*., 1961). Furthermore, the disease seriously debilitates seedlings used as rootstock and complicates the safe national and international movement of germplasm (Ploetz, 2001).

Inflorescence malformation occurs in mature trees at flowering. This form of malformation results in an enlargement of the inflorescence, increased panicle growth and the abortion of fruit production (Kumar *et al*., 1993), thus it is a more important problem than vegetative malformation. Primary or secondary axes on affected panicles are often shortened, thickened and greatly branched (Figure 1). Malformed panicles produce up to three times the normal number of flowers, ranging from half to two times normal size, and have an increased proportion of male vs. perfect flowers (Singh *et al*., 1961). Malformed panicles may also produce dwarfed and distorted leaves.

Figure 1. Symptoms of mango malformation disease on mango trees. A, Vegetative malformation; B, Floral malformation.

The affected inflorescences, if are not quickly withdrawn constitute an important source of secondary inoculum and contribute to disseminate the disease among nearby trees.

Internal symptoms include: the development of hyperplastic and hypertrophied cells in malformed tissues (Hifni *et al*., 1978); malformed flowers with inflated disks; undifferentiated of poorly developed ovules in infected hermaphrodite flowers; degenerating or undeveloped embryos in diseased fruit (Kumar *et al*., 1993).

1.2.2. Aetiology

Numerous studies have attempted to determine the nature of the disease since its recognition in India in 1981 (Srivastaba *et al*., 1982). The aetiology of mango malformation has been approached from many angles- physiological, viral, acarological, and fungal. Suggested causes include mites (Narasimhan, 1954), nutritional problems (Prasad *et al*., 1965), physiological and hormonal imbalances (Dang & Daulta, 1982; Singh & Dhillon, 1989), viruses (Kauser, 1959) and unknown causes (Kumar & Beniwal, 1991). Summanwar *et al*., (1996) identified the fungal pathogen commonly associated with the disease as *Fusarium subglutinans* (Wollenweber and Reinking) Nelson, Toussoun and Marasas (= *F. moniliforme* Sheldon *var. subglutinans* Wollenweber and Reinking), residing in section Liseola. In 1999, Freeman *et al*., (1999) demonstrated that isolates identified as *F. subglutinans*

induced typical mango malformation symptoms on mango trees, originally collected from mango inflorescences in Israel. In 2002, twenty-nine strains of this fungus pathogen isolated from Egypt, Florida, Israel, Malaysia, and South Africa were described as a new species in the *Gibberella fujikuroi* species complex, *Fusarium mangiferae* Britz, Wingfield and Marasas (Britz *et al.*, 2002). The new *Fusarium* species *F. mangiferae*, was established based on β-tubulin and histone H3 DNA sequences, morphology, and because most of the examined strains had been shown in previous studies to cause malformation on artificially inoculated mango. Based on DNA sequence data (O´Donnell *et al*., 1998a; 2000; Steenkamp *et al*., 1999; 2000), *F. mangiferae* is related to a lineage that includes *Fusarium fujikuroi* Nirenberg, *Fusarium proliferatum* (Matsushima) Nirenberg, and *Fusarium sacchari* (Butler) Gams (Marasas *et al*., 2006), and corresponds to the "Asian Clade" described by O´Donnell *et al*., (1998a). Based on combined sequence data for five housekeeping genes, the closest known relative of *F. mangiferae* is an isolate from tropical rainforest soil in Papua-New Guinea (Marasas *et al*., 2006). *F. mangiferae* produces white, floccose mycelium on PDA with light to dark-purple pigments in the medium (Leslie & Summerell, 2006). Cream-coloured sporodochia are produced on carnation leaf agar (CLA); mycelium contained aerial conidiophores possessing three-to five-celled macroconidia, long, slender and straight to slightly curved, with curved apical cells and foot-shaped basal cells. Microconidia are produced in false heads

from monophialides, and polyphialides with two to five conidiogenous openings. These microconidia are ovoid, single-celled, and never form chains. Chlamydospores are absent.

After the discovery of *F. mangiferae* as the casual agent of MMD, a second species *Fusarium sterilihyphosum* Britz, Wingfield and Marasas, was described originally for isolates from a small area in South Africa (Britz *et al*., 2002). In subsequent work, Lima *et al*., (2009b) detected and reported this species as the casual agent of malformation in Brazil after artificial inoculation. On PDA, colonies of *F. sterilihyphosum* produce white, floccose mycelium with rose to light purple pigmentation in the agar (Leslie & Summerell, 2006). Uncommon, cream- to orange-coloured sporodochia are produced on CLA that produce rare, long, slender, three to five-septate macroconidia. On mono- and polyphialides, ovoid, oval to allantoid microconidia that are usually single-celled are produced on false heads. Sterile coiled hyphae are produced by some isolates of this species.

Since the year 2002 when *F. mangiferae* and *F. sterilihyphosum* were described (Britz *et al*., 2002), three new species of *Fusarium* have been reported as causal agents of MMD, two of them novel species described based on morphology, direct sequencing of two or more genes and/or sexual cross fertility studies. *Fusarium proliferatum* have been reported to cause the disease in Egypt, South China and Malaysia (Haggag *et al*., 2009; Zhang *et al*., 2010; Mohamed Nor *et al*., 2013). *F. proliferatum* is the only species associated with MMD that produces microconidia in chains

growing on PDA, all the rest of the *Fusarium* spp. associated with the disease produce them in false heads. *Fusarium mexicanum* (a novel species) has been described to cause malformation on mango in Mexico (Otero-Colina *et al*., 2010), and its morphological characteristics are close to those described for *F. sterilihyphosum*. *Fusarium tupiense*, the latest species described associated with this disease in Brazil (Lima *et al*., 2012), has also been reported in Senegal (Senghor *et al*., 2012), and in this work in Spain. Morphological features of *F. tupiense* are similar to those described for *F. sterilihyphosum* and *F. mexicanum*, including microconidia aggregated in false heads, mono- and polyphialidic conidiophores, sporodochia present and absence of chlamydospores. *F. proliferatum* and *F. tupiense* are the only two species of the *Gibberella fujikuroi* species complex with a known sexual stage (teleomorph) (Leslie & Summerell, 2006).

All of these five different *Fusarium* species have been associated conclusively with the disease in different growing areas in both hemispheres. *F. mexicanum* have been detected only in Mexico (Otero-Colina *et al*., 2010); *F. proliferatum* in Egypt, China and Malaysia (Haggag *et al*., 2009; Zhan *et al*., 2010; Nor *et al*., 2012), and *F. mangiferae*, the most prevalent species in the Northern hemisphere, have been reported to be the casual agent of MMD in at least South Africa, Egypt, Israel, Oman, USA (Youssef *et al*., 1997; Britz *et al*., 2002; Kvas *et al*., 2008), and in the present work in Spain (Crespo *et al*., 2012, 2014). Nevertheless, in the Southern hemisphere other *Fusarium* species associated with

this disease are more prevalent; *F. sterilihyphosum* in South Africa and Brazil (Steemkamp *et al*., 2000; Britz *et al*., 2002; Lima *et al*., 2008) and *F. tupiense*, in Brazil and Senegal (Lima *et al*., 2012; Senghor *et al*., 2012), and also it is described in Spain in this study.

The population diversity of these species can be differentiated and characterized using several genetic and molecular tools, among them vegetative compatibility groups (VCGs), amplified length polymorphisms (AFLPs) (Lima *et al*., 2009b), random amplified polymorphic DNA (RAPD-PCR), multilocus DNA sequence data (O´Donnell *et al*., 1998a, 2000; Steenkamp *et al*., 2000; Marasas *et al*., 2006; Lima *et al*., 2009b; Crespo *et al*., 2014) and identification of mating –type idiomorphs based on polymerase chain reaction (PCR) assays (Britz *et al*., 2000).

1.2.3. Epidemiology

Microconidia are infective propagules since they are the primary spores that are produced by the fungus (macroconidia are less common) and form profusely on dead malformed tissues. Conidia of the pathogen are dispersed by wind and may disseminate over distances of up to 35 m over a limited time period according to epidemiological studies of the disease (Gamliel-Atinsky *et al*. 2009b), and survival of conidia on the soil surface or when buried is limited (Youssef *et al*., 2007). Thus, dissemination across long distances is most likely to occur via

propagation material (Lima *et al*., 2009b). The pathogen is spread by grafting, and also in infected nursery stocks. Spread on a smaller scale is clearly evident in nurseries (Prakash & Srivastava, 1987). Although malformation can be severe in seedlings in nurseries, this only occurs when they are produced underneath affected trees (Ploetz, 2001). Wounding enhances infection and subsequent disease development (Ploetz & Gregory, 1993).

The mango mite, *Aceria* (*Eriophyes*) *mangiferae* Sayed, has been associated with the disease in the way of moving spores of *F. mangiferae* to infections courts in mango buds via external contamination of its body, and increasing infection of buds by the pathogen (Gamliel-Atinsky *et al*., 2009a and b). Presumably, the mites feeding on buds facilitates infection, although it does not appear to play a significant role in disseminating the pathogen among trees.

1.2.4. Management and control

Obviously, the first question is to control the plant material movement worldwide to avoid introductions of primary inoculum into new regions. Several approaches have been used to manage malformation, but most have been ineffective. The best way to avoid problems with the disease is to establish new plantings with pathogen-free nursery stock. Scion material should never be taken from an affected orchard, and affected plants that are observed in the nursery should be removed and

destroyed. Nurseries should also not be established in orchards, especially when they are affected by malformation.

Once the disease is found in an orchard, control is possible, but time-consuming. In these cases, cultural management has been most effective (Narasimhan, 1959; Singh *et al*., 1974; Manicon, 1989; Freeman *et al*., 2014). Affected terminals and the subtending tree nodes are cut from trees removed from the field and burned or solarized (Figure 2). Unfortunately, pruning to manage malformation is not practical for all producers, some of whom are unable, or unwilling, to devote the effort that is required to ensure that this approach succeeds. In addition, it may be difficult, or impossible, to impose this treatment on large trees.

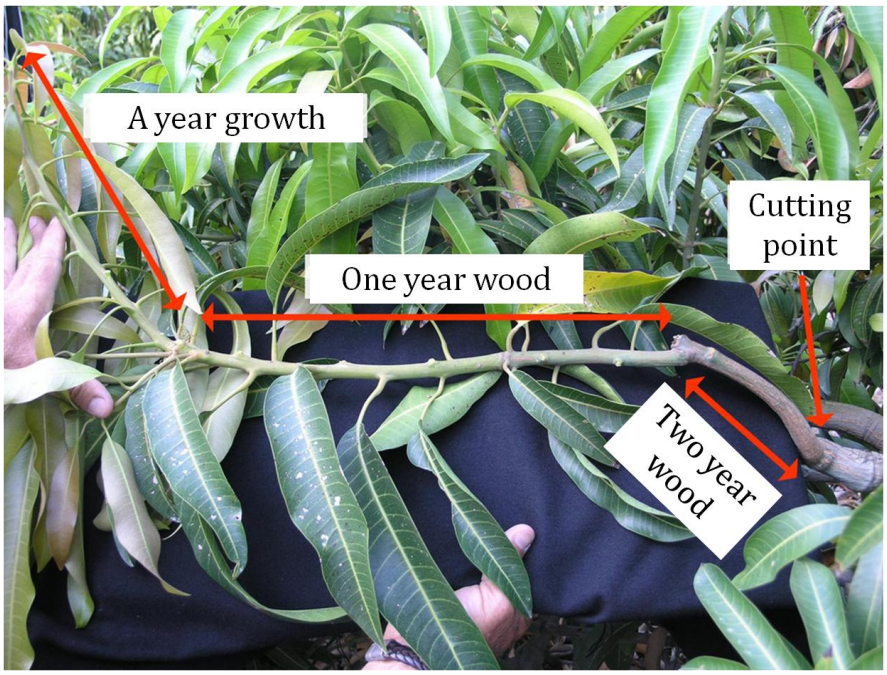


Figure 2 Sanitary pruning. Cutting point below two year wood.

A diverse array of fungicides, hormones and growth regulators has been tested for the control of malformation. These measures have been, at best, marginally effective. Singh *et al*., (1994) tested sulphates of cobalt, cadmium and nickel for malformation control in India. It is unlikely, though, that these compounds could be used safely on this food crop due to their toxicity to humans. In general, the protected, internal location of the pathogen in affected trees makes control of this disease a difficult proposition. Prakash and Srivistava (1987) indicated that there is a great variation in the susceptibility of the existing varieties. Unfortunately, controlled inoculations have not been used to determine cultivar resistance, and these reports have come from nonreplicated tests; cultivars listed as "resistant" may have come from healthy nursery stock or may have escaped infection once planted in the field (Ploetz, 2001).

Symptoms of malformation suggest that a hormone imbalance occurs in the affected tissues. Singh and Dhillon (1989) assayed levels of indol acetic acid (IAA), giberellic acid (GA3) and zeatin in malformed and healthy mango seedlings. Whereas IAA, and GA3 levels were, respectively, between five to ten times lower in malformed plants, levels of the cytokinin zeatin were found to be between five to ten times higher. Whether productions of hormones by the pathogen directly causes the noted changes or whether hormone production by the host is somehow altered in the presence of the pathogen is not clear.

As mentioned, the pathogen is frequently spread by grafting and in infected nursery stock, and spread on a small scale is clearly evident in nurseries (Prakash & Srivastava, 1987). In this context, heat treatments have been shown to kill pathogenic microorganisms Buschaert *et al*., (1978). Freeman and Katan (1988) carried out experiments involving heating within a temperature range that caused a partial reduction of conidial and chlamidospore viability. Nevertheless, additional work needs to be done in the development of strategies to control the fungi as thermotherapy, or generating propagating material free of the pathogen.

AIMS

2. AIMS

Mango malformation is a limiting disease affecting this crop worldwide. Several *Fusarium* species have been associated with this disease. Recently, mango trees with MMD symptoms were observed in the Axarquía region in Southern Spain (Cazorla *et al*., 2009). In this study we plan to collect data about the presence of MMD in Spain and about its causal agent in this area. This study aims to:

1-. Confirm the presence and distribution of MMD in Southern Spain.

2.- Identify the *Fusarium* species associated with this disease in the Axarquía region and confirm their pathogenicity.

3.- Analyze the population diversity of the *Fusarium* species found associated with this disease in Spain, and determine the relationship amongst these strains and other *Fusarium* spp. associated with MMD worldwide.

OBJETIVOS

OBJETIVOS

La malformación es una de las enfermedades más importantes del mango en el mundo y causa importantes pérdidas económicas. Esta enfermedad ha sido atribuida a varias especies de hongos del género *Fusarium*. En la región de la Axarquía (Málaga) se han observado recientemente árboles con síntomas de malformación (Cazorla *et al*., 2009). En este estudio se pretende recoger información sobre la presencia de esta enfermedad en España y su agente causal en la zona de estudio. De forma más específica, esta tesis doctoral se plantea los siguientes objetivos:

1-. Confirmar la presencia de la malformación del mango en el Sur de España y conocer su distribución geográfica.

2.- Identificar las especies de *Fusarium* asociadas a esta enfermedad en la región de la Axarquía y confirmar su patogenicidad.

3.- Analizar la diversidad de las poblaciones de *Fusarium* spp., y determinar las relaciones filogenéticas entre estos aislados y otros *Fusarium* spp. asociados con la malformación del mango a nivel mundial.

MATERIAL AND METHODS

3. MATERIALS AND METHODS

3.1. Surveys: Detection and isolation of *Fusarium* strains

In this work, surveys were conducted between 2009 to 2012 during the blossom seasons (April to July) in different mango orchards, located in different districts of the Axarquía region (Málaga, Spain): Algarrobo, Almáchar, Benamargosa, Benamocarra, Cajiz, Cútar, Frigiliana and Vélez-Málaga. The orchards were selected after reports by agricultural engineers or farmers of suspicious symptoms of mango malformation. Samples of mango malformation disease (MMD) were taken from mango trees of the main cultivars in this area, Keitt, Kent, Osteen, Tommy Atkins, and a variety of minor commercial cultivars from a total of 43 different orchards (Table 1). In thirteen of these orchards monitoring was carried out in different years to evaluate dispersion of the pathogen and the suitability of sanitary measurements. Additionally, a collection of *Fusarium* spp. reference isolates were used as control in different experiments (Table 2), and were kindly donated by different collections (Dr. S. Freeman, Volcani Center, Israel; Dr. R. Ploetz, University of Florida, Tropical and Education Center, FL, USA; and Dr. L. H. Pfenning (Universidade Federal de Lavras, Brazil).

Table 1. Information concerning the isolation of *Fusarium* strains from symptomatic mango trees of mango malformation disease at different time periods and from different orchards and locations in the Axarquía region (Spain).

Origin	Orchards	Cultivar	Survey´s dates	Symptoms[a]		Number of Samples	Number of isolates *Fusarium sp.*	Incidence[c]
Algarrobo	Casa alta	Osteen	Jun 2009	FM	Typical	1	1	Very low
				VM	Unclear	1	0	
	Chicano	Osteen	July 2009	FM	nd	2[b]	0	nd
			June 2010	FM	Unclear	1[b]	0	
	González	Keitt	May 2009	FM	nd	1[b]	0	nd
	Lupiáñez	Osteen	April 2011	FM	Typical	1	1	Low
				VM	Unclear	1	0	
				FM	Unclear	1	0	
	Melgares	Osteen	May 2010	FM	Typical	3	3	Low
				VM	Typical	1	1	
	Peláez	Osteen	April 2011	FM	Typical	1	1	Very low
	Portillo	Keitt	June 2010	FM	Typical	2	2	Medium-low
		Osteen	April 2011	FM	Typical	1	1	Very low
				FM	Unclear	1	0	
		Gomera 3	May 2012	FM	Unclear	1	0	Null
Almáchar	Martín	Osteen	May 2012	FM	Typical	1[b]	1	Low
Almayate (V-M)	Parra	Osteen	May 2011	FM	Typical	1[b]	1	Very low
			June 2011	FM		1[b]	1	
Benajarafe (V-M)	Pintao	Osteen	June 2009	FM	Typical	2	4	Low
				FM	Unclear	1	1	
			June 2012	FM	Typical	2	2	Very low
Benamargosa	Arcas	Keitt	June 2010	FM	Typical	2	2	Low
	Barranco	Keitt	June 2009	FM	Typical	1	1	Low
		Keitt		-	No symptoms	1	0	
		Osteen		FM	Typical	1	1	
		Osteen	May 2010	FM	Unclear	1	0	Low
					Typical	1	1	

Table 1. (Continued)

Origin	Orchards	Cultivar	Survey´s dates	Symptoms[a]		Number of Samples	Number of isolates *Fusarium sp.*	Incidence[c]
Benamargosa	Barranco II	Keitt	June 2009	FM	Typical	1	1	nd
	Encantá	Tommy Atkins	June 2010	FM	Typical	1	1	Medium
			April 2011	FM	Typical	1	1	High
			May 2012	FM	Typical	2	2	High
	Huerta	Keitt/Kent/Osteen	June 2009	FM	Typical	4	4	Very high
		Keitt		VM	Typical	1	1	
		Tommy Atkins	April 2011	FM	Typical	2	2	Very high
				VM	Typical	1	1	
		Osteen	May 2012	FM	Typical	1	1	Very high
		Keitt		VM	Typical	2	2	
Benamocarra	Garzón	Tommy Atkins/Osteen	June 2012	FM	Typical	3	3	Very high
		Sensation		VM	Typical	1	1	
	Matao	Tommy Atkins	June 2012	FM	Typical	1	1	Very high
	Palomo	Keitt	July 2010	VM	Typical	1	1	Very low
	Sarmiento	Kent	June 2011	FM	Typical	3	3	Medium
			June 2012	FM	Typical	2	2	Low
Cajiz	Cerrillo	Osteen/Tommy Atkins	May 2009	FM	Unclear	8	0	nd
		Kent	May 2010	FM	nd	2[b]	0	nd
	Cerro	Tree ungrafted/Tommy Atkins	May 2009	FM	Unclear	7	0	nd
Cútar	Botín	Tommy Atkins/ Dusheri/ Otts	June 2009	FM	Typical	3	3	Low
		Tommy Atkins	June 2012	FM	Typical	1	1	Medium-
		Dusheri		VM	Typical	2	2	high

Table 1. (Continued)

Origin	Orchards	Cultivar	Survey´s dates	Symptoms[a]		Number of Samples	Number of isolates *Fusarium sp.*	Incidence[c]
Frigiliana	Acosta	Osteen	June 2010	FM	Unclear	1[b]	*Fusarium. sp* non pathogenic	nd
Vélez-Málaga	Arroyo	Kent	June 2009	FM	Typical	2	2	Very high
				VM	Typical	1	1	
		Keitt	Mayo 2010	FM	Typical	1	1	Medium-high
				VM	Typical	1	1	
		Keitt	April 2011	FM	Typical	2	2	Medium-high
		Keitt	June 2011	FM	Typical	2	2	
		Kent		VM	Typical	1	1	
		Keitt	May 2012	FM	Typical	1	1	Medium-high
		Gomera 3		VM	Unclear	1	0	
	Braun	Tommy Atkins	July 2010	FM	Typical	1	1	Very low
	Cabrera	Osteen	June 2009	FM	Typical	1[b]	1	Low
		Manzanillo	June 2010	FM	Typical	1	1	High
	Cabrilla	Keitt/ Criollo	Spring 2006	FM	nd	7	7	nd
		Mexicano/Osteen	April 2009	FM	nd	7	0	
	Carauta	Osteen	June 2010	FM	Typical	1	1	Very low
	Carril	Kent	May 2010	FM	Typical	4	2	High
	Casillas	Tommy Atkins	May 2010	FM	Unclear	1	0	Very low- null
	Córdoba	Tommy Atkins	May 2010	FM	Typical	3	3	Very high
	Cortijo	Keitt /Osteen	June 2009	FM	Typical	3	3	High
		Keitt		VM	Typical	2	2	
		Keitt	April 2011	FM	Typical	3	3	Low
		Keitt /Osteen	May 2012	FM	Typical	3	3	Low

Table 1. (Continued)

Origin	Orchards	Cultivar	Survey´s dates	Symptoms[a]		Number of Samples	Number of isolates *Fusarium sp.*	Incidence[c]
Vélez-Málaga	Fondos	Osteen	April 2009	FM VM	Unclear Unclear	1[b] 1[b]	0 0	Very low-null
	Gámez	Osteen	May 2012	FM	Typical	1[b]	1	Low
	Granaíno	Kent Keitt	May 2010	FM VM	Typical Typical	3 1	3 1	High
	Hijano	Keitt	June 2011	FM VM	Typical Typical	2 2	2 2	Very high
	Lara	Osteen Ataúlfo/Osteen	June 2009 May 2010 June 2011 May 2012	FM FM FM VM FM VM	Typical Typical Typical Typical Typical Unclear	2 1 6 1 5 1	2 1 6 1 5 0	Low Medium Medium Medium
	Lorca	Osteen	June 2012	FM	Typical	3[b]	3	Very low
	Moneda	Kent	May 2012	FM	Typical	3	3	nd
	Pinar alto	nd	June 2009	FM	nd	1[b]	0	nd
	Potril	Keitt	June 2009 June 2011 June 2012	FM FM -	Typical Typical No symptoms	3 1 0	3 1 0	Low Low Null
	Tejares	Keitt	June 2009	FM	Typical	2	2	nd
	Triana	Osteen	May 2012	FM	Typical Unclear	1 1	1 0	Medium

Table 1. (Continued)

Origin	Orchards	Cultivar	Survey´s dates	Symptoms[a]		Number of Samples	Number of isolates *Fusarium sp.*	Incidence[c]
Vélez-Málaga	Zingg	Tommy Atkins/ Osteen/ Tree ungrafted	June 2009	FM	No symptoms	0	0	nd

[a] Symptoms: FM, floral malformation; VM, vegetative malformation.
[b] Sample provided by the farmer and processed over 24 hours.
[c] In some orchards the incidence was observed during more than one season.
nd: no data.

Table 2. Reference isolates of different *Fusarium* species used in this study. Strains marked with (X) were used as control in the different experiments carried out in this study.

Species	Isolate[a]	Origin (reference)	Used in experiments of					
			Pathogenicity	VCGs	Arbitrary primer-PCR	PCR diagnosis	MLSTs	Cross-fertility
F. mangiferae	CG-1-4	USA/(Zheng & Ploetz 2002)		X	X	X		
F. mangiferae	CG-2-7	USA/(Zheng & Ploetz 2002)		X				
F. mangiferae	EM22B	Egypt/(Zheng & Ploetz 2002)		X				
F. mangiferae	EM32E	Egypt/(Zheng & Ploetz 2002)		X				
F. mangiferae	EM42C	Egypt/(Zheng & Ploetz 2002)		X				
F. mangiferae	EM43C	Egypt/(Zheng & Ploetz 2002)		X				
F. mangiferae	EM44F	Egypt/(Zheng & Ploetz 2002)		X				
F. mangiferae	EM50B	Egypt/(Zheng & Ploetz 2002)		X	X	X		
F. mangiferae	EM73C	Egypt/(Zheng & Ploetz 2002)		X				
F. mangiferae	MRC7560	Israel/(Volcani Center Collection)	X	X	X	X	X	
F. mangiferae	NRRL25226[b]	Israel/(Volcani Center Collection)					X	
F. mangiferae	X3875-2	South Africa/(Zheng & Ploetz 2002)		X				
F. mangiferae	X3875-5	South Africa/(Zheng & Ploetz 2002)		X				
F. mangiferae	X4707	Egypt/(Zheng & Ploetz 2002)		X				
F. mexicanum	NRRL47473	Israel/(Volcani Center Collection)		X		X	X	
F. mexicanum	NRRL47485	Israel/(Volcani Center Collection)				X	X	
F. mexicanum	NRRL53147[b]	Mexico/(Otero-Colina et al. 2010)					X	
F. mexicanum	NRRL53571	Israel/(Volcani Center Collection)				X	X	
F. mexicanum	NRRL53575	Israel/(Volcani Center Collection)				X	X	
F. mexicanum	NRRL53580	Mexico/(Otero-Colina et al. 2010)		X	X	X	X	
F. oxysporum	NRRL22902[b]	Brazil/(Lima et al. 2012)					X	

Table 2. (Continued)

Species	Isolate[a]	Origin (reference)	Used in experiments of					
			Pathogenicity	VCGs	Arbitrary primer-PCR	PCR diagnosis	MLSTs	Cross-fertility
F. proliferatum	NRRL22944	Israel/(Volcani Center Collection)				X	X	
F. pseudocircinatum	NRRL53570	Israel/(Volcani Center Collection)			X			
F. pseudocircinatum	NRRL22946[b]	Brazil/(Lima et al. 2012)					X	
F. sterilihyphosum	CML283[b]	Brazil/(Lima et al. 2012)					X	
F. sterilihyphosum	NRRL25623 (MRC2802)	South Africa/(Wingfield et al. 2000)		X	X	X	X	
F. sterilihyphosum	NRRL53569 (MRC7602)	South Africa/ (Wingfield et al. 2000)		X		X		
F. subglutinans	NRRL22016[b]	USA/(Leslie et al. 1998)					X	
F. tupiense	CML345[b]	Brazil/(Lima et al. 2012)					X	
F. tupiense	CML1000[c]	Brazil/(Lima et al. 2012)						X
F. tupiense	CML1843[c]	Brazil/(Lima et al. 2012)						X
F. tupiense	NRRL53984[b] (CML262)	Brazil/(Lima et al. 2009)			X	X	X	
F. tupiense	NRRL53986 (CML266)	Brazil/(Lima et al. 2009)		X	X			
F. tupiense	NRRL53992 (CML350)	Brazil/(Lima et al. 2009)		X	X			
F. tupiense	NRRL53993 (CML383)	Brazil/(Lima et al. 2009)		X	X	X		
F. tupiense	NRRL53994 (CML385)	Brazil/(Lima et al. 2009)		X	X			
F. tupiense	NRRL53995 (CML386)	Brazil/(Lima et al. 2009)		X	X			
F. tupiense	NRRL53996[b] (CML389)	Brazil/(Lima et al. 2012		X	X	X	X	
F. udum	NRRL22949[b]	Brazil/(Lima et al. 2012)					X	
F. verticillioides	NRRL22172[b]	Brazil/(Lima et al. 2012)					X	

[a]Accession prefixes: EM and CG (University of Florida, Tropical and Education Center, FL, USA); X (Kansas State University, Manhattan, KS, USA); MRC = Medical Research Council, Tygerberg, South Africa; NRRL =Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA; CML = Coleção Micológica de Lavras (Universidade Federal de Lavras, Brazil).

[b]TEF and β-tubulin sequences of these isolates were obtained from those available in GenBank.

[c]*F. tupiense* isolates kindly gifted by Prof. Ludwig H. Pfenning (Universidade Federal de Lavras, Brazil).

3.2. Samples collection and processing

Samples were taken following a strict hygienic protocol to avoid possible propagation of the pathogen to other trees or nearby orchards, as fungal spores can be transported attached to clothes and footwear. Therefore, and to minimize the risk of pathogen dispersal, disposable working clothes and latex gloves were worn, and sodium hypochlorite and 70 º ethanol solution were used for disinfecting pruning tools and soles of shoes. Infected mango shoots were collected from the tree by cutting with disinfected pruning scissors and carried in refrigerated plastic bags to ensure maximum recovery of fungi. Samples were processed as soon as possible, generally within 24 hours. Once in the lab, samples from floral panicles and vegetative shoots were processed by randomly cutting tissue fragments of approximately 5 mm long. Each sample was surface disinfected in a 1:2:7 alcohol, bleach, water solution, 100 ml final volume, stirring for three minutes. Five or six fragments per isolate of malformed or suspicious samples were plated on Petri dishes with potato dextrose agar (PDA) medium acidified by adding 1ml of 25 % citric acid to 1 l volume (Figure 3). The plates were incubated at 25 ºC in darkness for three to five days. Afterwards, fungal colonies were reisolated on PDA medium for its subsequent identification and preservation. A collection of 127 isolates of *Fusarium* sp. were obtained from samples collected in this work. In addition, seven isolates previously obtained in our laboratory in 2006 were incorporated to this collection (Table 3).

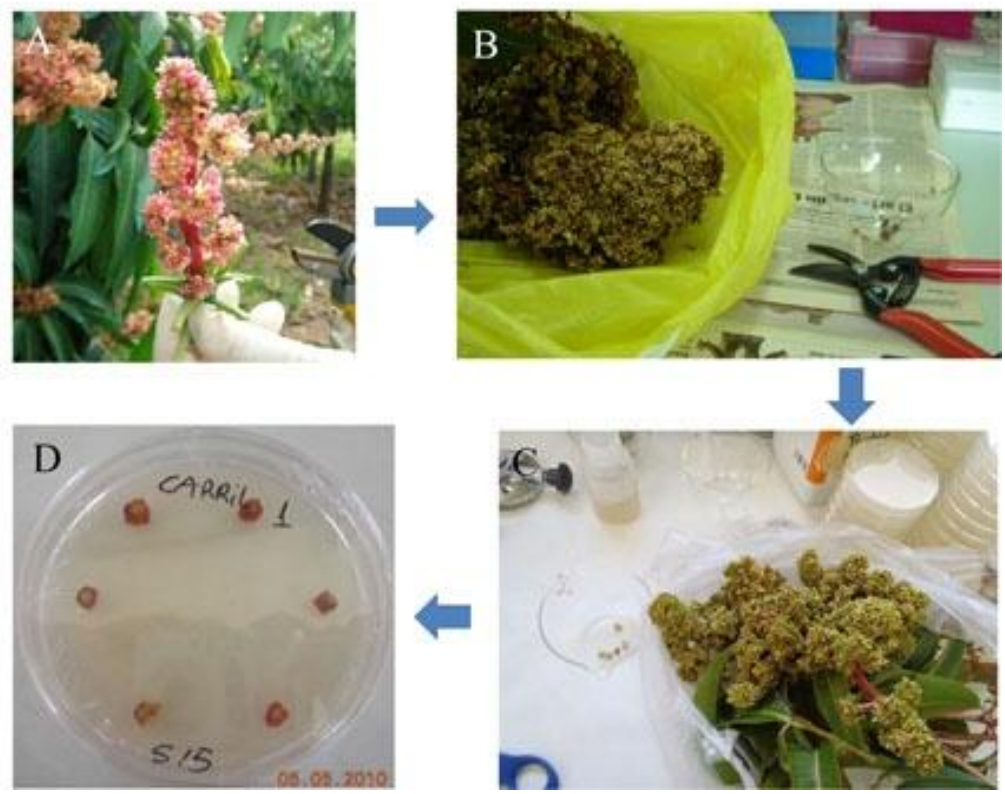


Figure 3. Diagram of samples processing. A, Symptomatic tree showing floral malformation; B, Sample processing in the laboratory; C, Tissue fragments disinfection; D, Tissue fragments plated on acidified PDA.

Table 3. *Fusarium* strains isolated from mango and studied in this work. Isolates marked with (X) were used on pathogenicity, Vegetative Compatibility Group (VCG), phylogenetic analysis and cross fertility assays.

				Used in experiments of			
				Pathogenicity	VCGs	Phylogenetic analysis	Cross-Fertility
Isolate[a]	Origin	Orchard-sample	Year				
UMAF F0601	Vélez-Málaga	Cabrilla 1	2006				X
UMAF F0602	Vélez-Málaga	Cabrilla 2	2006	X		X	
UMAF F0603	Vélez-Málaga	Cabrilla 3	2006				X
UMAF F0604	Vélez-Málaga	Cabrilla 4	2006	X	X	X	X
UMAF F0605	Vélez-Málaga	Cabrilla 5	2006				X
UMAF F0606	Vélez-Málaga	Cabrilla 6	2006		X	X	X
UMAF F0607	Vélez-Málaga	Cabrilla 7	2006				X
UMAF F0908	Benamargosa	Huerta 1	2009		X		X
UMAF F0909	Benamargosa	Huerta 2	2009		X		X
UMAF F0910[b]	Benamargosa	Huerta 3	2009	X	X	X	X
UMAF F0911	Benamargosa	Huerta 4	2009		X	X	X
UMAF F0912	Benamargosa	Huerta 5	2009		X		X
UMAF F0913	Vélez-Málaga	Arroyo 1	2009		X	X	X
UMAF F0914	Vélez-Málaga	Arroyo 2	2009				X
UMAF F0915	Vélez-Málaga	Arroyo 3	2009	X	X	X	X
UMAF F0916	Vélez-Málaga	Lara 1	2009	X	X	X	X
UMAF F0917[b]	Vélez-Málaga	Lara 2	2009	X	X	X	X
UMAF F0918	Benajarafe (V-M)	Pintao 1	2009				X
UMAF F0919	Benajarafe (V-M)	Pintao 3	2009		X		X
UMAF F0920	Benajarafe (V-M)	Pintao 3	2009	X	X	X	X
UMAF F0921	Benajarafe (V-M)	Pintao 2	2009				X
UMAF F0922	Benajarafe (V-M)	Pintao 2	2009				X

Table 3 (Continued)

Isolate[a]	Origin	Orchard-sample	Year	Used in experiments of			
				Pathogenicity	VCGs	Phylogenetic analysis	Cross-Fertility
UMAF F0923	Cútar	Botín 3	2009	X	X	X	
UMAF F0924[b]	Vélez-Málaga	Potril 3	2009	X	X	X	X
UMAF F0925	Cútar	Botín 2	2009	X	X	X	X
UMAF F0926	Vélez-Málaga	Potril 2	2009		X		X
UMAF F0927[b]	Vélez-Málaga	Tejares 2	2009	X	X	X	
UMAF F0928	Vélez-Málaga	Tejares 1	2009		X	X	
UMAF F0929	Vélez-Málaga	Cabrera 1	2009		X		X
UMAF F0930	Algarrobo	Casa alta 1	2009	X	X	X	X
UMAF F0931	Vélez-Málaga	Cortijo 1	2009		X		X
UMAF F0932	Vélez-Málaga	Cortijo 2	2009				X
UMAF F0933[b]	Vélez-Málaga	Cortijo 2	2009	X	X	X	X
UMAF F0934	Vélez-Málaga	Cortijo 3	2009				X
UMAF F0935	Vélez-Málaga	Cortijo 4	2009				X
UMAF F0936	Benamargosa	Barranco 1	2009		X		X
UMAF F0937	Benamargosa	Barranco 3	2009		X		X
UMAF F0938	Benamargosa	Barranco II	2009	X	X	X	X
UMAF F0939	Vélez-Málaga	Potril 1	2009		X		X
UMAF F0940	Cútar	Botín 1	2009		X		X
UMAF F1041	Vélez-Málaga	Carril 3	2010		X	X	X
UMAF F1042	Vélez-Málaga	Carril 4	2010				X
UMAF F1043	Vélez-Málaga	Lara 3	2010				X
UMAF F1044	Vélez-Málaga	Arroyo 4	2010				
UMAF F1045	Vélez-Málaga	Arroyo 5	2010				
UMAF F1046	Vélez-Málaga	Córdoba 1	2010		X		X

Table 3 (Continued)

Isolate[a]	Origin	Orchard-sample	Year	Used in experiments of: Pathogenicity	VCGs	Phylogenetic analysis	Cross-Fertility
UMAF F1047	Vélez-Málaga	Córdoba 2	2010		X		X
UMAF F1048	Vélez-Málaga	Córdoba 3	2010				X
UMAF F1049	Benamargosa	Barranco 1	2010		X		X
UMAF F1050	Vélez-Málaga	Granaíno 1	2010				X
UMAF F1051	Vélez-Málaga	Granaíno 2	2010		X		X
UMAF F1052	Vélez-Málaga	Granaíno 3	2010				X
UMAF F1053	Vélez-Málaga	Granaíno 4	2010				X
UMAF F1054	Vélez-Málaga	Cabrera 2	2010		X		X
UMAF F1055	Benamargosa	Encantá 1	2010				X
UMAF F1056	Algarrobo	Portillo 1	2010		X		X
UMAF F1057	Algarrobo	Portillo 2	2010				X
UMAF F1058	Algarrobo	Melgares 1	2010				X
UMAF F1059	Algarrobo	Melgares 2	2010				X
UMAF F1060	Algarrobo	Melgares 2	2010		X	X	X
UMAF F1061	Algarrobo	Melgares 4	2010				X
UMAF F1062	Frigiliana	Acosta	2010	X	X		
UMAF F1063	Vélez-Málaga	Carauta	2010		X		X
UMAF F1064	Benamargosa	Arcas 1	2010		X	X	X
UMAF F1065	Benamargosa	Arcas 2	2010		X		X
UMAF F1066	Vélez-Málaga	Braun	2010		X		X
UMAF F1067	Benamocarra	Palomo	2010		X		X
UMAF F1168	Benamargosa	Encantá 2	2011				X
UMAF F1169	Vélez-Málaga	Arroyo 4	2011				X
UMAF F1170	Vélez-Málaga	Arroyo 5	2011				X

Table 3 (Continued)

Isolate[a]	Origin	Orchard-sample	Year	Used in experiments of: Pathogenicity	VCGs	Phylogenetic analysis	Cross-Fertility
UMAF F1171	Vélez-Málaga	Cortijo 5	2011	X	X	X	X
UMAF F1172	Vélez-Málaga	Cortijo 6	2011				X
UMAF F1173	Vélez-Málaga	Cortijo 7	2011				X
UMAF F1174	Benamargosa	Huerta 6	2011	X	X	X	X
UMAF F1175	Benamargosa	Huerta 7	2011		X		X
UMAF F1176	Benamargosa	Huerta 8	2011		X		X
UMAF F1177	Algarrobo	Portillo 3	2011		X		X
UMAF F1178	Algarrobo	Peláez 1	2011		X		X
UMAF F1179	Algarrobo	Lupiáñez	2011		X		
UMAF F1180	Almayate (V-M)	Parra 1	2011		X	X	X
UMAF F1181	Vélez-Málaga	Lara 4	2011	X	X	X	X
UMAF F1182	Vélez-Málaga	Lara 5	2011		X		X
UMAF F1183	Vélez-Málaga	Lara 6	2011				X
UMAF F1184	Vélez-Málaga	Lara 7a	2011				
UMAF F1185	Vélez-Málaga	Lara 7b	2011				X
UMAF F1186	Vélez-Málaga	Lara 8	2011				X
UMAF F1187	Vélez-Málaga	Lara 9	2011				X
UMAF F1188	Almayate (V-M)	Parra 2	2011				X
UMAF F1189	Vélez-Málaga	Arroyo 6	2011				X
UMAF F1190[b]	Vélez-Málaga	Arroyo 7	2011	X	X		X
UMAF F1191	Vélez-Málaga	Arroyo 8	2011				X
UMAF F1192	Vélez-Málaga	Potril 7	2011	X	X	X	X
UMAF F1193	Benamocarra	Sarmiento 1	2011				X
UMAF F1194[b]	Benamocarra	Sarmiento 2	2011	X	X	X	X

Table 3 (Continued)

Isolate[a]	Origin	Orchard-sample	Year	Used in experiments of			
				Pathogenicity	VCGs	Phylogenetic analysis	Cross-Fertility
UMAF F1195	Benamocarra	Sarmiento 3	2011				X
UMAF F1196	Vélez-Málaga	Hijano 1	2011				X
UMAF F1197	Vélez-Málaga	Hijano 2	2011		X	X	
UMAF F1198	Vélez-Málaga	Hijano 3	2011				X
UMAF F1199	Vélez-Málaga	Hijano 4	2011	X	X	X	X
UMAF F12100	Vélez-Málaga	Lara 10	2012				X
UMAF F12101	Vélez-Málaga	Lara 11	2012				X
UMAF F12102	Vélez-Málaga	Lara 12	2012				
UMAF F12103	Vélez-Málaga	Lara 13	2012				
UMAF F12104	Vélez-Málaga	Lara 14	2012		X		X
UMAF F12105	Benamargosa	Encantá 3	2012				X
UMAF F12106	Benamargosa	Encantá 4	2012				X
UMAF F12107	Vélez-Málaga	Arroyo 9	2012		X		X
UMAF F12108	Benamargosa	Huerta 9	2012				X
UMAF F12109	Benamargosa	Huerta 10	2012				X
UMAF F12110	Benamargosa	Huerta 11	2012		X		X
UMAF F12111	Vélez-Málaga	Triana	2012		X		X
UMAF F12112	Vélez-Málaga	Gámez	2012		X		X
UMAF F12113	Almáchar	Martín 1	2012		X		X
UMAF F12114	Benamocarra	Sarmiento 4	2012				X
UMAF F12115	Vélez-Málaga	Cortijo 8	2012				X
UMAF F12116	Vélez-Málaga	Cortijo 9	2012				X
UMAF F12117	Vélez-Málaga	Cortijo 10	2012		X		
UMAF F12118	Vélez-Málaga	Moneda 1	2012				X

Table 3 (Continued)

				Used in experiments of			
Isolate[a]	Origin	Orchard-sample	Year	Pathogenicity	VCGs	Phylogenetic analysis	Cross-Fertility
UMAF F12119	Vélez-Málaga	Moneda 2	2012				X
UMAF F12120	Vélez-Málaga	Moneda 3	2012		X		X
UMAF F12121	Benamocarra	Sarmiento 5	2012		X		
UMAF F12122	Benamocarra	Matao	2012		X		
UMAF F12123	Benajarafe (V-M)	Pintao 4	2012		X		X
UMAF F12124	Benajarafe (V-M)	Pintao 5	2012		X		X
UMAF F12125	Cútar	Botín 4	2012		X		X
UMAF F12126	Cútar	Botín 5	2012		X		X
UMAF F12127	Cútar	Botín 6	2012		X		X
UMAF F12128	Benamocarra	Garzón 1	2012		X		X
UMAF F12129	Benamocarra	Garzón 2	2012				X
UMAF F12130	Benamocarra	Garzón 3	2012				X
UMAF F12131	Benamocarra	Garzón 4	2012		X		X
UMAF F12132	Vélez-Málaga	Lorca 1	2012		X		X
UMAF F12133	Vélez-Málaga	Lorca 2	2012		X		
UMAF F12134	Vélez-Málaga	Lorca 3	2012		X		

[a]UMAF= Microbiology and Plant Pathology Laboratory collection, University of Málaga, Spain.
[b]Phylogenetic analysis also performed in the Bacterial and Foodborne Pathogen and Mycology Research Unit, United States Department of Agriculture-Agricultural Research Service-NCAUR, University of Peoria, USA.

3.2.1. Generating and preserving single-spore isolates.

Single-spore isolates were obtained by adding a small scraping of culture material to 10 ml of sterile water in a test tube, serial dilutions were prepared until 1-10 conidia were seen in a drop viewed under an optic microscope 10X. Spore dilutions were plated on PDA media and grown for a few days until the isolated colonies appeared. Single-spore colonies were then transferred to grow on PDA for further identification and conservation (Leslie & Summerell, 2006).

Fungal single-spore isolates were preserved in two different ways. For long term conservation, a mycelial disk derived from colony margins was introduced on 2 ml tubes and filled with glycerol 20 % solution. These tubes were kept in the ultrafreezer at -80 ºC temperature. For short term conservation and with the aim of using fungal material with certain frequency, mycelium from colony margins was introduced on 5 ml test tubes filled with sterile distilled water, and stored at room temperature and in darkness (Castellani, 1939; Panizo *et al*., 2005) where they can be preserved morphologically stable for 4 to 7 years.

In the Figure 4 is summarized the work flow, from isolation of the fungi from plant affected tissues, to pathogenicity, fungal identification and diversity analysis.

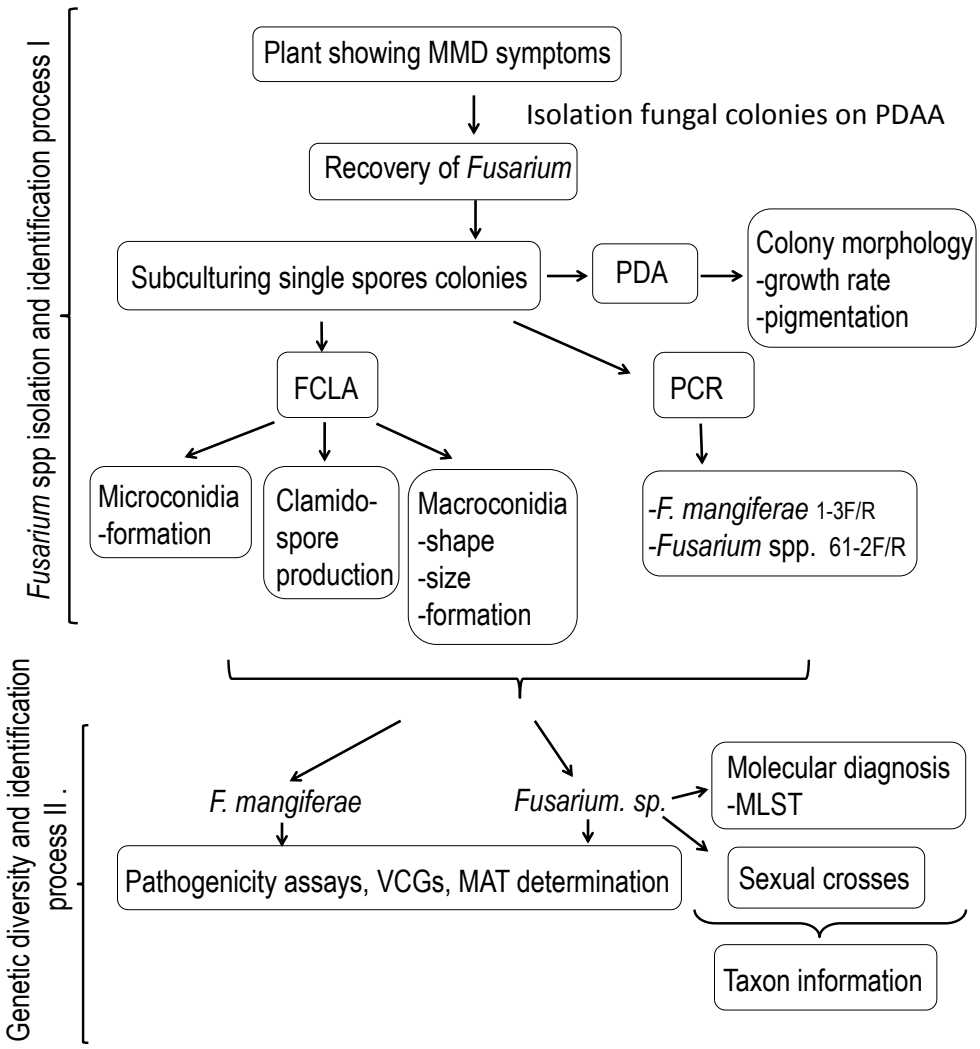


Figure 4. Diagram flow of identification protocol used for identifying and characterizing *Fusarium* isolates in this work. Modified from Leslie & Summerell, 2006.

3.3. Morphological and molecular diagnostic.

3.3.1. Morphological identification

To correctly asses the fungal genus of the isolates, the colony characteristics on PDA such as colour, texture, and the morphology of the asexual spores (conidia) were considered. Single-spore isolates were identified as members of the *Fusarium* genera by the observation under an optic microscope, of the typical fusiform shaped conidia (Figure 5).

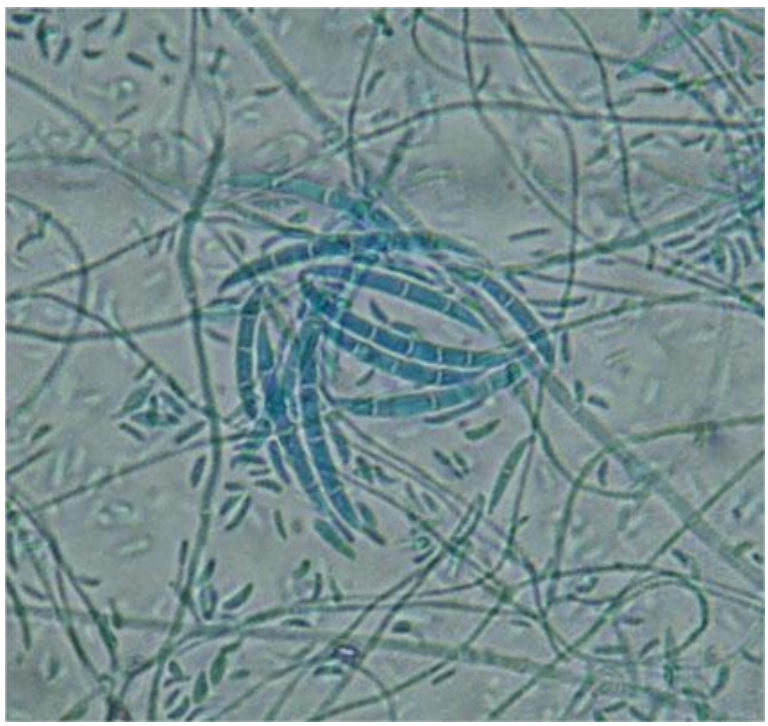


Figure 5. Typical fusiform *Fusarium* macroconidia

For a more accurate attempt at identifying the isolates to species level, colonies from single-spore isolates were grown on PDA and fresh carnation leaf agar (FCLA) (Iqbal *et al.,* 2005, Annex I.) PDA medium was mainly used to determine cultural features such as colony colour and texture.

Cultures grown on FCLA produce macroconidia that are more uniform in size and shape, than do cultures grown on carbohydrate-rich media such as PDA or Czapek-Dox (Leslie & Summerell, 2006); therefore, FCLA medium has been used mainly to determine the morphological features. The morphology and disposition of the asexual spores (micro and macroconidia) as well as the morphological features of the specialized cells that produce them (conidiogenous cells or phialides) were considered (Figures 6 and 7).

Observations under an optic microscope were performed following the technique previously described by Butler and Mann (1959), by which a piece of adhesive tape, on the sticky side, gently touches the margin colony surface. By doing so, we collect a fine fungal layer to be deposited in the same direction that was collected over a microscope slide, in which a drop of lactophenol cotton blue (LPCB), was previously deposited (Crespo *et al.,* 2006; Annex I) The adhesive tape also works as coverslip and the preparation can be observed directly under the microscope. The tape collects the surface of the fungal colony, where the reproductive structures usually are, and conidia maintain their

original position so details of their formation can be observed, which is one of the advantages of this procedure (Crespo, 1995).

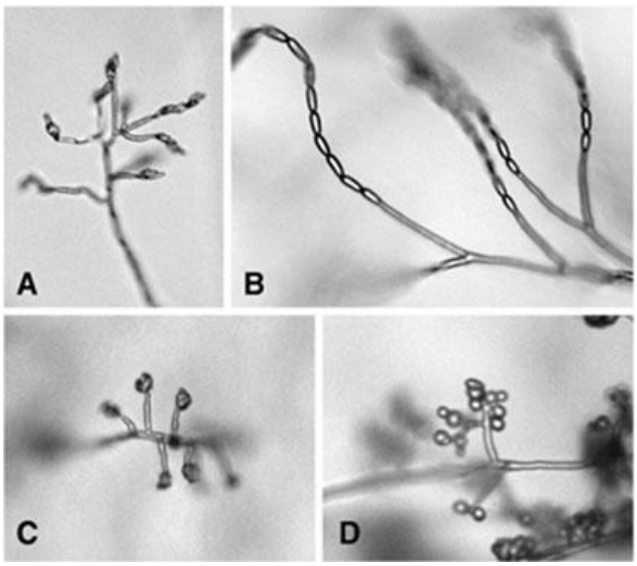


Figure 6. Ontogeny and disposition of conidia in *Fusarium* species. A, microconidia arranged in short chains; B, microconidia arranged in long chains; C and D, microconidia arranged in false heads. Taken from Leslie & Summerell, 2006.

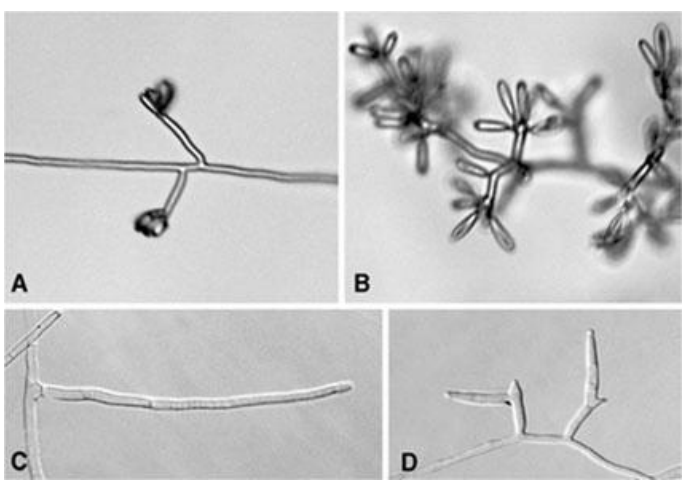


Figure 7. Conidiogenous cells in *Fusarium* species. A, microconidia produced on monophialides in false heads; B, microconidia produced on polyphialides; C, monophialide; D, polyphialides. Taken from Leslie &Summerell, 2006.

3.3.2. PCR diagnosis.

3.3.2.1. Fungal DNA extraction.

Fungal isolates grown on PDA were harvested and ground to fine powder using liquid nitrogen. Two methods for extracting fungal DNA were used. A summarized protocol of the first method follows:

1. Add 400 µl extraction buffer to the grounded mycelia (0.4 M NaCl; 10 mM Tris-HCl pH 8; 2 mM EDTA pH 8).
2. Add 80 µl SDS 10% and 8 µl of protein K (20 mg/ml).
3. Agitate vigorously and keep at 60ºC for 1 hour.
4. Add 350 µl of NaCl 5M.
5. Agitate for 30 seconds.
6. Centrifuge 30 minutes at 9000 rpm. Transfer the flow-through fraction to a new sterilized tube.
7. Add a volume of chloroform: isoamylic alcohol (24:1) equivalent to the volume of sample. Centrifuge 10 minutes at 9000 rpm. Transfer the flow-through fraction to a new sterilized tube.
8. Add a volume of isopropyl alcohol equivalent to the volume of sample and mix thoroughly.
9. Centrifuge 20 minutes at 9000 rpm.
10. Wash the pellet with ethanol 70% twice. Centrifuge for 5 minutes at 9000 rpm.

11. Dry and resuspend the pellet in 50 µl of sterile bidistilled water.

12. Quantify 3-5 µl in an agarose gel 0.8%.

Alternatively, fungal DNA was also extracted using DNeasy Plant Mini Kit® following the manufacturer´s instructions (Qiagen, USA) with minor variations.

DNA samples were quantified on spectrophotometer Nanodrop® ND-1000, and the different concentrations obtained were diluted to a final concentration of 20 ng/µl approximately to use in PCR assays.

3.3.2.2. Polymerase Chain Reaction (PCR).

For the specific diagnosis of *Fusarium* isolates such as *Fusarium mangiferae*, one of the main causal agent of MMD, a set of specific primers were used (primer pair 1-3 F/R) that amplified a 608-bp DNA fragment included in the ITS region of the ribosomal DNA of *F. mangiferae* (Zheng & Ploetz, 2002). The sequence of these primers is ("forward": 5´-TGCAGATAATGAGGGTCTGC-3´; "reverse": 5´GGAACATTGGGCAAAACTAC-3´). All PCR reactions were performed in a total volume of 25 µl containing approximately 1 µl of genomic DNA; 1 µl of each primer; 2.5 µl reaction Buffer (10X) containing: 200 mM Tris-HCl (pH 8.4), 500 mM KCl; 0.2 µl 10mM DNTPs; 0.75 µl 50mM Mg Cl_2 and 0.5 µl *Taq* DNA Polymerase (Invitrogen, USA). The total volume of 25 µl was

completed adding bidistilled sterilized water. The reactions were incubated in a MJ Mini™ (Bio-Rad, UK) Thermal cycler or by TC-412 Thermal cycler (TECHNE, UK) starting with 1 min denaturation step at 95 ºC followed by 40 cycles consisting of 15 s denaturing at 94 ºC, 30 s annealing at 68 ºC, and 60 s at 72 ºC; and ending with a final elongation step for 5 min at 72ºC.

A second primer set was also included in this study to check the diagnosis of alternative *Fusarium* spp. associated with MMD. Primers 61-2F/R, originally developed to diagnose *Fusarium subglutinans* from maize (Möller *et al*., 1999), were reported later to amplify a 445 pb fragment from *Fusarium sterilihyphosum* and *Fusarium mexicanum* (Rodriguez-Alvarado *et al*., 2007), and from some *Fusarium* mango isolates from Brazil, later named as *F. sterilihyphosum* (Zheng & Ploetz 2002; Marasas *et al*., 2006). The sequence of these primers is: (forward: 5´-GGCCACTCAAGAGGCGAAAG-3´; reverse: 5´-GTCAGACCAGAGCAATGGGC-3´) (Möller *et al.,* 1999). PCR were performed in a total volume of 25 µl containing approximately 1 µl of genomic DNA; 1 µl of each primer; 2.5 µl reaction Buffer (10X) containing: 200 mM Tris-HCl (pH 8.4), 500 mM KCl; 0.2 µl 10 mM DNTPs; 0.75 µl 50mM Mg Cl_2 and 0.5 µl *Taq* DNA Polymerase. The total volume of 25 µl was completed adding bidistilled sterilized water. The reactions were incubated in a MJ Mini™ Thermal cycler starting with 2 min denaturation step at 95 ºC followed by 25 cycles consisting of 60 s denaturing at

95 ºC, 60 s annealing at 65 ºC, and 90 s at 72 ºC; and ending with a final elongation step for 5 min at 72 ºC.

In both cases, aliquots of 3-4 µl were loaded onto 1% Agarose D1 Low EEO (Pronadisa, Conda Laboratorios, Spain) gels and run in 1X Tris-acetate-EDTA buffer (40 mM Tris-acetate and 1mM EDTA) at 90 V for 35 min. Gels were subsequent staining with ethidium bromide at 0.4 µg/ml and documented with a Gel Doc ™ XR+ imaging system (Bio-Rad, UK). Molecular weight analysis of patterns was performed with Quantity One version 4.2.1 software (Bio-Rad, UK), using Low DNA Mass Ladder (2-Kb) (Invitrogen, USA) as molecular weight markers.

3.4. Pathogenicity assays

In order to complete Koch´s postulates, and to demonstrate conclusively the pathogenic role of these *Fusarium* spp. isolates from mango malformed trees in Spain; pathogenicity assays were carried out on young mango trees with 22 Spanish representative isolates, eight identified as *F.* mangiferae, but one of them showing atypical morphological features (UMAF F0923), twelve identified later as *F. tupiense*, one as *Fusarium phyllophilum*-like and one *Fusarium sp*. (Table 3), as well as the *F. mangiferae* isolate MRC7560 from Israel included as reference.

The selected isolates were previously grown on PDA at 25 ºC and in darkness for a week, three replicates per isolate. Spores were collected from the surface of the colony, filtered and diluted

to a final volume of 5×10^7 conidia per ml. Pathogenicity assays were performed on a total of forty-six 2 year-old healthy mango seedlings cv. Keitt by inoculating five dormant buds per isolate on separate branches per tree with a 20-µl conidial suspension (Freeman *et al*., 1999). Sterile water was used as negative control (Figure 8). These experiments were conducted in March and November of 2010 and November of 2011. Trees were kept in a plant growth chamber at 25 ^{o}C and light cycle of 12 h day/night during the inoculation process and the first two-three days post-inoculation. Afterwards, inoculated trees were carried to a restricted area where environmental conditions were appropriate for floral induction, until bud break. MMD symptoms in the artificially inoculated plants were observed in March 2011 or March 2012, respectively. Finally from this symptomatic shoots, *Fusarium* spp. were reisolated and they were confirmed by PCR assays and morphological observation in comparison with the previously inoculated isolates.

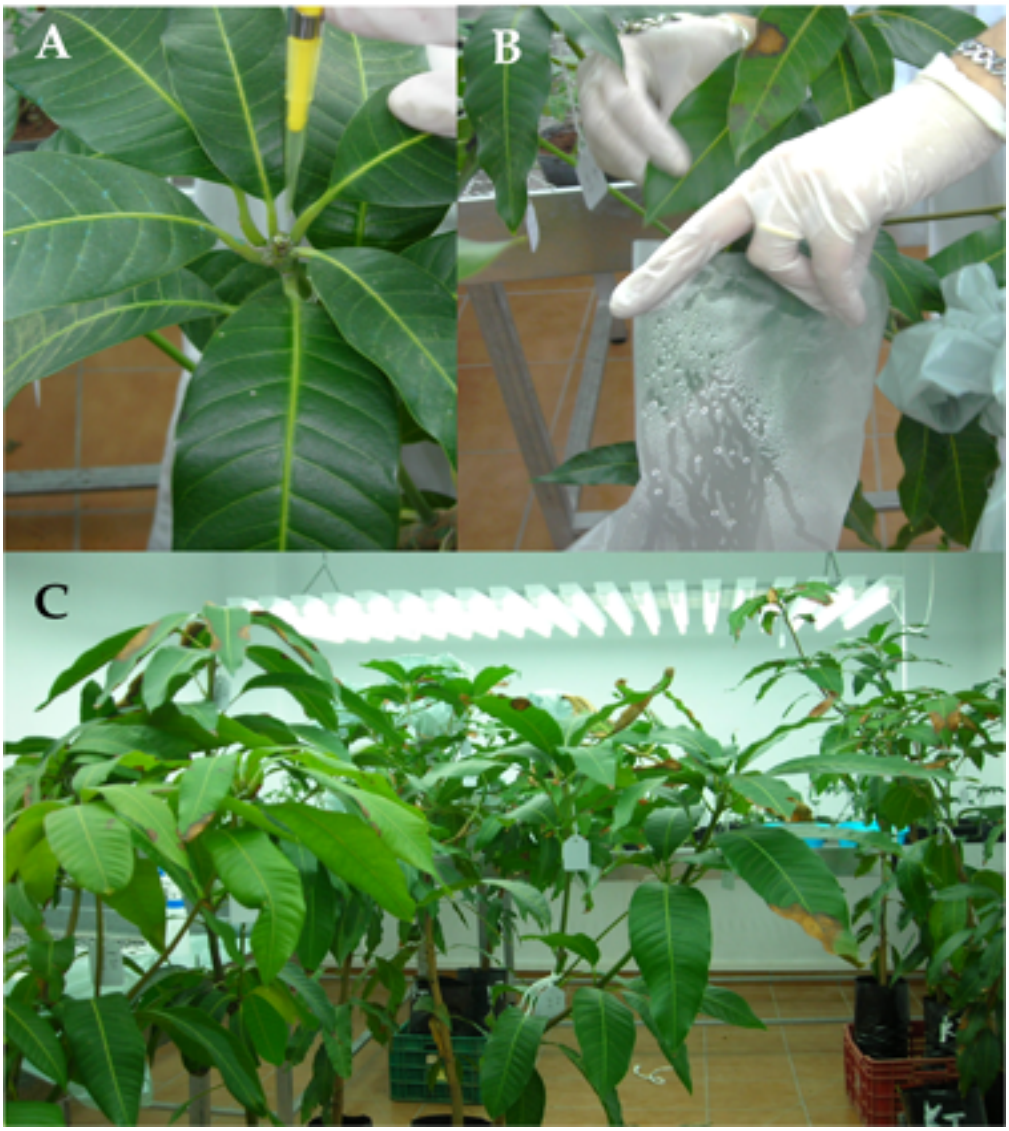


Figure 8. Pathogenicity assays. A, Inoculation of mango dormant buds; B, Covering buds with plastic bags; C, Inoculated mango trees maintained in a greenhouse.

3.5. *Fusarium* population diversity analysis

Determining the structure of an unknown fungal population, in our case *Fusarium* spp. causing MMD in Spain, can be very useful to know more about the introduction and dispersion of the pathogen in a restricted area, and subsequently, develop more effective control strategies. To determine the population diversity of *Fusarium* spp. Spanish isolates, several methodological approximations were taken into account.

3.5.1. Arbitrary primer Polymerase Chain Reaction (ap-PCR) and Random Amplified Polymorphism (RAPD-PCR)

Both of these molecular methods have been widely used to evaluate the genetic diversity within different fungal species as well as to identify different races or pathotypes, and also have proven to be useful for characterizing some populations of fungal pathogens (Dobinson *et al.*, 1998; Zheng & Ploetz, 2002).

To determine genetic diversity of the *Fusarium* Spanish isolates pathogenic on mango, ap-PCR analysis was performed using DNA extracted from 131 *Fusarium* Spanish isolates (Table 3), and 13 representative isolates from different *Fusarium* species causing MMD worldwide used as reference: *F. mangiferae* from Israel (MRC7560), Egypt (EM50B) and Florida (CG-1-4), *F. sterilihyphosum* from South Africa (NRRL25623), *F. mexicanum* from Mexico (NRRL53580), *F. pseudocircinatum* (NRRL53570) and *F. tupiense* from Brazil (NRRL53984, NRRL53986, NRRL53992,

NRRL53993, NRRL53994, NRRL53995, NRRL53996) (Table 2). Fungal DNA was extracted using DNeasy Plant Mini Kit following the manufacturer´s instructions (Qiagen, USA). Analysis by ap-PCR of these isolates were conducted with three repeat motif primers- GACACGACACGACAC, CAGCAGCAGCAGCAG and GACAGACAGACAGACA, designated as $(GACAC)_3$, $(CAG)_5$ $(GACA)_4$, respectively. All PCR reactions were performed as previously described (Otero-Colina *et al*. 2010) with subtle variations, in a total volume of 20 µl, containing approximately 1 µl of genomic DNA; 2 µl Buffer (10X) containing 50 mM KCl, 10mM Tris-HCl; 2 µl 1.5 mM $MgCl_2$; 0.2 µl of *Taq* DNA Polymerase (Promega Corp., Madison, USA); 2 µl of dNTPs solution 0.2 mM each dATP, dCTP, dGTP, dTTP, and 1 µl of each primer. The reactions were incubated on a PYC-1000 thermocycler (MJ Research, Inc., USA) starting with 1 min of denaturation at 95 ^{o}C followed by 30 cycles consisting of 30 s at 95 ^{o}C, 30 s at either 60 ^{o}C for $(CAG)_5$, or 48 ^{o}C for $(GACAC)_3$ and $(GACA)_4$, and 90 s at 72 ^{o}C, and ending with a final elongation step for 15 min at 72 ^{o}C for $(CAG)_5$, or 10 min at 72 ^{o}C for $(GACAC)_3$ and $(GACA)_4$.

RAPD-PCR analysis was determined on a set of 14 representative *Fusarium* spp. Spanish isolates (UMAF F 0910, UMAF F0915, UMAF F0916, UMAF F0917, UMAF F0924, UMAF F0926, UMAF F0927, UMAF F0933, UMAF F0938, UMAF F0939, UMAF F1074, UMAF F1190, UMAF F1192, UMAF F1194) and the representative isolates of *F. mangiferae*, *F. sterilihyphosum*, *F.*

mexicanum, and *F. tupiense* mentioned above (Table 2). RAPD-PCR analysis was conducted in similar reaction mixtures as described for ap-PCR analysis using 10-base primer OPF-08 (GGGATATCGG) and OPF-13 (GGCTGCAGAA) (Kit F; Operon Technologies, USA). For RAPD-PCR, the initial denaturation step at 95 ºC was followed by 45 cycles consisting of 1 min at 94 ºC, 1 min at 34 ºC, and 2 min at 72 ºC, and ending with a final elongation step for 15 min at 72 ºC.

Amplification products from ap-PCR or RAPD-PCR were separated in agarose gels (1.5%) in Tris-acetate-EDTA buffer electrophoresed at 90 V for 90 min. In both cases, aliquots of 3-4 µl were loaded onto 1.5% agarose gel and run in Tris-acetate-EDTA buffer electrophoresed at 90 V for 1.5 min. Gels were subsequently stained with ethidium bromide and documented with a Gel Doc XR+ imaging system (Bio-Rad, UK). Molecular weight analysis of patterns was performed with Quantity One version 4.2.1 software (Bio-Rad, UK), with GeneRuler (1-kb DNA ladder, Invitrogen, USA) as molecular weight marker. PCR experiments were conducted at least four times to ensure reproducibility of the experiment.

Taking into account the population variability of the *F. tupiense* isolates from Brazil (Lima *et al.*, 2009a), six *F. tupiense* reference isolates; each one belonging to a different VCG (NRRL53986, NRRL53992, NRRL53993, NRRL53994, NRRL53995, NRRL53996) (Tables 2 and 7), jointly with four *F. tupiense* Spanish isolates and one *F. phyllophilum*-like Spanish isolate were included

in a second ap-PCR and RAPD trial, with the same primers and conditions as mentioned above, to determine banding pattern similarities among the *F. tupiense* Spanish and Brazilian isolates.

3.5.2. Vegetative Compatibility Groups (VCGs)

Vegetative Compatibility Group (VCG) analysis has been shown to be a useful technique to examine genetic diversity among populations of plant pathogenic fungi. Two physiologically distinct individuals of the same species belong to the same VCG if they can fuse asexually, forming a stable heterokaryon. Puhalla (1985) found that mutants resistant to $KClO_3$ were usually nitrate-nonutilizing (*nit*) mutants. He proposed a model using *nit* mutants to subdivide fungal populations into different VCGs. VCGs are well-suited for measuring the frequency of different genotypes within a population and to determine if two strains are alike to one another (Leslie, 1996).

VCGs of 77 *Fusarium* representative Spanish isolates were studied in two independent experimental assays. In a first trial VCGs were evaluated with 33 *F. mangiferae* Spanish isolates; including two *F. tupiense*, two *F. phyllophilum*-like and one *Fusarium* sp. Spanish isolates as control strains (Table 3). Also were included, a collection of six *F. mangiferae* reference isolates (12 *nit* mutants) representative of the six described VCGs in *F. mangiferae* (Zheng & Ploetz, 2002); and five additional reference

isolates, *F. mangiferae* (MRC7560), *F. sterilihyphosum* (NRRL25623, NRRL53569), and *F. mexicanum* (NRRL47473, NRRL53580) (Table 2).

In a second trial, VCGs were evaluated with 41 *F. tupiense* Spanish isolates including three *F. mangiferae* and two *F. phyllophilum*-like Spanish isolates as control strains (Table 3); and also the reference isolates of *F. mangiferae*, *F. sterilihyphosum*, and *F. mexicanum* above mentioned. In this second trial were also included six *F. tupiense* reference isolates; each one belonging to a different VCG previously described by (Lima *et al.,* 2009a), (NRRL53986, NRRL53992, NRRL53993, NRRL53994, NRRL53995, NRRL53996) (Table 2). For this purpose, in both cases, nitrate-nonutilizing (*nit*) auxotrophic mutants were generated in this work for all of the isolates referred above with the exception of the auxotrophic mutants from the six *F. mangiferae* VCGs reference strains (Zheng & Ploetz, 2002), kindly provided by Dr. R. Ploetz (University of Florida, USA), and utilized as described previously (Correll *et al.*, 1987). The *nit* mutants were generated by growing the isolate on PDA and transferring 4 small plugs from the edge of the colony to Petri dishes (90-mm diameter) containing minimal medium (MM)+ClO^-_3 (Annex I). The initial level of $KClO^-_3$ in the MM for producing *nit* mutants (MM+ClO^-_3) was 15 g/l, but when isolates failed to form mutants on this media, chlorate concentration was increased to 25 g/l. $KClO^-_3$ resistant sectors were subcultured to MM with $NaNO_3$ as soon as they were detected (Figure 9). Strains that grow thinly on the

minimal medium were scored as *nit* mutants and were saved in distilled water for later analysis. Strains with heavy growth that resembles wild type were discarded. The *nit* mutants were assigned to the different phenotypic classes based on their growth on media containing different nitrogen sources: MM+ $NaNO_3$, MM+ $NaNO_2$, MM+ hypoxanthine, and MM+ ammonium tartrate (Figure 10). As result from growing patterns in these media, isolates were assigned into categories, *nit* 1, *nit* 3 or Nit M defined by Correll *et. al* (1987) (Table 4).

Figure 9. Colonies of *Fusarium* sp. on MM+$KClO_3$ sectoring to produce a ClO_3 resistant, NO_3 non-utilizing (*nit*) mutant.

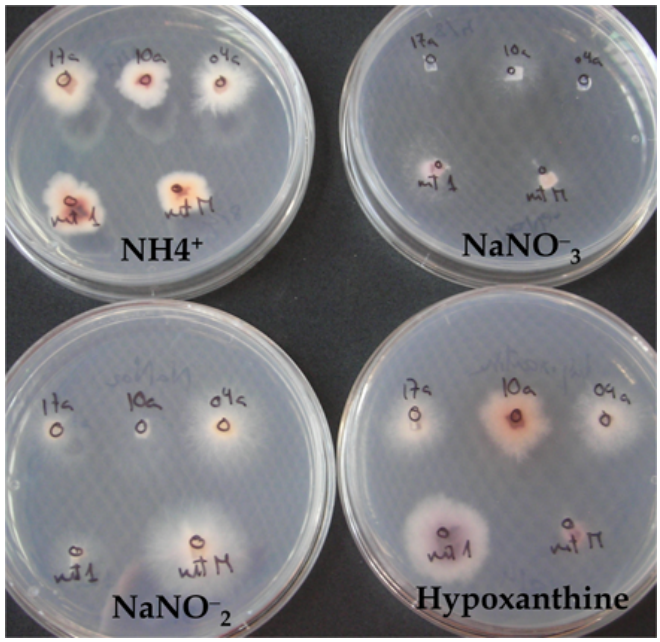


Figure 10. *Fusarium* isolates growing on media containing different nitrogen sources to characterize the NO_3-non-utilizing (*nit*) mutants.

Table 4. Utilization of nitrogen sources in a standard phenotype screening by *nit* mutants that commonly results after growth on Minimal Medium + CLO^-_3. Taken from Leslie & Summerell (2006)

	Medium Supplement				
Strain Type	NH_4^+	NO^-_3	NO^-_2	Hypoxanthine	ClO^-_3
Wild Type	+	+	+	+	−
nit 1	+	−	+	+	+
nit 3	+	−	−	+	+
Nit M	+	−	+	−	+

Figure 11. Prototrophic heterokaryon formed on MM+$NaNO_3$ by two *nit* mutants (*nit*-1 X Nit M) derived from vegetatively compatible strains of *Fusarium* sp.

Initially, in the minimal medium to produce *nit* mutants 1.6 g/l of L-asparagine was added as an alternative nitrogen source, but in later experiments L-asparagine was replaced by 1.4 g/l of L-threonine. The change from L-asparagine to L-threonine resulted in an increase in the percentage of recovered Nit M mutants (Klittich & Leslie, 1988).

VCGs were determined through the complementation of nitrate non-utilizing (*nit*) mutants of different isolates as a visual indicator of heterokaryon formation (Figure 11). Complementation tests were carried out on MM with $NaNO_3$ as nitrogen source by mycelial pairings from different phenotypic classes, *nit* 1/Nit M in Petri dishes (90×15mm). Inoculated plates were incubated at 25 ^{o}C (Leslie & Summerell, 2006). Heterokaryon

formation was evaluated after 5 to 7 days. All vegetative compatibility tests were conducted at least twice.

3.5.3. Mating type determination by PCR

Within the ascomycetes, sexually reproducing species usually follow one of three basic sexual reproductive strategies: homothallic, pseudohomothallic (also termed secondary homothallic), and haploid heterothallic with each species limited to a single reproductive strategy (Fincham *et al.*, 1979; Nelson, 1996). Species whose life cycle requires a sexual interaction between two physiologically distinct strains arising from separate spores are termed heterothallic. Most of the *Fusarium* species are heterothallic. The physiological difference between heterothallic strains that is of important usually is the difference in mating type. The genetically simplest fungal mating type is the dimictic system found in most heterothallic ascomycetes, including all heterothallic *Fusarium* species. In the dimictic mating system, there is a single mating type locus, termed *MAT* in *Fusarium* that has two functional alleles, termed *MAT*-1 and *MAT*-2 (Leslie & Summerell, 2006).

The *MAT* locus determines the sexual compatibility of heterothallic fungi. It has been hypothesized that the *MAT* genes have the potential to delimit the frontiers amongst species (Yun *et al.*, 2000) and its utility has been demonstrated in evolutionary and phylogenetic analyses (Leslie & Klein, 1996).

We analyzed mating-type idiomorphs (*MAT*-1 or *MAT*-2) for 132 *Fusarium* spp. Spanish isolates and two reference strains of *F. mexicanum* (NRRL 47473 and NRRL53580), with PCR-based assays (Kerényi *et al*., 1999; Steenkamp *et al*., 2000). PCR amplification of *MAT*-1 and *MAT*-2 were analyzed using the respective primer pairs GFmat1a and GFmat1b (forward 5′-GTTCATCAAAGGGCAAGCG-3′; reverse 5′-TAAGCGCCCTCTTAACGCCTTC-3´); and GFmat2c and GFmat2d (forward 5′-AGCGTCATTATTCGATCAAG-3′; reverse 5′-CTACGTTGAGAGCTGTACA-3′) (Steenkamp *et al*., 2000). All PCR reactions were performed in a total volume of 20 µl containing approximately 1.5 µl of genomic DNA; 0.5 µl of each primer; 2 µl reaction Buffer (10X) containing: 200 mM Tris-HCl (pH 8.4), 500 mM KCl; 2 µl DNTPs solution 0.2 mM each dATP, dCTP, dGTP, dTTP; 2 µl 1.5 mM Mg Cl_2 and 0.2 µl *Taq* DNA Polymerase (Invitrogen, USA). The total volume of 20 µl was completed adding bidistilled sterilized water. The reactions were incubated on a PYC-1000 thermocycler starting with a denaturation step at 94 ºC, 1 min was followed by 34 cycles consisting of 30 s at 92 ºC, 30 s at 67 ºC, and 30 s at 72 ºC, and ending with a final elongation step for 5 min at 70ºC. Amplification products were separated in agarose gels (1.2%) in Tris-acetate-EDTA buffer electrophoresed at 100 V for 20-30 min. Gels were subsequent stained with ethidium bromide and documented with a Gel Doc XR+ imaging system (Bio-Rad, UK). Molecular weight analysis of patterns was performed with

Quantity One version 4.2.1 software (Bio-Rad, UK), with GeneRuler (100 DNA ladder, Invitrogen, USA) as molecular weight marker.

3.6. Phylogenetic analysis of *Fusarium* Spanish isolates

Direct sequencing of one or more housekeeping genes is usually done to make species assignments and to get insight of phylogenetic relations (Leslie & Summerell, 2006). The translation elongation factor 1-α (TEF) gene, which encodes an essential part of the protein translation machinery, has high phylogenetic utility because it is (1) highly informative at the species level in *Fusarium*; (2) non-orthologous copies of the gene have not been detected in the genus; and (3) universal primers have been designated that work across the phylogenetic breadth of the genus (Geiser *et al*., 2004). Also, previous studies have also demonstrated the phylogenetic utility of the β-tubulin (O´Donnell & Cigelnik, 1997; Schardl *et al*., 1994) at the interspecific level in fungi. In this study, portions of TEF and β-tubulin genes were selected to infer phylogenetic relationships among *Fusarium* Spanish isolates and the other *Fusarium* associated with MMD, and to help resolving the identification of the *Fusarium* spp. Spanish isolates, based on sequences available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and/or in *Fusarium*-ID (O´Donnell *et al*., 1998a; 2000; 2008; 2011; Geiser *et al*., 2004).

Concerning the unsolved identity of the *Fusarium* spp. Spanish isolates pathogenic on mango, as well to confirm the identification of *F. tupiense* Spanish isolates, a phylogenetic analysis with several housekeeping genes was carried out in two independent assays. In our lab, the translation elongation factor 1-α (TEF) and β-tubulin DNA partial sequences of 29 representative Spanish *Fusarium* spp. isolates, were obtained and compared with similar sequences from strains of other species in the *G. fujikuroi* species complex already available in GenBank, National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genbank/) or in *Fusarium*-ID (http://isolate.fusariumdb.org)

For this purpose, DNA of selected Spanish isolates: 10 *F. mangiferae*, 17 *F. tupiense* and 2 *F. phyllophilum*-like comprising the maximum diversity of isolates included in this work (Table 3), and DNA from several reference strains of *F. mangiferae*, *F. sterilihyphosum*, and *F. mexicanum* worldwide (Table 2) were amplified and sequenced. Portions of the TEF gene were amplified with the primers Ef-1 (forward; 5´–ATGGGTAAGGAGGACAAGAC–3´) and Ef-2 (reverse; 5´–GGAAGTACCAGTGATCATGTT–3´) (Geiser *et al*., 2004). All PCR reactions were performed in a total volume of 25 µl containing approximately 1 µl of genomic DNA; 1 µl of each primer; 2.5 µl reaction Buffer (10X) containing: 200 mM Tris-HCl (pH 8.4), 500 mM KCl; 0.2 µl 10mM DNTPs; 0.75 µl 50mM Mg Cl_2 and 0.5 µl *Taq* DNA Polymerase (Invitrogen, USA). The total volume of 25 µl was completed adding bidistilled sterilized water. The reactions

were incubated in a MJ Mini™ (Bio-Rad, UK) Thermal cycler or by TC-412 Thermal cycler (TECHNE, UK) with an initial denaturation step at 95 ºC, 2 min, followed by 30 cycles consisting of 60 s at 95 ºC, 60 s at 55.5 ºC, and 90 s at 72 ºC, and ending with a final elongation step for 7 min at 72 ºC. Portions of the β-tubulin gene were amplified with a set of three primers, T1 (5´-AACATGCGTGAGATTGTAAGT-3´), T2 (5´-TAGTGACCCTTGGCCCAGTTG-3´) and T22 (5´-TCTGGATGTTGTTGGGAATCC-3´) (Figure 12), and used as pairs T1 and T2, or T1 and T22 in a PCR reaction using similar mixtures reaction as above described, with an initial denaturation step at 95 ºC, 2 min, followed by 35 cycles consisting of 60 s at 95 ºC, 60 s at 56.5 ºC, and 90 s at 72 ºC, and ending with a final elongation step for 7 min at 72 ºC. In both cases, amplifications were done in a MJ Mini™ or TC-412 Thermal cycler.

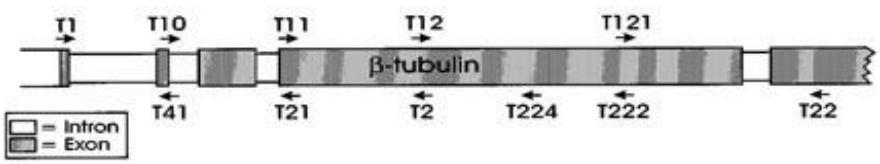


Figure 12. Map of the β-tubulin gene. Stripped boxes indicate β-tubulin exons; introns are unstripped. Labelled arrows indicate the primers used for PCR amplification and sequencing, in this case primers T1, T2 and T22. Taken from O´Donnell & Cigelnik (1997).

Amplified fragments, previously visualized on a 1% agarose gel, were cleaned with a GFX™ PCR DNA and gel purification kit (Healthcare, UK), and sequenced in both directions by Macrogen Europe (The Netherlands). The ABI DNA sequences chromatograms were edited and aligned with ContigExpress Vector NTI Advance 10 (Invitrogen, USA), and compared with those already in GeneBank using BLAST (Zang *et al.,* 2000). Additional sequences of TEF and β-tubulin of some of the reference isolates were obtained from the sequences available in GenBank (Table 2).

Multiple sequence alignments of representative Spanish *Fusarium* spp. and reference isolates were generated with Clustal W (Thompson *et al.,* 1994). Phylogenetic trees were performed using MEGA 5.0 (Tamura *et al.,* 2011), using the Neighbor-Joining method (Saito and Nei, 1987). Confidence levels of the branching points were determined using 10.000 bootstrap replicates.

Regarding the conclusive identification and phylogenetic relatedness of *F. tupiense* and *F. phyllophilum*-like Spanish isolates with the other *Fusarium* spp. included in the *Gibberella fujikuroi* species complex, a set of 4 *F. tupiense,* 2 *F. mangiferae* and 1 *F. phyllophilum*-like Spanish isolates (Table 3) were selected for a broader phylogenetic analysis including DNA samples and sequences of different members of the *Gibberella fujikuroi* species complex. Accession numbers from the different DNA sequences used in this study are included in the Annex II. This study was partially carried out in the Bacterial and Foodborne Pathogen and

Mycology Research Unit, U.S. Department of Agriculture-Agricultural Research Service (USA) using DNA sequence data from portions of seven genes, TEF, β-tubulin, calmodulin, histone H3, nuclear ribosomal intergenic spacer region (IGS rDNA), and the RNA polymerase subunits RPB1 and RPB2. For DNA extraction for multilocus analyses, isolates were grown in 100 ml of yeast-malt broth in 300 ml Erlenmeyer flasks of a rotary shaker at 100 rpm for 2 to 3 days. Mycelium was harvested and them freeze dried. A hexadecyltrimethyl-ammonium bromide protocol was used to extract total genomic DNA from freeze-dried mycelium (O´Donnell *et al.*, 1998). Platinum *Taq* DNA Polymerase was used for all PCR reactions and DNA sequencing was conducted as previously described in Otero-Colina *et al.*, (2010). ABI DNA sequence chromatograms were edited and aligned with Sequencher version 4.1.2 (Gene Codes; Ann Arbor, USA). TextPad version 5.1.0 for Microsoft Windows was used to manually improve the alignments prior to phylogenetic analysis. Maximum parsimony (MP) analyses were implemented in PAUP* 4.0b10 (Swofford, 2002) and maximum likelihood (ML) in GARLI version 0.951 (Zwickl, 2006) as previously described in O´Donnell *et al.*, (2008).

3.7. Cross fertility assays

Most ascomycetes, including *Fusarium* are usually vegetatively haploid and can propagate vegetatively or reproduce sexually. Typically, only the anamorph or the asexual stage of the fungus is found in the field and in culture, and the fungus is identified on this basis; the teleomorph or sexual stage (perfect stage) is unknown in many cases or have not been described yet. Nevertheless, sexual stages are known for a number of *Fusarium* species. All the studied *Fusarium* species are monoecious (an individual produces both male a female sexual structures), with an idealized strain in a heterothallic species described as "self-sterile" hermaphrodite. This idealized strain can serve as male and as female, but it cannot fertilize itself because different mating types are required for a sexual cross to be successful. For a sexual cross to occur, both strains have to be in the same biological species and carry different *MAT* allele. In general, male-fertile/female-sterile strains are relatively common under field conditions. The ability to cross and to produce the teleomorph (sexual or perfect stage) with standard tester of defined species groups is the ultimate assurance of a correct species identification (Leslie & Summerell, 2006).

Sexual crosses between Spanish *Fusarium* spp. were conducted via the method developed by Klittich & Leslie (1988). High fertile female *F. mangiferae* tester strains are not available for using in crossing protocols as the teleomorph of this species have

not been found. For that reason, *F. mangiferae* Spanish isolates with opposite mating type were intercrossed ensuring all strains were tested as female parents in crosses with all strains of the opposite mating type as the male parent (Leslie & Klein 1996; Covert *et al*., 1999, Leslie & Summerell, 2006). On the other hand, the teleomorph of *F. tupiense* have been previously described (Lima *et al*., 2012) and female/male fertile tester strains CLM1000 (*MAT*-1) and CLM1843 (*MAT*-2) were kindly gifted by Dr. Pfenning, Universidade Federal de Lavras, Brazil, and included in this study (Table 2). Isolates serving as female parents were inoculated on plates containing carrot agar (Annex I), and male parent strains were inoculated on slants containing complete medium (Annex I, Figure 13). After seven days, the spore suspensions of male parent strains in 0.2-0.25% Tween 60 solution were spread onto the surfaces of the female cultures. Fertilized plates were incubated at 22ºC and 12h light/12 h dark cycle using fluorescent and near-ultraviolet light. All crosses were examined weekly for the presence of perithecia.

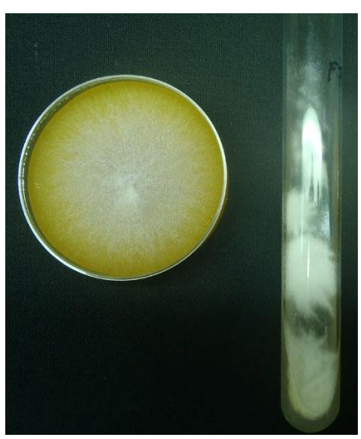


Figure 13. *Fusarium* sp. isolates growing on a plate containing carrot agar and on a slant containing complete medium.

RESULTS

4. RESULTS

4.1. Isolation and identification of *Fusarium* spp. from mango malformed trees in Spain.

4.1.1. Detection and isolation of *Fusarium.*

Mango malformation disease (MMD) symptoms were first observed in the Axarquía region in 2006 where few samples of symptomatic trees were collected and *Fusarium* sp. isolated without further identification. Afterwards, subsequent surveys were conducted in the years 2009 to 2012 during the blossom seasons (April to July) in different mango orchards located in different districts of the Axarquía region and showing symptoms of MMD (Figure 14, Table 1). From 36 of these orchards *Fusarium sp.* was isolated from vegetative shoots and floral tissue of symptomatic mango trees (Figure 15 A). In 7 orchards *Fusarium* sp. was not isolated from samples that showed, in any case, unclear symptoms; growing instead saprophytic fungi as *Alternaria, Ulocladium* or *Aureobasidium*.

As a result of these surveys, a total of 127 *Fusarium* isolates were obtained from mango tissues with apparent symptoms of MMD from 9 different districts of the Axarquía region (Table 5, Figure 16). The seven isolates, previously obtained in 2006, were added to this collection. The 134 isolates were purified as single conidial subcultures (Figure 15 B). Therefore, in this study we confirm conclusively the presence of MMD in the Axarquía

region, showing a relevant dispersion and affecting the majority of mango cultivars, in particular the most prevalent in the Axarquía region: Osteen, Keitt, Kent and Tommy Atkins. Some of the orchards affected with MMD were supervised for incidence of the disease during the following years, and samples of MMD were obtained in the majority of them. Only in one orchard MMD was totally eradicated, but in the others the apparent incidence was reduced (Table 1).

Figure 14. Symptoms of mango malformation disease caused by some *Fusarium* species on mango trees from Southern Spain. Commercial orchards: A, vegetative malformation, B, branch showing floral and vegetative malformation, C-D, floral malformation.

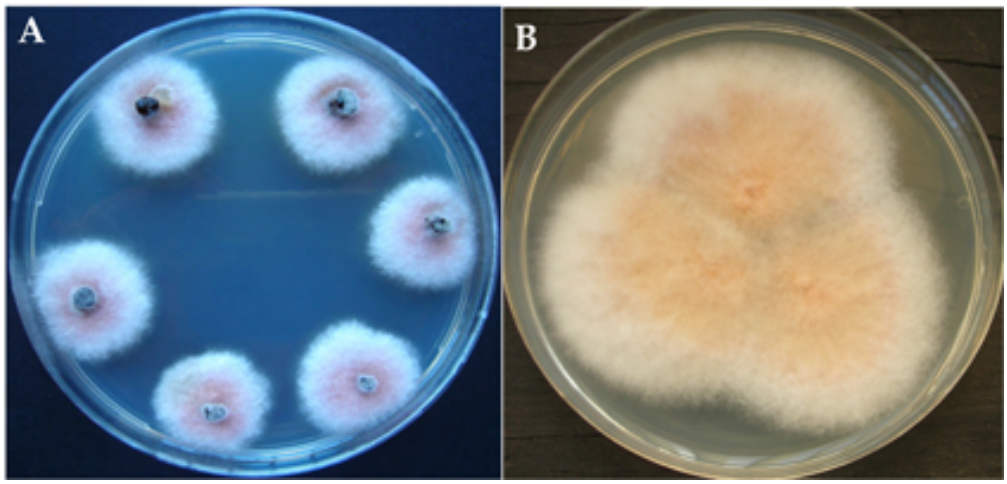


Figure 15. A, Typical colonies of *Fusarium* growing from symptomatic mango tissue on PDA acidified; B, *Fusarium* sp. colonies isolated from mango growing on Potato dextrose agar.

Table 5. Identification of *Fusarium* spp. isolates according to morphological characteristics and PCR diagnosis; and determination of mating type.

Isolate (species) [a]	Origin (Orchard or reference)	Texture on PDA and FCLA	Morphological characteristics on FCLA and PDA[b]	PCR diagnosis 61-2F/R	PCR diagnosis 1-3F/R	Identification based on morphology and PCR	MAT 1/2[c]
Spanish isolates							
UMAF F0601	Vélez-Málaga (Cabrilla)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0602	Vélez-Málaga (Cabrilla)	Floccose	Resembling those of *F. mangiferae*	+	−	*F. tupiense*	2
UMAF F0603	Vélez-Málaga (Cabrilla)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0604	Vélez-Málaga (Cabrilla)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0605	Vélez-Málaga (Cabrilla)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0606	Vélez-Málaga (Cabrilla)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0607	Vélez-Málaga (Cabrilla)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0908	Benamargosa (Huerta)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0909	Benamargosa (Huerta)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0910	Benamargosa (Huerta)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0911	Benamargosa (Huerta)	Floccose	Typical of *F. mangiferae*.	−	+	*F. mangiferae*	2
UMAF F0912	Benamargosa (Huerta)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0913	Vélez-Málaga (Arroyo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0914	Vélez-Málaga (Arroyo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2

Table 5. (Continued)

Isolate (species) [a]	Origin (Orchard or reference)	Texture on PDA and FCLA	Morphological characteristics on FCLA and PDA[b]	PCR diagnosis 61-2F/R	PCR diagnosis 1-3F/R	Identification based on morphology and PCR	MAT 1/2[c]
Spanish isolates							
UMAF F0915	Vélez-Málaga (Arroyo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0916	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0917	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0918	Benajarafe (V-M) (Pintao)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0919	Benajarafe (V-M) (Pintao)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0920	Benajarafe (V-M) (Pintao)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0921	Benajarafe (V-M) (Pintao)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0922	Benajarafe (V-M) (Pintao)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0923[d]	Cútar (Botin)	Floccose	Untypical of *F. mangiferae*	−	+	*F. mangiferae*? [d]	2
UMAF F0924	Vélez-Málaga (Potril)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0925	Cútar (Botín)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0926	Vélez-Málaga (Potril)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0927	Vélez-Málaga (Tejares)	Woolly	Untypical	−	−	*F. phyllophilum*-like	2
UMAF F0928	Vélez-Málaga (Tejares)	Woolly	Untypical	−	−	*F. phyllophilum*-like	2
UMAF F0929	Vélez-Málaga (Cabrera)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2

Table 5. (Continued)

Isolate (species)[a]	Origin (Orchard or reference)	Texture on PDA and FCLA	Morphological characteristics on FCLA and PDA[b]	PCR diagnosis 61-2F/R	PCR diagnosis 1-3F/R	Identification based on morphology and PCR	MAT 1/2[c]
Spanish isolates							
UMAF F0930	Algarrobo (Casa Alta)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0931	Vélez-Málaga (Cortijo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0932	Vélez-Málaga (Cortijo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0933	Vélez-Málaga (Cortijo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0934	Vélez-Málaga (Cortijo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0935	Vélez-Málaga (Cortijo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F0936	Benamargosa (Barranco)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0937	Benamargosa (Barranco)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0938	Benamargosa (Barranco II)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0939	Vélez-Málaga (Potril)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F0940	Cútar (Botín)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F1041	Vélez-Málaga (Carril)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1042	Vélez-Málaga (Carril)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1043	Vélez-Málaga (Lara)	Woolly	Typical of *F. mangiferae*	+	−	*F. tupiense*	2
UMAF F1044	Vélez-Málaga (Arroyo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	negative
UMAF F1045	Vélez-Málaga (Arroyo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	negative

Table 5. (Continued)

Isolate (species) [a]	Origin (Orchard or reference)	Texture on PDA and FCLA	Morphological characteristics on FCLA and PDA[b]	PCR diagnosis 61-2F/R	PCR diagnosis 1-3F/R	Identification based on morphology and PCR	MAT 1/2[c]
Spanish isolates							
UMAF F1046	Vélez-Málaga (Córdoba)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1047	Vélez-Málaga (Córdoba)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1048	Vélez-Málaga (Córdoba)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1049	Benamargosa (Barranco)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F1050	Vélez-Málaga (Granaíno)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1051	Vélez-Málaga (Granaíno)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1052	Vélez-Málaga (Granaíno)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1053	Vélez-Málaga (Granaíno)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1054	Vélez-Málaga (Cabrera)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1055	Benamargosa (Encantá)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1056	Algarrobo (Portillo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1057	Algarrobo (Portillo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1058	Algarrobo (Melgares)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1059	Algarrobo (Melgares)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1060	Algarrobo (Melgares)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2

Table 5. (Continued)

Isolate (species) [a]	Origin (Orchard or reference)	Texture on PDA and FCLA	Morphological characteristics on FCLA and PDA[b]	PCR diagnosis 61-2F/R	PCR diagnosis 1-3F/R	Identification based on morphology and PCR	MAT 1/2[c]
Spanish isolates							
UMAF F1061	Algarrobo (Melgares)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1062[e]	Frigiliana (Acosta)	Velvety/woolly	Untypical. Macroconidia absent.	−	−	*Fusarium* sp. [e]	U2[e]
UMAF F1063	Vélez-Málaga (Carauta)	Woolly	Typical of *F. mangiferae*	+	−	*F. tupiense*	2
UMAF F1064	Benamargosa (Arcas)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F1065	Benamargosa (Arcas)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F1066	Vélez-Málaga (Braun)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1067	Benamocarra (Palomo)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1168	Benamargosa (Encantá)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	1
UMAF F1169	Vélez-Málaga (Arroyo)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1170	Vélez-Málaga (Arroyo)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1171	Vélez-Málaga (Cortijo)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1172	Vélez-Málaga (Cortijo)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1173	Vélez-Málaga (Cortijo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1174	Benamargosa (Huerta)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F1175	Benamargosa (Huerta)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2

Table 5. (Continued)

Isolate (species) [a]	Origin (Orchard or reference)	Texture on PDA and FCLA	Morphological characteristics on FCLA and PDA[b]	PCR diagnosis 61-2F/R	PCR diagnosis 1-3F/R	Identification based on morphology and PCR	MAT 1/2[c]
Spanish isolates							
UMAF F1176	Benamargosa (Huerta)	Floccose	Typical of *F. mangiferae*.	−	+	*F. mangiferae*	2
UMAF F1177	Algarrobo (Portillo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1178	Algarrobo (Peláez)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1179	Algarrobo (Lupiáñez)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	negative
UMAF F1180	Almayate (V-M) (Parra)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1181	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1182	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1183	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1184	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	negative
UMAF F1185	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1186	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1187	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1188	Almayate (V-M) (Parra)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1189	Vélez-Málaga (Arroyo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1190	Vélez-Málaga (Arroyo)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2

Table 5. (Continued)

Isolate (species) [a]	Origin (Orchard or reference)	Texture on PDA and FCLA	Morphological characteristics on FCLA and PDA[b]	PCR diagnosis 61-2F/R	PCR diagnosis 1-3F/R	Identification based on morphology and PCR	MAT 1/2[c]
Spanish isolates							
UMAF F1191	Vélez-Málaga (Arroyo)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1192	Vélez-Málaga (Potril)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F1193	Benamocarra (Sarmiento)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1194	Benamocarra (Sarmiento)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1195	Benamocarra (Sarmiento)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1196	Vélez-Málaga (Hijano)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1197	Vélez-Málaga (Hijano)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	negative
UMAF F1198	Vélez-Málaga (Hijano)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F1199	Vélez-Málaga (Hijano)	Floccose/woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12100	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12101	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12102	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	negative
UMAF F12103	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	negative
UMAF F12104	Vélez-Málaga (Lara)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12105	Benamargosa (Encantá)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2

Table 5. (Continued)

Isolate (species) [a]	Origin (Orchard or reference)	Texture on PDA and FCLA	Morphological characteristics on FCLA and PDA[b]	PCR diagnosis 61-2F/R	PCR diagnosis 1-3F/R	Identification based on morphology and PCR	MAT 1/2[c]
Spanish isolates							
UMAF F12106	Benamargosa (Encantá)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12107	Vélez-Málaga (Arroyo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12108	Benamargosa (Huerta)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F12109	Benamargosa (Huerta)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F12110	Benamargosa (Huerta)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F12111	Vélez-Málaga (Triana)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12112	Vélez-Málaga (Gámez)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12113	Almáchar (Martín)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12114	Benamocarra (Sarmiento)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12115	Vélez-Málaga (Cortijo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12116	Vélez-Málaga (Cortijo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12117	Vélez-Málaga (Cortijo)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	negative
UMAF F12118	Vélez-Málaga (Moneda)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12119	Vélez-Málaga (Moneda)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12120	Vélez-Málaga (Moneda)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2

Table 5. (Continued)

Isolate (species) [a]	Origin (Orchard or reference)	Texture on PDA and FCLA	Morphological characteristics on FCLA and PDA[b]	PCR diagnosis 61-2F/R	PCR diagnosis 1-3F/R	Identification based on morphology and PCR	MAT 1/2[c]
Spanish isolates							
UMAF F12121	Benamocarra (Sarmiento)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	negative
UMAF F12122	Benamocarra (Matao)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	negative
UMAF F12123	Benajarafe (V-M) (Pintao)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	1
UMAF F12124	Benajarafe (V-M) (Pintao)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F12125	Cútar (Botín)	Floccose	Typical of *F. mangiferae* Macroconidia absent	−	+	*F. mangiferae*	2
UMAF F12126	Cútar (Botín)	Floccose	Typical of *F. mangiferae* Macroconidia absent	−	+	*F. mangiferae*	1
UMAF F12127	Cútar (Botín)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F12128	Benamocarra (Garzón)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12129	Benamocarra (Garzón)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12130	Benamocarra (Garzón)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12131	Benamocarra (Garzón)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	2
UMAF F12132	Vélez-Málaga (Lorca)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	2
UMAF F12133	Vélez-Málaga (Lorca)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	negative
UMAF F12134	Vélez-Málaga (Lorca)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	negative

Table 5. (Continued)

Isolate (species) [a]	Origin (Orchard or reference)	Texture on PDA and FCLA	Morphological characteristics on FCLA and PDA[b]	PCR diagnosis 61-2F/R	PCR diagnosis 1-3F/R	Identification based on morphology and PCR	MAT 1/2[c]
Reference isolates							
F. mangiferae (CG-1-4)	USA/(Zheng & Ploetz 2002)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	nd
F. mangiferae (EM50B)	Egypt/(Zheng & Ploetz 2002)	Floccose	Typical of *F. mangiferae*	−	+	*F. mangiferae*	nd
F. mexicanum (NRRL47473)	Mexico/(Otero-Colina et al. 2010)	Woolly	Typical of *F. mexicanum*	+	−	*F. mexicanum*	1
F. mexicanum (NRRL47485)	Mexico/(Otero-Colina et al. 2010)	Woolly	Typical of *F. mexicanum*	+	−	*F. mexicanum*	nd
F. mexicanum (NRRL53571)	Mexico/(Otero-Colina et al. 2010)	Woolly	Typical of *F. mexicanum*	+	−	*F. mexicanum*	nd
F. mexicanum (NRRL53575)	Mexico/(Otero-Colina et al. 2010)	Woolly	Typical of *F. mexicanum*	+	−	*F. mexicanum*	nd
F. mexicanum (NRRL53580)	Mexico/(Otero-Colina et al. 2010)	Woolly	Typical of *F. mexicanum*	+	−	*F. mexicanum*	2
F. proliferatum (NRRL22944)	Israel/(Volcani Center Collection)	Floccose	Typical of *F. proliferatum*	−	−	*F. proliferatum*	nd
F. sterlihyphosum (NRRL25623)	South Africa/(Wingfield et al. 2000)	Woolly	Typical of *F. sterilihyphosum*	+	−	*F. sterlihyphosum*	nd
F. sterlihyphosum (NRRL53569)	Israel/(Wingfield et al. 2000)	Woolly	Typical of *F. sterilihyphosum*	+	−	*F. sterlihyphosum*	nd
F. tupiense (NRRL53984)	Brazil/(Lima et al. 2009)	Woolly	Typical of *F. tupiense*	+	−	*F. tupiense*	nd

[a]UMAF= Microbiology and Plant Pathology Laboratory collection, University of Málaga, Spain; MRC = Medical Research Council, Tygerberg, South Africa; NRRL (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA); CML = Coleção Micológica de Lavras, Universidade Federal de Lavras, Brazil.

[b]Morphological characteristics of *F. mangiferae*: mycelium contained aerial conidiophores possessing three- to five-celled macroconidia and abundant microconidia in false heads from mono- and polyphialides. Cream-orange-colored sporodochia produced on CLA and FCLA. Chlamydospores absent (Britz *et al*., 2002) (see Figure 17). Morphological characteristics of *F. tupiense*: aerial mycelium containing microconidia aggregated in false heads. Mono- and polyphialidic conidiophores, sympodially branching, often associated with coiled sterile hypha. Sporodochia cream to orange. Chlamydospores absent (Lima *et al*., 2012) (see Figure 18).

[c]Mating type according to MAT gene amplification.

[d]Isolate UMAF F0923 diagnosed as *F. mangiferae* according to PCR, and enclosed in VCG 5; but non pathogenic according to Koch's postulates and showed morphological characteristics distinct from *F. mangiferae*.

nd: not determined.

[e]Isolate UMAF F1062 was non pathogenic on mango.

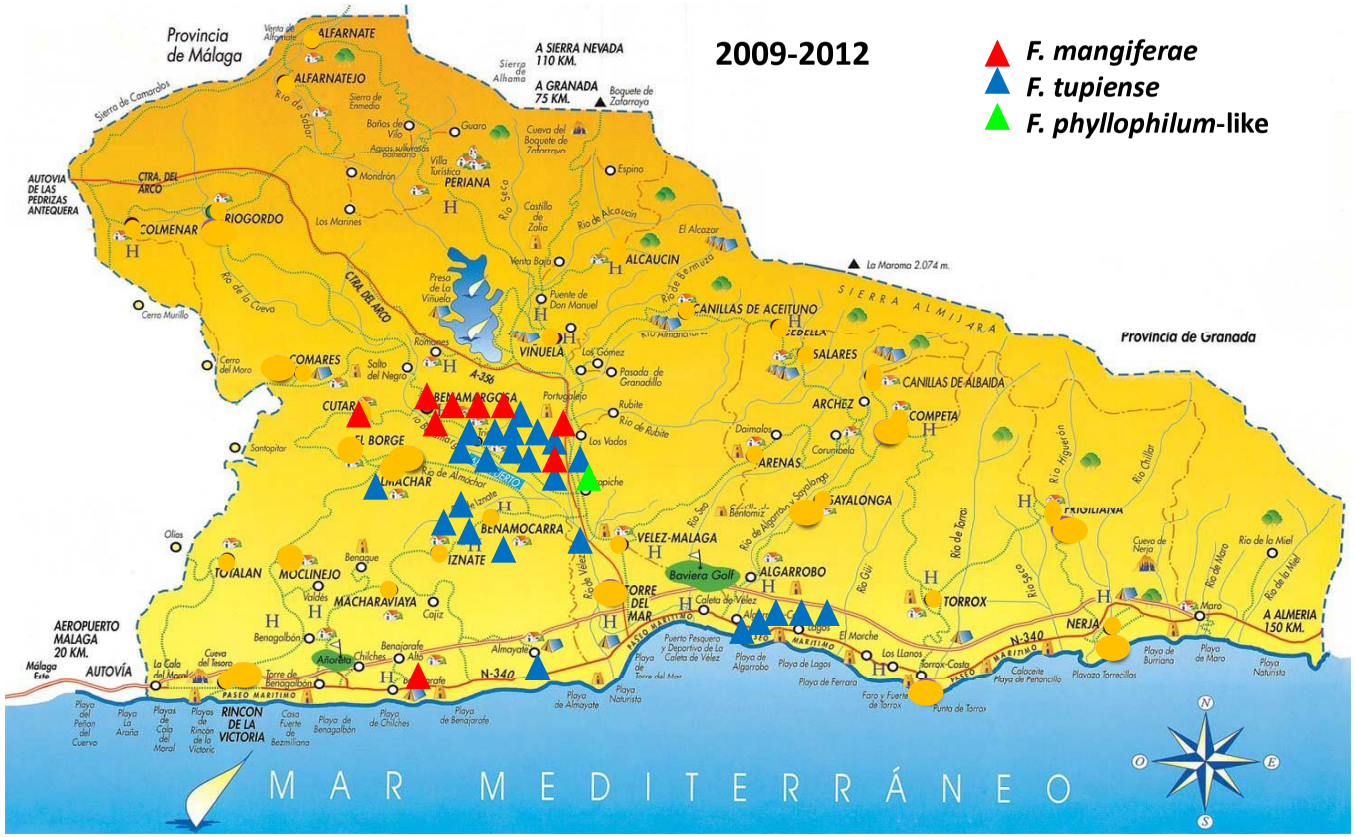


Figure 16. Map of the Axarquía region. Geographic distribution of isolates of the three *Fusarium* species associated with mango malformation disease in Spain.

4.1.2. Morphological description.

One hundred and thirty-four single-spore isolates grown on PDA (Figure 15B) were identified as members of the *Fusarium* genus by the observation under an optic microscope, of the typical fusiform conidia that gives name to this genus (Figure 5).

Forty of these isolates possessed dark purple-to-salmon-colored mycelium when grown on potato dextrose agar (PDA) medium, and on fresh carnation leaf agar (FCLA) medium, mycelium contained aerial conidiophores possessing three- to five-celled macroconidia and abundant microconidia in false heads from mono- and polyphialides; while cream-orange-colored sporodochia were produced on the surface of the medium, typical features of *Fusarium mangiferae* (Britz *et al*., 2002; Iqbal *et al.* 2005; Leslie & Summerell, 2006), (Figure 17). On the other hand, 90 of the total isolates from the Axarquía presented microconidia in false heads, mono- and polyphialides and the absence of chlamidospores, but macroconidia were shorter and wider compared to those produced by *F. mangiferae* isolates (Figure 18) these morphological characteristics were compatible with the description of *Fusarium tupiense* (Lima *et al*., 2012). The isolate UMAF F0602 that presented cultural degeneration in the way of lacking aerial mycelium and subsequently, its morphology was difficult to assess. These *Fusarium* sp. Spanish isolates, jointly with three isolates (UMAF F0602, UMAF F1043 y UMAF F1063) that showed morphological features closer to *F. mangiferae*, were afterwards identified as *F. tupiense*, based on additional molecular

techniques described further in this work (Table 5). Three other isolates (UMAF F0927, UMAF F0928 and UMAF F1062) showed atypical morphological characteristics, different of those described for the several *Fusarium* species associated with MMD, and were initially identified as *Fusarium* spp. based on morphological characteristics, but the species level was not conclusively determined (Table 5).

Regarding the morphological characteristics of the *F. tupiense* Spanish isolates; a more detailed study has been recently carried out in cooperation with Dr. T. Aoki (Laboratory Genetic Resources Center, National Institute of Agrobiological Sciences, Japan) to obtain a more precise morphological description of the *F. tupiense* Spanish isolates(Annex III)

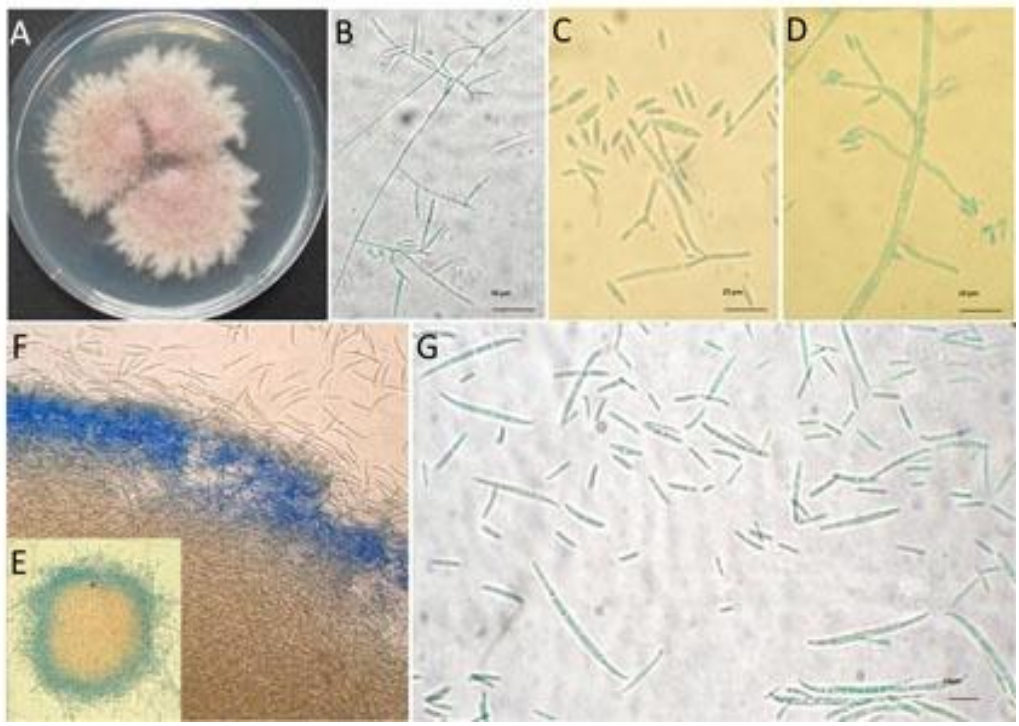


Figure 17. Macro- and microscopic characteristics of *Fusarium mangiferae* typical isolates from Spain: A, Seven-day-old colony of *F. mangiferae* on PDA. B-G Microscopic characteristics observed from *F. mangiferae* growing on FCLA; B, Branched conidiophores bearing polyphialides, Bar = 50 µm. C, Mono-and polyphialides, Bar = 25 µm. D, Microconidia in false heads, Bar = 10 µm. E-F, Sporodochia. G, Macroconidia, Bar = 10 µm.

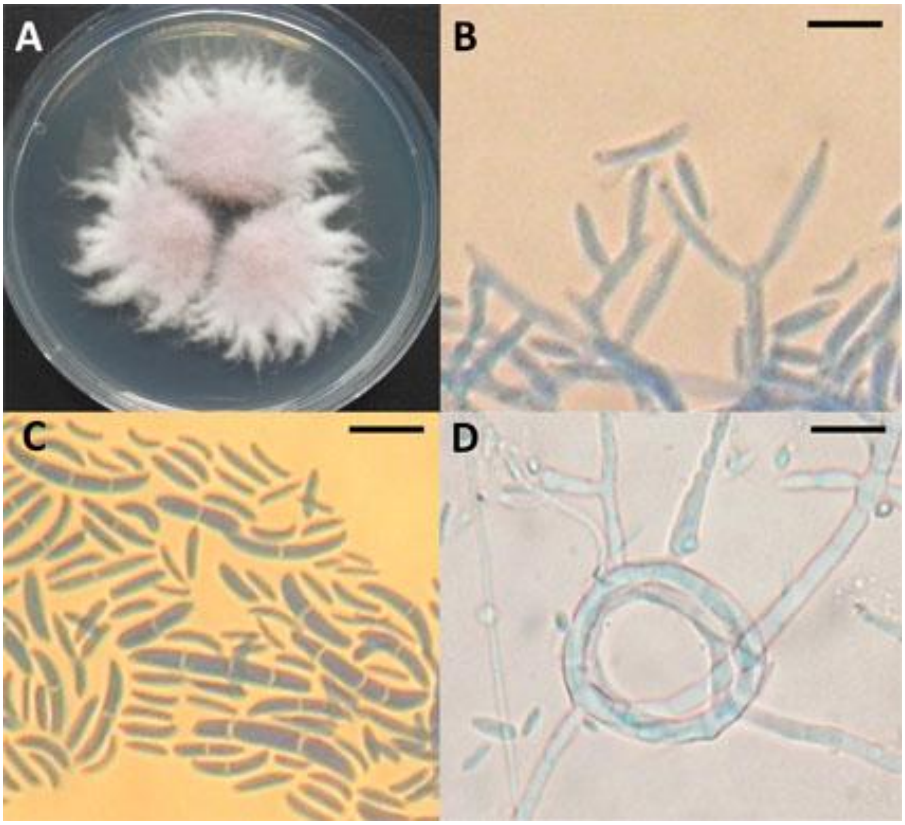


Figure 18. Macro- and microscopic characteristics of *Fusarium tupiense* typical isolates from Spain: A, Seven-day-old colony on PDA**.** B-D; Microscopic characteristics observed from *F. tupiense* growing on FCLA; B, Mono-and polyphialides in the aerial mycelium. C, Macro and microconidia. D, Coiled hyphae. Bar = 10 µm.

4.1.3. Confirmed diagnosis by PCR

From the collection of 134 isolates obtained from symptomatic mango plants in Spain, 38 isolates were diagnosed as *F. mangiferae* by a specific PCR assay with the primer pair 1-3 F/R that amplified a 608-bp DNA fragment (Zheng & Ploetz 2002; Crespo *et al*., 2012) (Figure 19); 37 of these isolates showing also typical morphology of this species. Only, the isolate UMAF F0923 amplified with F1-3 F/R primer pair, but showed morphological characteristics distinct from *F. mangiferae* (Table 5).

None of the rest of the *Fusarium* spp. Spanish isolates amplified with the primer pair mentioned above. However, all of the *F. tupiense* Spanish isolates (n=93), 90 with typical morphological characteristics of this species, and also three isolates with morphological features closed to *F. mangiferae* (UMAF F0602, UMAF F1043 and UMAF F1063) amplified a 445 pb fragment with 61-2F/R primer pair (Figure 20). Similarly, the *Fusarium sterilihyphosum*, *Fusarium mexicanum* and *F. tupiense* reference strains also amplified with 61-2F/R primer pair. These primers were originally developed to diagnose *Fusarium subglutinans* from maize (Möller *et al*., 1999), but later reported to amplify a 445 pb fragment from *F. sterilihyphosum* and *F. mexicanum* (Rodriguez-Alvarado *et al*., 2008; Zheng &Ploetz, 2002). No amplification was observed with the *F. mangiferae* Spanish isolates and reference strains, neither with the *Fusarium*

sp. isolates (UMAF F1062, UMAF F0927 and UMAF F0928) (Figure 20) (Table 5).

Summarizing, 131 *Fusarium* isolates were identified in two main species: 38 conclusively diagnosed as *F. mangiferae,* with the exception of the isolate UMAF F0923 that showed untypical morphological features, and 93 diagnosed as *F. tupiense*; not conclusively identified by PCR but through the use of additional molecular techniques described further in this thesis; additionally three undetermined isolates of *Fusarium* sp. (UMAF F0927, UMAF F0928 and UMAF F1062).

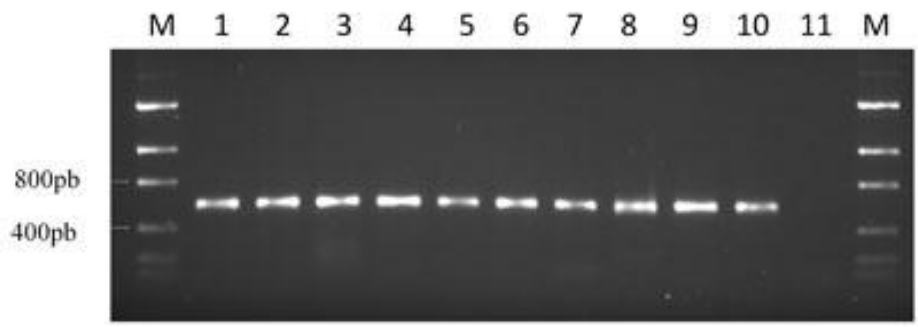


Figure 19. PCR amplification of a 608-pb DNA fragment from *Fusarium* spp. isolates from mango malformation in Spain with the F1/3 primer pair. Lanes 1 to 7, *Fusarium* spp. Spanish isolates identified as *Fusarium mangiferae* (UMAFF0910, UMAFF1174, UMAFF0938, UMAFF0924, UMAFF0926, UMAFF0939, UMAFF1192); lane 8, 9, and 10, *F. mangiferae* reference isolates MRC7560, EM50B and CG-1-4 from Israel, Egypt and Florida, (USA), respectively; lane 11 Spanish isolate UMAF F0916 of *Fusarium tupiense*. M: Marker lanes are 2000 bp ladders.

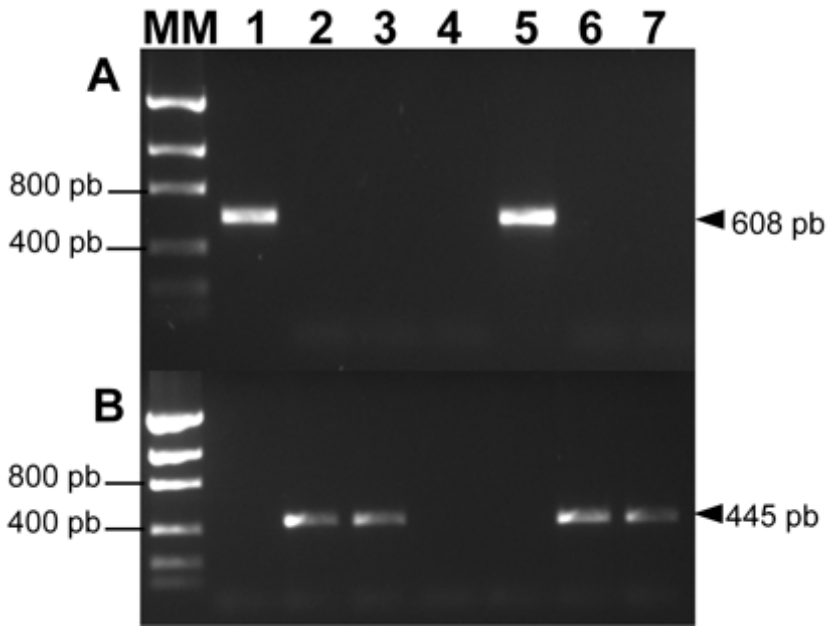


Figure 20. PCR amplification of DNA from *Fusarium* spp. isolates from mango malformation. A, Using 1-3F/R primer pair. B, Using 61-2F/R primer pair. Lane 1, *F. mangiferae* (MRC7560); lane 2 and 3, *F. tupiense* (UMAF F0916, NRRL53984); lane 4, *Fusarium phyllophilum*-like (UMAF F0927); lane 5, *F. mangiferae* (UMAF F0910); lane 6, *F. sterilihyphosum* (NRRL25623); line 7, *F. mexicanum* (NRRL 53580). Marker lane is 2 Kb ladder.

4.2. Pathogenicity assays

To confirm the role of these *Fusarium* Spanish isolates as the causal agent of MMD in the Axarquía region, some experiments of artificial inoculation on mango trees were carried out with some *F. mangiferae* (n=8), *F. tupiense* (n=12), and two *Fusarium* sp. representative isolates (Table 3).

MMD symptoms were detected after bud break in March 2011 (from trees inoculated in 2010), and March 2012 (from trees inoculated in 2011), in the majority of the experimental inoculations. Seven Spanish isolates of *F. mangiferae* assayed on mango trees as well as the reference strain MRC 7560 showed typical symptoms of MMD (Table 6, Figure 21) with the only exception of isolate UMAF F0923 that was nonpathogenic and also showed untypical morphological features. These isolates were newly recovered from the artificially infected floral or vegetative malformed buds, and they were identical morphologically to those inoculated; also the specific 608-bp fragment described for *F. mangiferae* was amplified in all of the isolates recovered.

On the other hand, 12 *F. tupiense* isolates inoculated on mango were all positive and reproduced the typical symptoms of MMD floral and vegetative (Table 7, Figure 22). These recovered isolates were identical morphologically to those inoculated, and the 445-bp fragment was also amplified with PCR in all of them. Regarding the pathogenicity of the two *Fusarium* sp. isolates, the

isolate UMAF F0927, later termed as *Fusarium phyllophilum*-like; also reproduced the typical symptoms of MMD appearing as pathogenic on mango (Table 7, Figure 22). Also the recovered isolate resulted morphologically identical to the isolate inoculated, and did not amplified with F1-3 F/R and 61-2F/R primer sets; however, the isolate UMAF F1062 resulted nonpathogenic on mango inoculation assays (Table 7).

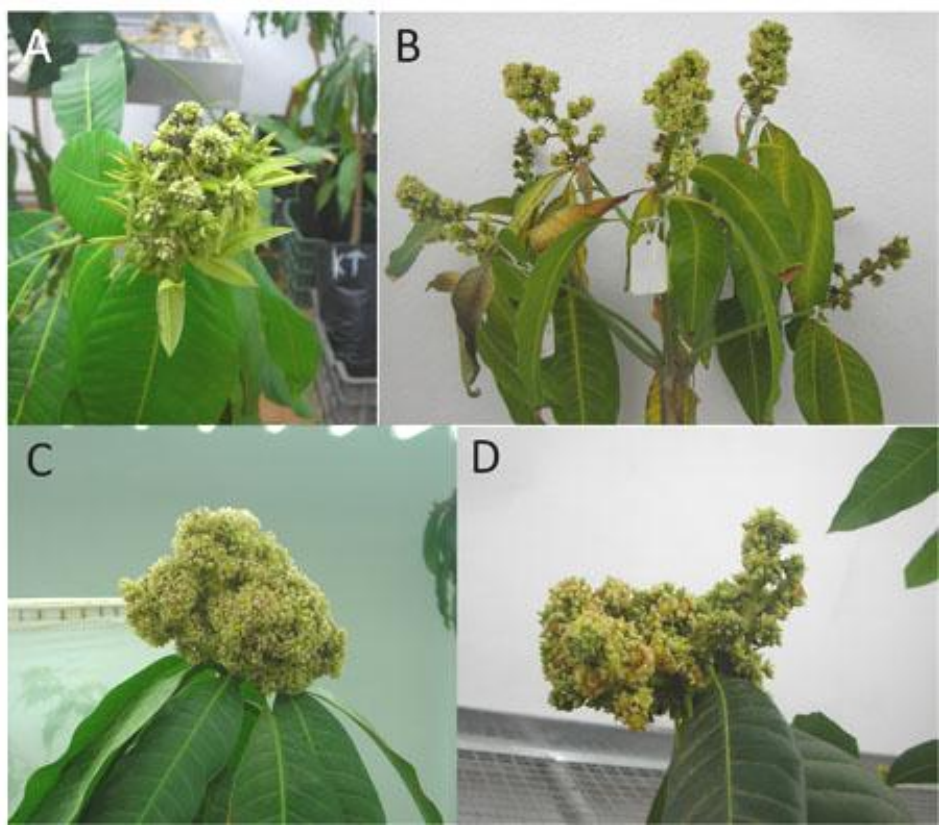


Figure 21. Pathogenicity test on mango trees artificially inoculated with some *Fusarium mangiferae* representative Spanish isolates (Table 6): A, Vegetative and floral malformation symptoms induced in inoculated buds, B-D, Floral malformation symptoms induced in inoculated buds.

Figure 22. Pathogenicity test on mango trees artificially inoculated with some *Fusarium tupiense* representative Spanish isolates (Table 7): A-B, Floral malformation symptoms induced in inoculated buds; C, Vegetative and floral malformation symptoms induced in inoculated buds.

4.3. Population diversity analysis of *Fusarium mangiferae*

Thirty eight *Fusarium* Spanish isolates identified as *F. mangiferae* by diagnostic PCR, including the isolate UMAF F0923 with atypical morphology and nonpathogenic (Tables 5 and 6), were included on a population study, with the aim of elucidate epidemiological aspects and design more efficient control strategies. Population diversity among the strains of *F. mangiferae* associated with MMD in Spain was determined by ap-PCR, RAPD-PCR, vegetative compatibility groups (VCGs), mating type and phylogenetic analyses.

4.3.1. Arbitrary Polymerase Chain Reaction (ap-PCR) and Random Amplified Polymorphism (RAPD-PCR)

To determine the population variability of the Spanish *F. mangiferae* isolates, ap-PCR was performed on DNA extracted from 43 Spanish isolates of *Fusarium* spp., and seven reference isolates from different *Fusarium* species causing MMD worldwide: *F. mangiferae* from Israel, Egypt and Florida (USA), *F. sterilihyphosum*, *F. mexicanum*, *Fusarium pseudocircinatum* and *F. tupiense* (Tables 2 and 3).

Among the tested isolates, identical banding patterns were observed in all of the 40 *F. mangiferae* assayed isolates (37 Spanish isolates and 3 reference isolates) with primers $(GACAC)_3$ and $(CAG)_5$, which was clearly different from the pattern of

representative isolates of *F. sterilihyphosum*, *F. mexicanum* and *F. tupiense*, and the other *Fusarium* spp. Spanish isolates (Figure 23). Different banding patterns were also observed for primer $(GACA)_4$ when comparing *F. mangiferae* isolates to those of the other tested *Fusarium* species isolates. However, additional differences were detected among some of the *F. mangiferae* isolates. Therefore, 37 *F. mangiferae* Spanish isolates, and 3 *F. mangiferae* reference strains (MRC7560, EM50B and CG-1-4) (Table 2), were tested for intraspecific genetic diversity using the repeat motif primers $(GACA)_4$, and a ten base RAPD primer OPF-13 (GGCTGCAGAA). With primer $(GACA)_4$, *F. mangiferae* Spanish isolates showed two different banding patterns (Figure 24). Isolates UMAF F0924, UMAF F0926, UMAF F0939 and UMAF F1192, collected in the same orchard (Potril) but in different years (2009 and 2011), and the reference isolates MRC7560 and EM50B from Israel and Egypt showed an identical profile (genotype 2) (Table 6; Figure 24). Likewise, a second group containing a different profile was observed with the remaining 33 Spanish isolates (genotype 1) (Table 6; Figure 24). The banding pattern of *F. mangiferae* isolate CG-1-4 (USA) was unique and different to the rest of the isolates included in this experiment (Figure 24). Similar results were also obtained when using RAPD primer OPF13. No differences in the banding patterns were detected when using the $(CAG)_5$ primer.

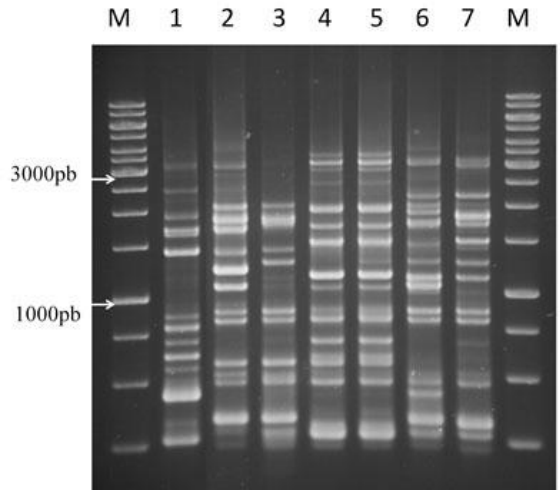


Figure 23. Band profiles generated from ap-PCR analysis with $(GAC)_5$ repeat motif primer. Lane 1 *Fusarium phyllophilum*-like (UMAF F0927); lanes 2 and 3 *F. tupiense* (UMAF F1194 and NRRL53984); lane 4, *F. mangiferae* (UMAF F0910, all the rest of the *F. mangiferae* Spanish isolates showed identical profile, Table 6); lane 5, *F. mangiferae* (MRC7560); lane 6, *F. sterilihyphosum* (NRRL25623); lane 7, *F. mexicanum* (NRRL53580). Outer marker lanes are 1Kb.

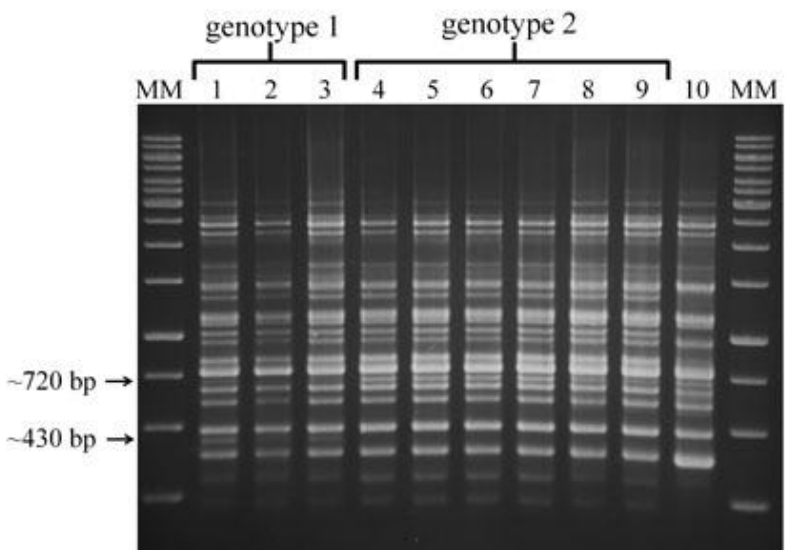


Figure 24. Band patterns generated from ap-PCR analysis with $(GACA)_4$ repeat motif. Lanes 1 to 3, *F. mangiferae* representative Spanish isolates genotype 1 (UMAF F0910, UMAF F1174, UMAF F0938); lanes 4 to 7, *F. mangiferae* Spanish isolates genotype 2 (UMAF F0924, UMAF F0926, UMAF F0939, UMAF F1192); lanes 8 to 10 *F. mangiferae* isolates MRC7560 from Israel; EM50B from Egypt and CG-1-4 from Florida, respectively. Outer marker lanes are 1Kb. Coincident band profile of lanes 1 to 3 (genotype 1) was observed in the rest of the 30 *F. mangiferae* Spanish isolates (Table 6).

4.3.2. Vegetative Compatibility Groups (VCGs)

Vegetative Compatibility Groups (VCGs) were evaluated with nearly all (33) *F. mangiferae* Spanish isolates, including the isolate UMAF F0923 with atypical morphological features and nonpathogenic on mango (Table 6) and a collection of 12 reference isolates of *F. mangiferae* representative of the 6 VCGs previously described by Zheng & Ploetz (2002); and the additional reference strains of *F. mangiferae* (MRC7560), *F. sterilihyphosum* (NRRL25623 and NRRL53569), and *F. mexicanum* (NRRL47437 and NRRL53580) (Tables 2 and 6). Three different VCGs were found among the *F. mangiferae* Spanish isolates. Two of them were not compatible with any of the reference strains of *F. mangiferae* of the six VCGs described by Zheng & Ploetz (2002), and were newly named is this work as VCG 7 grouping the majority of isolates (genotype 1), and VCG 8 grouping the four isolates (UMAF F0924, UMAF F0926, UMAF F0936 and UMAF F1192) also separated according to their unique ap-PCR profile (genotype 2) as we described above in this work (Table 6, Figures 24 and 25). These four isolates were collected from the same orchard (Potril) and in two different years as we mentioned before. Additionally, a third VCG grouping four Spanish isolates (UMAF F0923, UMAF F12125, UMAF F12126 and UMAF F12127) with the isolates X3875-5 and X3875-2 from South Africa in the VCG 5, previously described by Zheng & Ploetz (2002) (Table 6). The isolate UMAF F0923 identified by PCR as *F. mangiferae* but showing atypical

morphological features and being nonpathogenic on mango, did show vegetative compatibility with the *F. mangiferae* VCG 5 reference strains confirming its proposed identification as *F. mangiferae* by PCR, in spite of its atypical features. None of the 33 representative Spanish isolates complemented with the tested *F. mangiferae* reference isolates from Egypt, USA and Israel, nor with the isolates of *F. sterilihyphosum* or *F. mexicanum,* included as negative controls. Nevertheless, *F. mangiferae* isolate MRC7560 from Israel was included with *F. mangiferae* isolates EM50B and EM43C from Egypt in VCG 2, showing an identical profile (genotype 2) (Table 6; Figure 24).

4.3.3. Identification of mating type by PCR

PCR reactions containing primers GFmat1a and GFmat1b for the amplification of *MAT*-1, or GFmat2c and GFmat2d for the amplification of *MAT*-2, resulted in amplification of either the ~200-bp (*MAT*-1) or the ~800-bp (*MAT*-2) fragments (Figure 26). Among the 38 *F. mangiferae* Spanish isolates tested for PCR amplification of *MAT*-1 and *MAT*-2, two of them were identified as *MAT*-1 (UMAF F12123 and UMAF F12126) (Figure 26, Table 5), and the majority as *MAT*-2; however, isolates UMAF F12133 and UMAF F12134 did not amplified with the primer pairs described above.

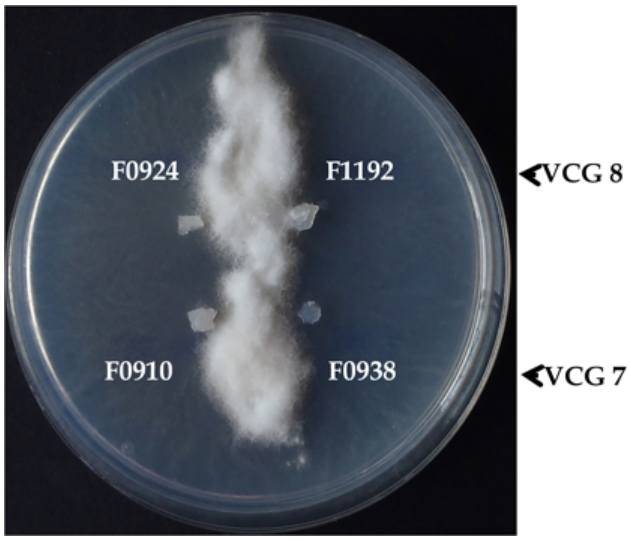


Figure 25. Complementation test among 4 different *nit* mutants of 4 *Fusarium mangiferae* Spanish isolates growing on minimal medium containing $NaN0_3$ as the sole nitrogen source. Isolates UMAF F0910 and UMAF F0938 belonging to VCG 7, isolates UMAF F0924 and UMAF F1192 belonging to VCG 8. Robust mycelial growth indicates complementation between isolates.

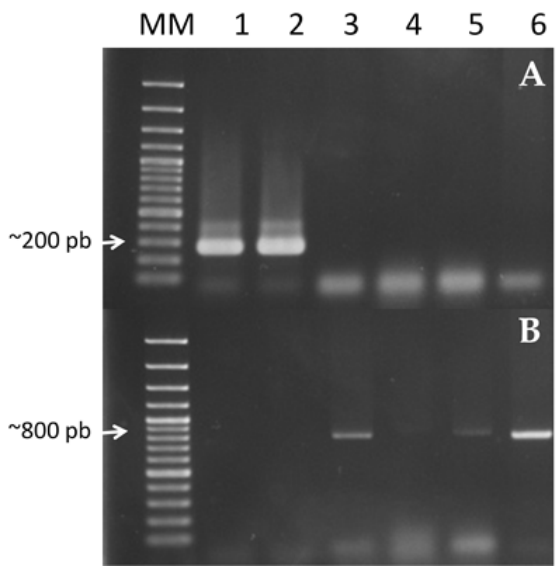


Figure 26. PCR amplification patterns of mating types from *Fusarium* spp. isolates from mango malformation. A, *MAT*-1 idiomorph, ~200-300 bp; B, *MAT*-2 idiomorph, ~ 800-900 bp. Lane 1, *F. mexicanum* (NRRL 47473); lane 2, *F. mangiferae* (UMAF F12123); lane 3, *F. mexicanum* (NRRL53580); lane 4 to 6, *F. tupiense* (UMAF F1045, UMAF F0933 and UMAF F0914). M: Marker lane is 1000 bp ladder.

Table 6. Characterization of *Fusarium mangiferae* isolates (n=35) obtained from mango malformed tissues in Spain: Pathogenicity on mango, Vegetative Compatibility Group (VCG) and ap-PCR genotype determination.

Species	Isolate[a]	Origin (reference)	Year	Pathogenicity assays[b]	VCGs[c]	Genotype[d]
Spanish isolates						
F. mangiferae	UMAF F0908	Spain (Benamargosa)	2009	nd	VCG 7	1
F. mangiferae	UMAF F0909	Spain (Benamargosa)	2009	nd	VCG 7	1
F. mangiferae	UMAF F0910	Spain (Benamargosa)	2009	positive	VCG 7	1
F. mangiferae	UMAF F0911	Spain (Benamargosa)	2009	nd	VCG 7	1
F. mangiferae	UMAF F0912	Spain (Benamargosa)	2009	nd	VCG 7	1
F. mangiferae	UMAF F0919	Spain (Benajarafe, V-M)	2009	nd	VCG 7	1
F. mangiferae	UMAF F0920	Spain (Benajarafe, V-M)	2009	positive	VCG 7	1
F. mangiferae	UMAF F0921	Spain (Benajarafe, V-M)	2009	nd	nd	1
F. mangiferae	UMAF F0922	Spain (Benajarafe, V-M)	2009	nd	nd	1
F. mangiferae?	UMAF F0923	Spain (Cútar)	2009	negative	VCG 5	nd
F. mangiferae	UMAF F0924	Spain (Vélez-Málaga)	2009	positive	VCG 8	2
F. mangiferae	UMAF F0925	Spain (Cútar)	2009	positive	VCG 7	1
F. mangiferae	UMAF F0926	Spain (Vélez-Málaga)	2009	nd	VCG 8	2
F. mangiferae	UMAF F0929	Spain (Vélez-Málaga)	2009	nd	VCG 7	1

Table 6. (Continued)

Species	Isolate[a]	Origin (reference)	Year	Pathogenicity assays[b]	VCGs[c]	Genotype[d]
Spanish isolates						
F. mangiferae	UMAF F0936	Spain (Benamargosa)	2009	nd	VCG 7	1
F. mangiferae	UMAF F0937	Spain (Benamargosa)	2009	nd	VCG 7	1
F. mangiferae	UMAF F0938	Spain (Benamargosa)	2009	positive	VCG 7	1
F. mangiferae	UMAF F0939	Spain (Vélez-Málaga)	2009	nd	VCG 8	2
F. mangiferae	UMAF F0940	Spain (Cútar)	2009	nd	VCG 7	1
F. mangiferae	UMAF F1049	Spain (Benamargosa)	2010	nd	VCG 7	1
F. mangiferae	UMAF F1064	Spain (Benamargosa)	2010	nd	VCG 7	1
F. mangiferae	UMAF F1065	Spain (Benamargosa)	2010	nd	VCG 7	1
F. mangiferae	UMAF F1174	Spain (Benamargosa)	2011	positive	VCG 7	1
F. mangiferae	UMAF F1175	Spain (Benamargosa)	2011	nd	VCG 7	1
F. mangiferae	UMAF F1176	Spain (Benamargosa)	2011	nd	VCG 7	1
F. mangiferae	UMAF F1192	Spain (Vélez-Málaga)	2011	positive	VCG 8	2
F. mangiferae	UMAF F12110	Spain (Benamargosa)	2012	nd	VCG 7	1
F. mangiferae	UMAF F12123	Spain (Benajarafe, V-M)	2012	nd	VCG 7	1

Table 6. (Continued)

Species	Isolate[a]	Origin (reference)	Year	Pathogenicity assays[b]	VCGs[c]	Genotype[d]
F. mangiferae	UMAF F12124	Spain (Benajarafe, V-M)	2012	nd	VCG 7	1
F. mangiferae	UMAF F12125	Spain (Cútar)	2012	nd	VCG 5	1
F. mangiferae	UMAF F12126	Spain (Cútar)	2012	nd	VCG 5	1
F. mangiferae	UMAF F12127	Spain (Cútar)	2012	nd	VCG 5	1
F. mangiferae	UMAF F12132	Spain (Vélez-Málaga)	2012	nd	VCG 7	1
F. mangiferae	UMAF F12133	Spain (Vélez-Málaga)	2012	nd	VCG 7	1
F. mangiferae	UMAF F12134	Spain (Vélez-Málaga)	2012	nd	VCG 7	1
Reference isolates						
F. mangiferae	CG-1-4	USA/(Zheng & Ploetz 2002)	–	nd	VCG6	3
F. mangiferae	CG-2-7	USA/(Zheng & Ploetz 2002)	–	nd	VCG6	nd
F. mangiferae	EM22B	Egypt/(Zheng & Ploetz 2002)	–	nd	VCG4	nd
F. mangiferae	EM32E	Egypt/(Zheng & Ploetz 2002)	–	nd	VCG4	nd
F. mangiferae	EM42C	Egypt/(Zheng & Ploetz 2002)	–	nd	VCG1	nd
F. mangiferae	EM43C	Egypt/(Zheng & Ploetz 2002)	–	nd	VCG2	nd
F. mangiferae	EM44F	Egypt/(Zheng & Ploetz 2002)	–	nd	VCG3	nd
F. mangiferae	EM50B	Egypt/(Zheng & Ploetz 2002)	–	nd	VCG2	2

Table 6. (Continued)

Species	Isolate[a]	Origin (reference)	Year	Pathogenicity assays[b]	VCGs[c]	Genotype[d]
Reference isolates						
F. mangiferae	EM73C	Egypt/(Zheng & Ploetz 2002)	–	nd	VCG3	nd
F. mangiferae	MRC7560	Israel/(Volcani Center Collection)	–	nd	VCG2	2
F. mangiferae	X3875-5	South Africa/(Zheng & Ploetz 2002)	–	nd	VCG5	nd
F. mangiferae	X3875-2	South Africa/(Zheng & Ploetz 2002)	–	nd	VCG5	nd
F. mangiferae	X4707	Egypt/(Zheng & Ploetz 2002)	–	nd	VCG1	nd
F. mexicanum	NRRL47437	Mexico/(Otero-Colina et al. 2010)	–	nd	VCGA	nd
F. mexicanum	NRRL53580	Mexico/(Otero-Colina et al. 2010)	–	nd	VCGB	4
F. pseudocircinatum	NRRL53570	Israel/(Volcani Center Collection)	–	nd	nd	7
F. sterlihyphosum	NRRL25623 (MRC2802)	South Africa/(Wingfield et al. 2000)	–	nd	VCGC	5
F. sterlihyphosum	NRRL53569	South Africa/(Wingfield et al. 2000)	–	nd	VCGC	nd
F. tupiense	NRRL53984 (CML262)	Brazil/(Lima et al. 2009)	–	nd	nd	6

[a]UMAF = Microbiology and Plant Pathology Laboratory collection, University of Málaga, Spain; MRC = Medical Research Council, Tygerberg, South Africa; NRRL (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois USA); CML = Coleção Micológica de Lavras, Universidade Federal de Lavras, Brazil.

[b]Pathogenicity tests were conducted using the designated isolates.

[c]Vegetative compatibility groups 7 and 8 were determined only in isolates from this study. Those from 1-6 were determined previously by Zheng and Ploetz (2002). VCGs A, B and C were also determined in this study.

[d]Genotypes were designated according to band patterns using three different repeat motif and two different RAPD primers, containing identical band patterns. nd= not determined

4.3.4. Phylogenetic analysis

Phylogenetic analysis of some *F. mangiferae* representative Spanish isolates was carried out using several housekeeping genes in two independent assays.

In a first trial, TEF and β-tubulin sequences obtained from ten selected *F. mangiferae* Spanish isolates comprising the maximum diversity (Table 3) were compared with similar sequences from strains of other species in the *Gibberella fujikuroi* species complex (GFSC) already available in GenBank or in *Fusarium*-ID (Table 2). A combined alignment of TEF and β-tubulin DNA sequences was used to generate phylogenetic trees to infer the phylogenetic relationship between *Fusarium* Spanish isolates and other species belonging to the GFSC. All of the *F. mangiferae* Spanish isolates grouped in a cluster with *F. mangiferae* reference isolates (MRC7560 and NRRL25226) from Israel and India respectively, in the Asian Clade, and including the isolate UMAF F0923 with atypical morphology and nonpathogenic (Figure 27).

A second phylogenetic analysis with DNA sequence data from portions of seven genes, TEF, β-tubulin, calmodulin, histone H3, nuclear ribosomal intergenic spacer region (IGS rDNA), and the RNA polymerase subunits RPB1 and RPB2 was carried out with two *F. mangiferae* representative Spanish isolates (UMAF F0910 and UMAF F0924) (Table 3), and included sequence data of several MMD-associated *Fusarium* spp. and other members of the

GFSC. In this second trial, similar results were obtained and the phylogenetic tree resulting grouped *F. mangiferae* Spanish isolates in the Asian Clade with the *F. mangiferae* reference strain from India mentioned above (Figure 28).

4.3.5. Cross fertility assays

High fertile female *F. mangiferae* tester strains are not available for using in crossing protocols because the teleomorph of this species have not been found. In this study, we also tested the fertility among the *MAT*-1 and *MAT*-2 *F. mangiferae* Spanish isolates (Table 5). In our experiments, any of the *F. mangiferae* tested isolates produced perithecia, possibly due to a reduced female fertility of the field isolates, or to unsuitable cross conditions.

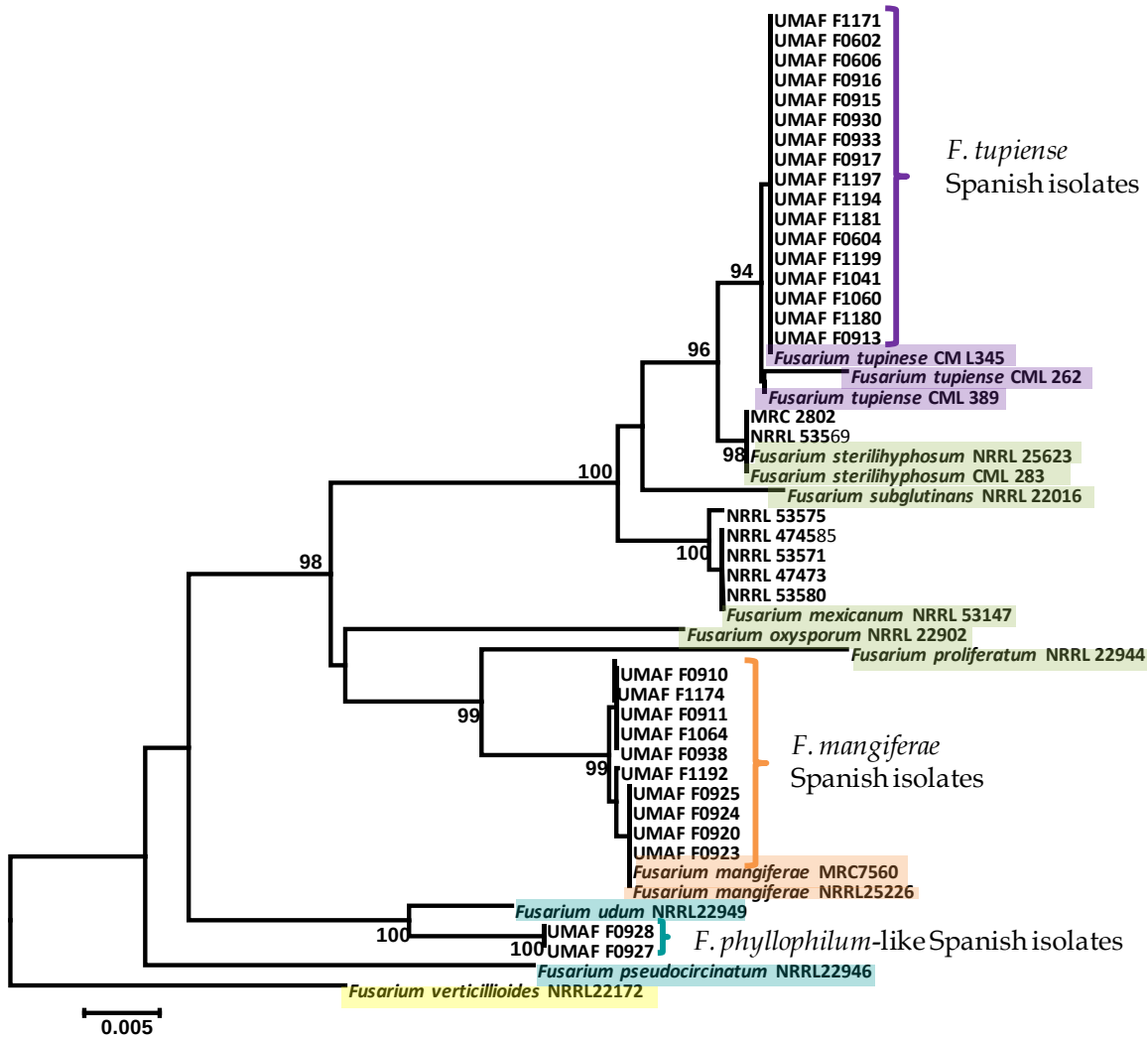


Figure 27. Phylogenetic tree based on combined partial sequences of TEF (*tef*1) and β-tubulin (*tub*2) genes. *Fusarium verticillioides* isolate NRRL 22172 was used as outgroup. Trees were conducted using MEGA 5.0. Bootstrap values (1.000 repetitions) are shown on branches. Sequences from strains with shaded names were extracted from published genome sequences. The biological and genetic characteristics of Fusarium spp. Spanish isolates appear in Tables 3, 5, 6 and 7.

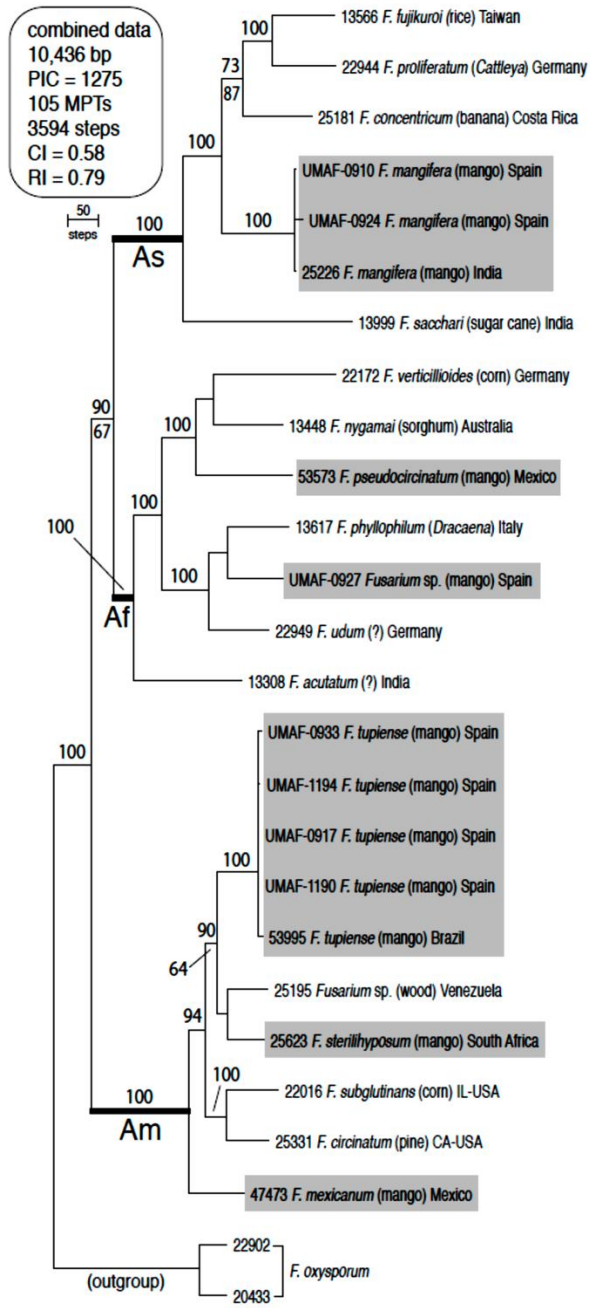


Figure 28. Analyses of the partial DNA sequences of seven genes: beta-tubulin, calmodulin, histone H3, IGS rDNA intergenic region, RPB1, RPB2, and TEF of members of the *Gibberella fujikuroi* species complex, including seven representative Spanish isolates.

4.4. Confirmed identification and population diversity of *Fusarium tupiense* and *Fusarium* sp. Spanish isolates

Forty-three representative Spanish isolates presumptively identified as *F. tupiense* based on morphology and PCR results, and two unidentifed *Fusarium* sp. isolates also pathogenic on mango (Tables 3 and 7), were included on a population study, with the aim of characterize and conclusively identify *Fusarium* spp. Spanish isolates. Population diversity was determined by ap-PCR, RAPD-PCR, vegetative compatibility groups (VCGs), mating type and phylogenetic analyses (MLST).

4.4.1. Arbitrary Polymerase Chain Reaction (ap-PCR) and Random Amplified Polymorphism (RAPD-PCR)

All *F. tupiense* Spanish isolates tested with the only exception of isolate UMAF F1182 (genotype 7, Table 7) presented an unique and uniform profile with the repeat motif primers $(GACAC)_3$, $(CAG)_5$, $(GACA)_4$ (genotype 1) (Table 7, Figure 29). This banding pattern was also reflected with OPF-08 and OPF-13 RAPD primers carried out with a set of 4 representative *F. tupiense* isolates (Figure 29). This band pattern (genotype 1) was different with all the primers mentioned above to the reference isolates of *F. sterilihyphosum* (NRRL25623), *F. mexicanum* (NRRL53580), *F. tupiense* (NRRL53984), and *F. mangiferae* (MRC7560) assayed in this trial (Figure 29, Table 7). *F. phyllophilum*-like isolates UMAF F0927 and UMAF F0928 showed and shared an specific and

identical profile with ap-PCR and RAPD primers, different to the rest of the *Fusarium* spp. tested isolates (genotype 8) (Figure 29, Table 7).

Taking into account the population variability of the *F. tupiense* isolates from Brazil, a second trial with the same primers mentioned above and including six *F. tupiense* reference isolates from Brazil, each one belonging to a different VCG (Table 7), was carried out with four representative *F. tupiense* and one *F. phyllophilum*-like Spanish isolates. As a result of this second trial, the *F. tupiense* Spanish isolates shared an identical and uniform profile with the *F. tupiense* reference isolate NRRL53995 (CLM386), with all the primers mentioned above (genotype 1) (Figure 30, Table 7). *F. phyllophilum*-like Spanish isolate UMAF F0927 showed again an unique profile different to the rest of the *Fusarium* spp. tested isolates (genotype 8) (Figure 30, Table 7).

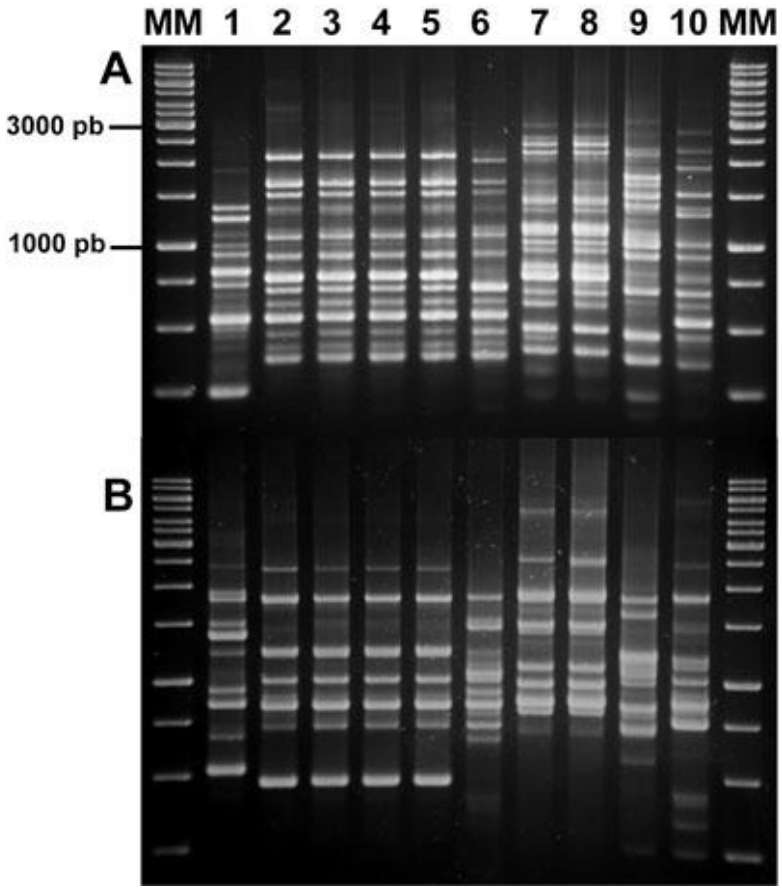


Figure 29. Comparative analysis of representative *Fusarium tupiense* Spanish isolates with some reference strains of different species pathogenic on mango. Band patterns generated from A: ap-PCR analysis. using $(GACA)_4$ repeat motif; B: RAPD-PCR analysis with OPF-08 primer pair. Lane 1 *Fusarium phillophilum*-like (UMAF F0927), lanes 2 to 5 *F. tupiense* isolates (UMAF F0917, UMAF F0933, UMAF F1190 and UMAF F1194, all the rest of the *F. tupiense* Spanish isolates showed identical profile, Table 7); lane 6, *F. tupiense* (NRRL53984); lane 7and 8, *F. mangiferae* (UMAF F0910, MRC7560); lane 9, *F. sterilihyphosum* (NRRL25623); lane 10, *F. mexicanum* (NRRL53580). Outer marker lanes are 1Kb.

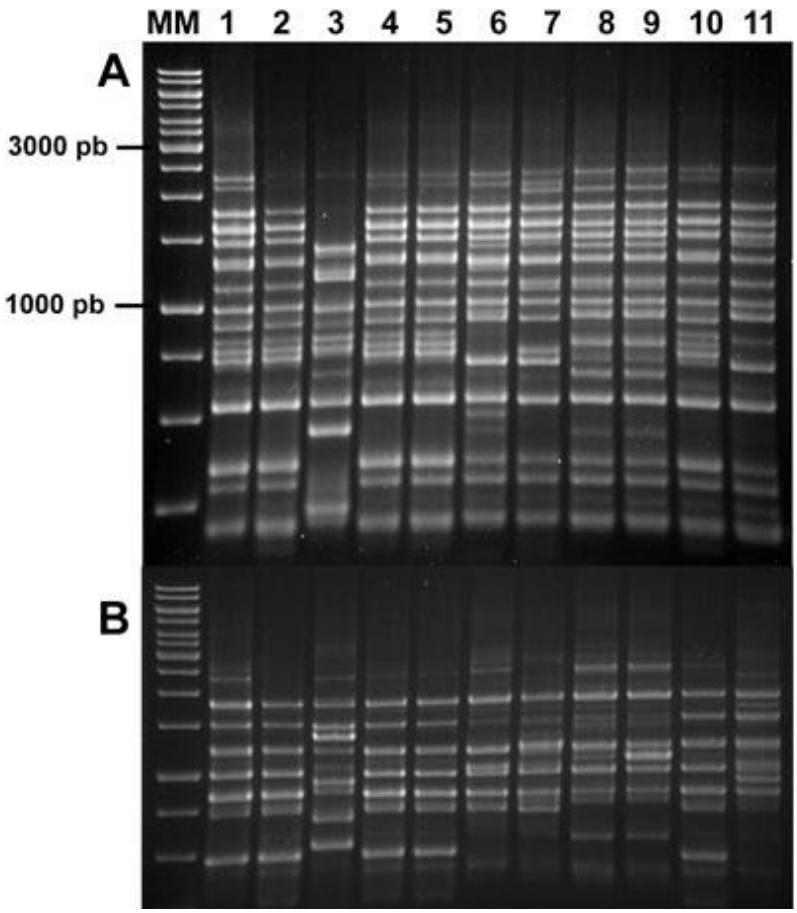


Figure 30. Comparative analysis of representative *Fusarium tupiense* isolates from Spain and Brazil. Band patterns generated from: A, ap-PCR analysis using $(GACA)_4$ repeat motif; B, RAPD-PCR analysis using OPF-08 primer pair. Lanes 1-2 and 4-5, *Fusarium tupiense* Spanish isolates (UMAF F0915, UMAF F0916, UMAF F0933, and UMAF F1194, all the rest of the *F. tupiense* Spanish isolates showed identical profile, Table 7); lane 3, *Fusarium phillophilum*-like (UMAF F0927), lane 6 to 11, *F. tupiense* isolates (NRRL53986, NRRL53992, NRRL53993, NRRL53994, NRRL53995, NRRL53996). Marker lane is 1Kb.

4.4.2. Vegetative Compatibility Groups (VCGs)

VCGs were evaluated with 41 *F. tupiense* representative isolates, two *F. phyllophilum*-like and one *Fusarium* sp. nonpathogenic on mango (UMAF F1062) Spanish isolates, and a collection of six *F. tupiense* reference isolates from Brazil each one belonging to a different VCG (Tables 2 and 7). The *nit* mutants were obtained from all of the *Fusarium* spp. Spanish isolates tested with the exception of *Fusarium* sp. isolate UMAF F1062.

All 41 Spanish *F. tupiense* isolates were grouped in the same VCG together with the *F. tupiense* Brazilian isolate CLM386 (VCG I) (Figure 31, Table 7). *F. phyllophilum*-like Spanish isolates were located in a single VCG (named as VCG-D) (Table 7). No complementation was observed with any of the other Brazilian reference strains. The *F. tupiense* isolate UMAF F1182 also grouped in VCG I in spite of the different band profile shown (genotype 7).

4.4.3. Identification of mating type by PCR

Similarly as described in section 4.3.3. for *F. mangiferae* isolates, PCR amplification of *MAT*-1 and *MAT*-2 DNA fragments were determined in the 93 *F. tupiense* and two *F. phyllophilum*- like Spanish isolates. Among the *F. tupiense,* all of the tested isolates were identified as *MAT*-2 (Figure 26), with the unique exception of the *F. tupiense* Spanish isolate UMAF F1168 identified as *MAT*-1 (Table 5); however, ten *F. tupiense* Spanish isolates did not

amplified with any of the primer pairs previously mentioned. The two *F. phyllophilum*- like Spanish isolates were determined as *MAT*-2 (Table 5).

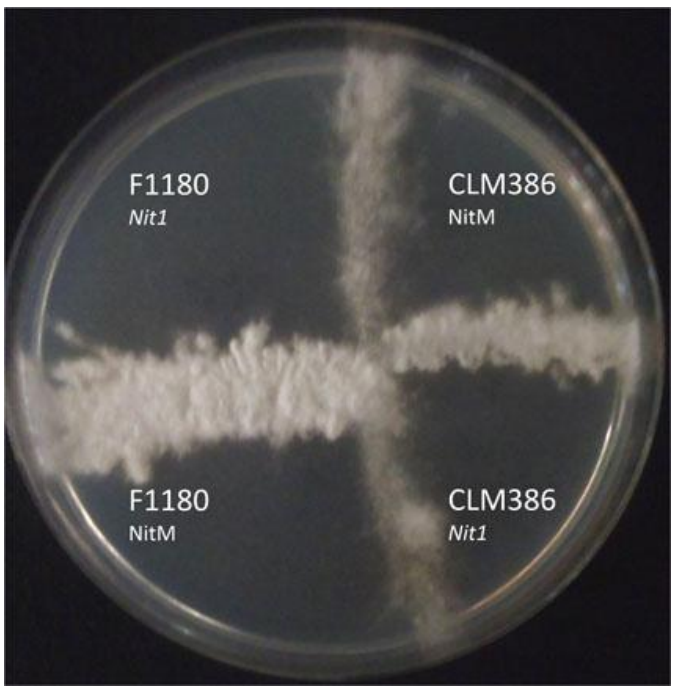


Figure 31. Complementation test among *Fusarium tupiense* Spanish isolate UMAF F1180 and *F. tupiense* isolate NRRL53995 (CML386) from Brazil growing on minimal medium containing $NaN0_3$ as the sole nitrogen source. Robust mycelial growth indicates complementation between isolates.

Table 7. Characterization of *Fusarium tupiense* and *Fusarium* spp. isolates obtained from mango malformed tissues in Spain: Pathogenicity on mango, Vegetative Compatibility Group (VCG) and ap-PCR genotype determination.

Species	Isolate[a]	Origin (reference)	Year	Pathogenicity assays[b]	VCGs[c]	Genotype[d]
Spanish isolates						
Fusarium sp.	UMAF F1062	Spain (Frigiliana)	2010	negative	nd	nd
F. phyllophilum-like	UMAF F0927[e]	Spain (Vélez-Málaga)	2009	positive	VCG D	8
F. phyllophilum-like	UMAF F0928[e]	Spain (Vélez-Málaga)	2009	nd	VCG D	8
F. tupiense	UMAF F0602	Spain (Vélez-Málaga)	2006	positive	nd	nd
F. tupiense	UMAF F0604	Spain (Vélez-Málaga)	2006	positive	VCG I	1
F. tupiense	UMAF F0606	Spain (Vélez-Málaga)	2006	nd	VCG I	1
F. tupiense	UMAF F0913	Spain (Vélez-Málaga)	2009	nd	VCG I	1
F. tupiense	UMAF F0915	Spain (Vélez-Málaga)	2009	positive	VCG I	1
F. tupiense	UMAF F0916	Spain (Vélez-Málaga)	2009	positive	VCG I	1
F. tupiense	UMAF F0917	Spain (Vélez-Málaga)	2009	positive	VCG I	1
F. tupiense	UMAF F0930	Spain (Algarrobo)	2009	positive	VCG I	1
F. tupiense	UMAF F0931	Spain (Vélez-Málaga)	2009	nd	VCG I	1
F. tupiense	UMAF F0933	Spain (Vélez-Málaga)	2009	positive	VCG I	1
F. tupiense	UMAF F1041	Spain (Vélez-Málaga)	2010	nd	VCG I	1

Table 7. (Continued)

Species	Isolate[a]	Origin (reference)	Year	Pathogenicity assays[b]	VCGs[c]	Genotype[d]
Spanish isolates						
F. tupiense	UMAF F1046	Spain (Vélez-Málaga)	2010	nd	VCG I	1
F. tupiense	UMAF F1047	Spain (Vélez-Málaga)	2010	nd	VCG I	1
F. tupiense	UMAF F1051	Spain (Vélez-Málaga)	2010	nd	VCG I	1
F. tupiense	UMAF F1054	Spain (Velez-Málaga)	2010	nd	VCG I	1
F. tupiense	UMAF F1056	Spain (Algarrobo)	2010	nd	VCG I	1
F. tupiense	UMAF F1060	Spain (Algarrobo)	2010	nd	VCG I	1
F. tupiense	UMAF F1063	Spain (Vélez-Málaga)	2010	nd	VCG I	1
F. tupiense	UMAF F1066	Spain (Vélez-Málaga)	2010	nd	VCG I	1
F. tupiense	UMAF F1067	Spain (Benamocarra)	2010	nd	VCG I	1
F. tupiense	UMAF F1168	Spain (Benamargosa)	2011	nd	nd	1
F. tupiense	UMAF F1171	Spain (Vélez-Málaga)	2011	positive	VCG I	1
F. tupiense	UMAF F1177	Spain (Algarrobo)	2011	nd	VCG I	1
F. tupiense	UMAF F1178	Spain (Algarrobo)	2011	nd	VCG I	1
F. tupiense	UMAF F1179	Spain (Algarrobo)	2011	nd	VCG I	1
F. tupiense	UMAF F1180	Spain (Almayate, V-M)	2011	nd	VCG I	1

Table 7. (Continued)

Species	Isolate[a]	Origin (reference)	Year	Pathogenicity assays[b]	VCGs[c]	Genotype[d]
Spanish isolates						
F. tupiense	UMAF F1181	Spain (Vélez-Málaga)	2011	positive	VCG I	1
F. tupiense	UMAF F1182[f]	Spain (Vélez-Málaga)	2011	nd	VCG I	7 [f]
F. tupiense	UMAF F1190	Spain (Vélez-Málaga)	2011	positive	VCG I	1
F. tupiense	UMAF F1194	Spain (Benamocarra)	2011	positive	VCG I	1
F. tupiense	UMAF F1197	Spain (Vélez-Málaga)	2011	nd	VCG I	1
F. tupiense	UMAF F1199	Spain (Vélez-Málaga)	2011	positive	VCG I	1
F. tupiense	UMAF F12104	Spain (Vélez-Málaga)	2012	nd	VCG I	1
F. tupiense	UMAF F12107	Spain (Vélez-Málaga)	2012	nd	VCG I	1
F. tupiense	UMAF F12111	Spain (Vélez-Málaga)	2012	nd	VCG I	1
F. tupiense	UMAF F12112	Spain (Vélez-Málaga)	2012	nd	VCG I	1
F. tupiense	UMAF F12113	Spain (Almáchar)	2012	nd	VCG I	1
F. tupiense	UMAF F12117	Spain (Vélez-Málaga)	2012	nd	VCG I	1
F. tupiense	UMAF F12120	Spain (Vélez-Málaga)	2012	nd	VCG I	1
F. tupiense	UMAF F12121	Spain (Benamocarra)	2012	nd	VCG I	1
F. tupiense	UMAF F12122	Spain (Benamocarra)	2012	nd	VCG I	1

Table 7. (Continued)

Species	Isolate[a]	Origin (reference)	Year	Pathogenicity assays[b]	VCGs[c]	Genotype[d]
Spanish isolates						
F. tupiense	UMAF F12128	Spain (Benamocarra)	2012	nd	VCG I	1
F. tupiense	UMAF F12131	Spain (Benamocarra)	2012	nd	VCG I	1
Reference isolates						
F. tupiense	NRRL 53984 (CML262)	Brazil/(Lima et al. 2009)	–	nd	nd	6
F. tupiense	NRRL 53986 (CML266)	Brazil/(Lima et al. 2009)	–	nd	VCG VI	2
F. tupiense	NRRL 53992 (CML350)	Brazil/(Lima et al. 2009)	–	nd	VCG II	3
F. tupiense	NRRL 53993 (CML383)	Brazil/(Lima et al. 2009)	–	nd	VCG III	4
F. tupiense	NRRL 53994 (CML385)	Brazil/(Lima et al. 2009)	–	nd	VCG IV	4
F. tupiense	NRRL 53995 (CML386)	Brazil/(Lima et al. 2009)	–	nd	VCG I	1
F. tupiense	NRRL 53996 (CML389)	Brazil/(Lima et al. 2009)	–	nd	VCG V	5

[a]UMAF = Microbiology and Plant Pathology Laboratory collection, University of Málaga, Spain; MRC = Medical Research Council, Tygerberg, South Africa; NRRL (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois USA); CML = Coleção Micológica de Lavras, Universidade Federal de Lavras, Brazil.

[b]Pathogenicity tests were conducted using the designated isolates.

[c]Vegetative compatibility groups of *Fusarium* spp. Spanish isolates were determined in this study. Those from I-VI were determined previously by Lima *et al.*, (2009).

[d]Genotypes were designated according to band patterns using three different repeat motif and two different RAPD primers, containing identical band patterns.

[e]The two isolates of *F. phyllophilum*-like shared identical ap-PCR banding pattern and were included in the same VCG (termed as genotype 8 and VCG D).

[f]The isolate of *F. tupiense* UMAF F1182 showed an specific ap-PCR profile (genotype 7), distinct from the rest of the *F. tupiense* Spanish isolates (genotype 1).

nd= not determined.

4.4.4. Phylogenetic analysis

Concerning the unsolved identity of the *F. phyllophilum*- like Spanish isolates pathogenic on mango (UMAF F0927 and UMAF F0928 isolates), as well to confirm the identification of the *F. tupiense* Spanish isolates, a phylogenetic analysis with several housekeeping genes was carried out in two independent assays.

In a first trial, similarly as it was described for *F. mangiferae* isolates (3.3.3 section), TEF and β-tubulin sequences obtained from 17 selected *F. tupiense* and the two *F. phyllophilum*- like Spanish isolates comprising the maximum diversity of them (Tables 3 and 7) were compared with similar sequences from strains of other species in the GFSC already available in GenBank (Table 2). A combined alignment of TEF and β-tubulin DNA sequences was used to generate phylogenetic trees to infer the phylogenetic relationship between this *Fusarium* spp. Spanish isolates and other species belonging to the GFSC. *F. tupiense* Spanish isolates were grouped with *F. tupiense* isolates from Brazil in the American Clade, and the 2 *F. phyllophilum*-like Spanish isolates (UMAF F0927 and UMAF F0928) were grouped together in a cluster in the African Clade, closer to *Fusarium udum* in this first study (Figure 27).

Due to the close relationship showed between *F. tupiense* Spanish isolates and sequences of strains of *F. tupiense* from Brazil, a more complete phylogenetic analysis was performed

using DNA sequence data from portions of seven genes. This phylogenetic analysis was carried out with DNA sequences from four *F. tupiense* and one *F. phyllophilum*-like representative Spanish isolates, and also two *F. mangiferae* Spanish isolates were considered (see section 4.2.4.) (Table 3). Sequence data of several MMD-associated *Fusarium* spp. and other members of the GFSC were also included. Similar results to the first trial were obtained, nevertheless *F. phyllophilum*-like isolate (UMAF F0927) was grouped on a separate cluster in the African Clade as well, but closer to *Fusarium phyllophilum* than to *Fusarium udum* (Figure 28). *F. tupiense* Spanish isolates were grouped with *F. tupiense* isolates from Brazil in the American Clade (Figure 28) and *F. mangiferae* Spanish isolates nested in the Asian Clade, as it was described above (Figure 28).

4.4.5. Cross fertility assays

In this study, we also tested the fertility of the *F. tupiense* Spanish isolates, by crossing the *MAT*-1 isolate with the rest of the isolates *MAT*-2 (Table 5), and including as positive control the two *F. tupiense* female/ male fertile tester strains CLM1000 (*MAT*-1) and CLM1843 (*MAT*-2) from Brazil. In any of the crosses of *F. tupiense* Spanish isolates with the reference strains of *F. tupiense* from Brazil we found the formation of perithecia. This most likely was as result of inappropriate cross conditions, impossible to solve after several unsuccessful attempts.

DISCUSSION

DISCUSSION

Mango malformation is the most important disease affecting mango trees (*Mangifera indica*), causing severe economic losses because of the reduction of productivity. Mango malformation disease (MMD) has been reported in nearly all areas worldwide where mango is cultivated (Marasas *et al*., 2006; Youssef *et al*., 2007; Kvas *et al*., 2008; Zhan *et al*., 2010; Kumar *et al*., 2011)), and in the present work is described for the first time in Spain (Crespo *et al*., 2012).

After four years of surveys carried out in this study, we confirm the presence of MMD in the Axarquía region, showing a relevant dispersion and affecting a great number of mango cultivars. Control of the disease is possible, but time-consuming. Cultural management has been the alternative most effective alternative (Narasimhan, 1959; Singh *et al*., 1974; Manicon, 1989). Unfortunately, pruning to manage malformation is not practical for all producers, some of whom are unable, or unwilling, to make the effort that is required to ensure that this approach succeeds. In spite of control measurements carried out in some of the visited orchards affected with MMD in the Axarquía region, the disease was very difficult to control, and only in two orchards was it totally eradicated. These results showed that cleaning labors of affected panicles and shoot should be done with a higher frequency and intensity.

Several species of *Fusarium* had been associated with this disease. The genus *Fusarium* includes many species that cause

plant diseases affecting the vascular system, roots and bulbs. The genus is taxonomically complex and accurate identification requires a suite of different morphological, biological and phylogenetic markers (Summerell & Leslie, 2011). *Fusarium* species associated with MMD in different areas of the world include *Fusarium mangiferae*, *F. sterilihyphosum*, *F. proliferatum*, *F. mexicanum*, and *F. tupiense*, all of them included in the *Gibberella fujikuroi* species complex (GFSP). Two of these species have been found associated with the disease in Southern Spain; *F. mangiferae* occurs in most of the production areas of the world, and is firmly established as one of the causal agents of mango malformation disease (MMD) (Freeman *et al.,* 1999; Ploetz, 2001), and *F. tupiense*, the latest novel species described in Brazil associated with this disease (Lima *et al*., 2009b), and have also been reported in Senegal (Senghor *et al.,* 2012). A third *Fusarium* species close to *Fusarium phyllophilum* and not previously associated with MMD, have also proved to cause MMD for the first time in South of Spain.

Morphological characteristics were studied in 134 isolates obtained from mango malformed tissues in the Axarquía region. *F. mangiferae, F. sterilihyphosum*, *F. mexicanum*, and *F. tupiense* all produce microconidia in false heads from mono and polyphialides; on the other hand *F. proliferatum* is the only *Fusarium* species associated with MMD that produces microconidia in chains. This morphological feature allowed us to exclude *F. proliferatum* as possible agent of the disease in Spain.

This early diagnosis was also confirmed afterwards by multilocus analysis. Nevertheless the morphological characteristics of *F. sterilihyphosum*, *F. mexicanum*, and *F. tupiense* are very alike, and by the time this study was carried out, *F. tupiense* was not formerly described as a species yet. Therefore other biological, genetic and phylogenetic markers were considered. Regarding the morphological characteristics of the *F. tupiense* Spanish isolates; a more detailed morphological study carried out in cooperation with Dr. T. Aoki (Laboratory Genetic Resources Center, National Institute of Agrobiological Sciences, Japan) (Annex III), allowed us to confirm conclusively the initial morphological diagnosis of the *F. tupiense* Spanish isolates. Three *Fusarium* sp. Spanish isolates (UMAF F0927, UMAF F0928 and UMAF F1062) that showed atypical morphological characteristics, different of those described for the several *Fusarium* species associated with MMD, were initially identified as *Fusarium* spp. based on morphological characteristics only.

PCR primer pairs have been used to quickly diagnose some of the Fusaria associated with MMD. Zheng and Ploetz (2002) developed a species-specific primer pair, 1-3F/R, that amplifies a 608 pb DNA fragment for *F. mangiferae*. On the other hand, the primer pair 61-2F/R developed to diagnose specifically *Fusarium subglutinans* from maize (Möller *et al.*, 1999), turned out to be unspecific at the temperature used by Möller *et al.* (1999) and amplified multiple DNA fragments for some *Fusarium* species (Zheng & Ploetz 2002). When the amplification protocol was

modified, amplified a 445 pb DNA fragment of *F. sterilihyphosum* isolates from Brazil, but no amplification was obtained from the *F. mangiferae* isolates from mango (Zheng & Ploetz 2002). Rodriguez-Alvarado *et al.* (2007) using also the 61-2F/R primer pair amplified a 445 pb DNA fragment from *F. sterilihyphosum* and *F. mexicanum* (Otero-Colina *et al*. 2010). In our lab, 61-2F/R primer pair amplified a 445 pb DNA fragment for *F. sterilihyphosum*, *F. mexicanum,* and *F. tupiense* reference isolates indistinctly; therefore, this set of primers was useful in a previous screening to differentiate the isolates morphologically diagnosed as *F. tupiense* from the other *Fusarium* sp. and *F. mangiferae* Spanish isolates. For an accurate molecular differentiation and identification of *F. sterilihyphosum*, *F. mexicanum,* and *F. tupiense* isolates other methodologies as PCR techniques with specific primers or multilocus DNA sequencing is needed.

Regarding the initial diagnosis combining data from morphology and PCR with specific primers there was, in general, correlation among morphological features and PCR diagnosis. A remarkable exception was the isolate UMAF F0923, which amplified with the specific primers for *F. mangiferae* but its morphology was atypical, and also resulted nonpathogenic in mango inoculation assays.

Considering the pathogenicity of the *Fusarium* spp. associated with MMD in Spain, three different *Fusarium* species have been proved to be pathogenic on mango: *F. mangiferae*, *F. tupiense* and an undescribed *Fusarium* sp. (isolate UMAF F0927)

close to *Fusarium phyllophilum,* and referred in this work as *F. phyllophilum*-like after the consideration of additional molecular and biological techniques. This species has been described as causal agent of MMD by the first time in this work. The confluence of several *Fusarium* spp. causing MMD in one country had also been reported, which is the case of Egypt where three different *Fusarium* spp., *F. mangiferae*, *F. sterilihyphosum* and *F. proliferatum,* have been described as causal agents of the disease.

Genetic variation has not been well studied in populations of *Fusarium* species that cause MMD. Population diversity among *Fusarium* spp. isolates associated with MMD in Spain was determined by ap-PCR, RAPD-PCR, vegetative compatibility groups (VCGs), mating type and phylogenetic analyses.

The ap-PCR and RAPD-PCR analysis showed clear differences in the banding pattern among the *Fusarium* Spanish isolates, differentiating clearly the three different species. Regarding *F. mangiferae* Spanish isolates, intraspecific diversity was also detected with ap-PCR and RAPD-PCR analysis, resulting in the detection of two different genotypic patterns among the Spanish isolates, genotype 1 comprising the majority of the isolates, and genotype 2 grouping only four isolates collected in the same orchard but in two different years, and the reference isolates MRC7560 and EM50B from Israel and Egypt.

In contrast, in the case of *F. tupiense* Spanish isolates, they all showed an identical banding pattern among them with all of the primers tested, with the unique exception of the atypical isolate

UMAF F1182. This general profile resulted also identical to one of the *F. tupiense* isolates from Brazil (CLM386), and located in VCG I according to Lima *et al*., (2009a).

Zheng and Ploetz (2002) evaluated VCG diversity in populations of *F. mangiferae* and *F. sterilihyphosum*, and Lima *et al*., (2009a) also of *F. tupiense*. Results from the present study corroborated those of these authors who reported the existence of several different VCGs among isolates of the mango malformation pathogenic species. Zheng and Ploetz (2002) identified six VCG within *F. mangiferae.* In our study three VCGs were found among the *F. mangiferae* Spanish isolates, two of them have not been previously described, VCG 7 grouping the majority of isolates, and VCG 8 grouping four isolates which originated from the same orchard, and also separated according to their unique ap-PCR profile (genotype 2). The third VCG grouping four Spanish isolates, including the atypical isolate UMAF F0923, from the same orchard with isolates from South Africa in VCG 5, previously described by Zheng and Ploetz (2002). The isolate UMAF F0923 identified by PCR as *F. mangiferae* but showing atypical morphological features and being nonpathogenic on mango, did show vegetative compatibility with the *F. mangiferae* VCG 5 reference strains, confirming its proposed identification as *F. mangiferae* by PCR, in spite of its atypical morphology and absence of pathogenicity, which demonstrates the idiosyncrasy of this particular isolate.

None of the *F. mangiferae* Spanish isolates complemented with the tested *F. mangiferae* reference isolates from Egypt, USA and Israel. These results suggest that *F. mangiferae* Spanish populations are different from those of Egypt, USA and Israel tested. Nevertheless, *F. mangiferae* isolate MRC7560 from Israel was included with *F. mangiferae* isolates EM50B and EM43C from Egypt in VCG 2, according to Zheng & Ploetz (2002). Although these isolates showed close genetic similarity according to the ap-PCR markers with the Spanish isolates in VCG 8 (genotype 2), they were located in a distinct VCG (VCG 2). Similarly, Spanish isolates in VCG 5 shared a similar ap-PCR profile with the 30 *F. mangiferae* Spanish isolates of VCG 7 (genotype 1), but were located in different VCGs, VCG 5. Similar RAPD profiles in different VCGs were also reported by Zheng and Ploetz (2002) who recognized among isolates of seven different VCGs, only four different RAPD profiles. These results could suggest that in some cases VCG analysis can be more discriminatory than ap-PCR and RAPD techniques as was previously reported by Crespo *et al*., (2014).

As mentioned before, Lima *et al.,* (2009a) evaluated VCG diversity in populations of *F. mangiferae*, *F. sterilihyphosum* and *F. tupiense* from Brazil. These authors identified 6 VCGs within *F. tupiense* isolates from Brazil, and found a different AFLP banding pattern for each of them. In our study we also found genetic diversity in the ap-PCR and RAPD banding patterns of the Brazilian isolates, as well as Lima *et al.,* (2009a) reported using

AFLP techniques, but two of the Brazilian isolates located in different VCGs shared similar ap-PCR and RAPD profile. These results suggest that at least in this case AFLP seems to be more sensitive technique than ap-PCR and RAPD. All *F. tupiense* Spanish tested isolates were grouped in VCG I together with *F. tupiense* isolate (CLM386), and all shared identical ap-PCR and RAPD profile with this Brazilian isolate with the unique exception of the atypical isolate UMAF F1182, nevertheless this isolate also grouped in VCG I; perhaps this could be the result of DNA contamination. These results manifest the need to combine several molecular and genetic techniques as VCGs, and AFLP or ap-PCR for characterizing populations of fungal pathogens.

F. phyllophilum-like Spanish isolates UMAF F0927 and UMAF F0928, were located, as expected, in a single VCG (named as VCG D), and shared an unique and identical band profile with all the primers tested, which also support the initial presumptive diagnosis as *Fusarium* sp. isolates different from the *Fusarium* species currently associated with MMD.

Based on VCGs, ap-PCR and RAPD results, *F. mangiferae* isolates from Spain clearly constitute several separated populations, one more widespread, consisting of a majority of the isolates; and other two minor populations located in restricted orchards, that may have been introduced in Spain from different sources (Crespo *et al*., 2014). In the case of *F. tupiense* Spanish isolates, the great uniformity in ap-PCR and RAPD profile supports the hypothesis that population of these pathogen

reproduce clonally. This limited variation could be due to a recent introduction of this species into the country, and also suggest that *F. tupiense* Spanish population has a clonal origin and reproduce primarily asexually; but this conclusion is based on a small population which could have been introduced only once to a limited geographic region.

Isolates possessing either *MAT*-1 or *MAT*-2 idiomorph were identified among the isolates of *F. mangiferae* and *F. tupiense* in the present study. Only three of the 134 Spanish isolates, two *F. mangiferae* and one *F. tupiense,* were identified as *MAT*-1, which also reflects the low diversity in the studied populations of these pathogens in Spain.

The VCGs results provide some clues to how this pathogen may have been spread internationally. Following the speculations made by Zheng and Ploetz (2002) suggesting the movement of isolates from Florida (USA), where many of the mango cultivars used commercially worldwide were developed, to Israel and South Africa; we can similarly speculate that *F. mangiferae* Spanish isolates in VCG 5 may have come from South Africa, but also possibly from USA or Israel, since from these countries at least one isolate was reported in VCG 5 (Zheng & Ploetz, 2002). The isolates from Israel and Egypt grouped in VCG 2 could have originated from Florida, as several Florida cultivars are grown in Israel. According to Zheng & Ploetz (2002), the greatest VCGs diversity was found in Egypt and USA. In the case of Egyptian isolates this possible origin is not so clear, as most of the cultivars

grown in this country are polyembryonic types selected in that country, and they appear to be land races different from those cultivated commercially in Israel, Florida and India.

On the other hand, the *F. tupiense* Brazilian isolates in VCG I are the most widespread group in Brazil, found in seven different collection sites of four different Brazilian States (Lima *et al.*, 2009a); although the origin of the *F. tupiense* Spanish population is unknown, their probable introduction most likely occurred through asymptomatic plant cuttings or seedling. No information about the genetic variability of the *F. tupiense* population recently described in Senegal has been reported, which could allow insight into the global epidemiology of this species. The origin of the *F. tupiense* Spanish population could be Brazil or Senegal. On the other hand, *F. phyllophilum*-like Spanish isolates pathogenic on mango could have also been introduced through asymptomatic plant cuttings or seedling; or might have jumped from a native host to mango, and in this case this pathogenic specie should have been more widespread. In any case the data we have is insufficient to establish further speculation. As Lima *et al.*, (2012) noted, the mango malformation pathosystem could be a good model for studying host jumping, because the disease occurs in different parts of the world and is apparently caused by locally adapted members of the GFSC.

Our study indicates at least five possible introductions of *Fusarium* spp. pathogenic on mango into the Axarquía region. All of the *Fusarium* isolates analyzed in the present work were

collected from a single and restricted geographic area, which had recently suffered the first incidence of MMD. It appears that exchange of nuclear material through sexual or parasexual recombination among isolates of the same VCG has still not occurred in Southern Spain where MMD occurs, and that these three species probably reproduce clonally. Conidia of the pathogen are dispersed by wind and may disseminate over distances of up to 35m over a limited time period according to epidemiological studies of the disease (Gamliel-Atinsky *et al.,* 2009b), and survival of conidia on the soil surface or when buried is limited (Youssef *et al.,* 2007). The pathogen is frequently spread by grafting and in infected nursery stock, and spread on a small scale is clearly evident in nurseries (Prakash & Srivastava, 1987). Thus, dissemination across large distances is most likely to occur via propagation material (Lima *et al.,* 2009a). Spatial distribution of the *Fusarium* genotypes in the Axarquía region also strengthens the hypothesis that in this area spread of the pathogen is most likely via propagation material. The exchange of propagation material amongst the different producing areas could also explain the widespread distribution as well as the presence of two different VCGs at a single location, which is the case of *F. mangiferae* VCG 7 and VCG 8. Thus, stronger sanitary measurements should be consider involving movement of propagation plant material into the country to avoid new introductions of primary inoculums, and through the nurseries stocks to prevent the spread of the pathogen in the region. As

mention, the pathogen is frequently spread by grafting and in infected nursery stock; in this context, heat treatments have been shown to kill pathogenic microorganisms (Buschaert *et al*., 1978). Freeman and Katan (1988) carried out experiments involving heating within a temperature range that caused a partial reduction of conidial and chlamidospore viability. Similarly, in our laboratory some preliminary assays involving heat treatment (40º-45ºC at different times, and up to three hours), of mango scions and *Fusarium* conidia suspensions have been carried out; unfortunately, these heat treatments did not sufficiently inactivate the fungal spores, but treatments at 45ºC or higher seriously affect the scion survival (data not shown). Nevertheless, additional work needs to be done in the development of strategies to control the fungi as thermotherapy, but at this stage, the alternative is generating propagating material free of the pathogen.

Considering the increase of global trade, and the reported presence in South of Spain of *F. mangiferae* (Crespo *et al*., 2014), *F. tupiense* and a third undescribed *Fusarium* sp. close to *F. phyllophilum* also pathogenic on mango, any plant material, plant cuttings or seedling, should go through strict quarantine to avoid additional introductions, because the control in the last years has been clearly ineffective.

CONCLUSIONS

6. CONCLUSIONS

1.- In this study we confirm the presence of MMD in Spain since the year 2006 or before, showing a considerable dispersion in the Axarquía region and affecting the majority of relevant cultivars.

2.- Isolates of three different *Fusarium* species have been proved to be causal agents of MMD in Spain: *F. mangiferae*, *F. tupiense* and a previously undescribed as pathogenic on mango *Fusarium* sp. phylogenetically close to *F. phillophylum*.

3.- After determination of population diversity based on several genetic and biological techniques, three different subpopulations were found among the *F. mangiferae* Spanish isolates, but only an homogeneous, and probably clonal population, was observed in *F. tupiense* isolates.

4.- Taking into account all of this data, there have been at least five different introductions of primary inoculum of *Fusarium* sp. pathogenic on mango into the Axarquía region.

5.- An additional *Fusarium* species (*F. phillophylum*-like) is proposed as a new causal agent of MMD.

CONCLUSIONES

CONCLUSIONES

1.- En este estudio se confirma la presencia de la malformación del mango en la Axarquía desde al menos 2006, alcanzando un importante grado de dispersión y afectando a la mayoría de cultivares relevantes en la zona.

2.- En este estudio se ha demostrado la implicación de tres especies diferentes de *Fusarium* como agentes causales de la MMD en España: *F. mangiferae*, *F. tupiense* y una nueva especie hasta ahora no descrita como patógena de mango y próxima filogenéticamente a *F. phillophylum*.

3.- Tras el estudio de la diversidad poblacional de los aislados de *Fusarium* de la Axarquía mediante varias técnicas genéticas y biológicas, se han observado tres subpoblaciones diferentes entre los aislados de *F. mangiferae*, mientras que la población de los aislados de *F. tupiense* ha resultado ser homogénea y posiblemente clonal.

4.- Teniendo en cuenta los resultados obtenidos, se deduce que se han producido al menos cinco entradas diferentes de inoculo primario de este patógeno de mango en España.

5.- Se propone una nueva especie de *Fusarium* (*F. phillophylum*-like) como agente causal de la malformación del mango.

REFERENCES

7. REFERENCES

Britz, H., Steenkamp, E.T, Coutinho, T.A. (2002) Two new species of *Fusarium* section *Liseola* associated with mango malformation. Mycologia 94: 722-730

Buschaert, S.C., Good, R.C., Szabocsik, J.M. (1978) Evaluation of thermal disinfection procedures for hydrophilic contact lenses. Applied and Environmental Microbiology 35: 618-621

Butler, E.E. & Mann, M.P. (1959) Use of cellophane tape for mounting and photographing phytopathogenic fungi. Phytopathology 49: 231-232

Castellani, A. *(*1939*)* The viability of some pathogenic fungi in sterile distilled water. Journal of Tropical Medicine and Hygiene 42**:** 225-226

Cazorla, F.M., Torés, J.A., Olalla, L., Pérez-García, A., Farré, J.M. y De Vicente, A. (1998) Bacterial apical necrosis of mango in Southern Spain: a disease caused by *Pseudomonas syringae* pv syringae. Phytopathology 88: 614-620

Cazorla, F.M., Arrebola, E., Olea, F., Velasco, L., Hermoso, J.M., et al. (2006) Field evaluation of treatments for control of the bacterial apical necrosis of mango (*Mangifera indica*) caused by *Pseudomonas syringae* pv syringae. European Journal of Plant Pathology 116: 279-288

Correll, J.C., Klittich, C.J.R., Leslie, J.F. (1987) Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. Phytopathology 77: 1640-1646

Covert, S.F., Briley, A., Wallace, M.M., McKinney, T. (1999) Partial *MAT*-2 gene structure and influence of temperature on mating success in *Gibberella circinata.* Fungal Genetics and Biology 28: 43-54

Crespo, M., Cazorla, F.M., Hermoso, J.M., Guirado, E., Maymon, M, Torés, J.A., Freeman, S., de Vicente, A. (2012) First report of mango malformation disease caused by *Fusarium mangiferae* in Spain. Plant Disease 96: 286-287

Crespo, M., Arrebola, E., Cazorla, F.M., Maymon, M., Freeman, S., Torés, J.A., de Vicente, A. (2014) Characterization of *Fusarium mangiferae* isolates from mango malformation disease in South of Spain. European Journal of Plant Pathology 139: 247-253

Crespo, V. (1995) Dermatomicosis. Diagnóstico microbiológico. International Marketing and Communications, S. A. Madrid

Crespo, V., Delgado, V., Martínez, S. (2006) Micología dermatológica. Ed. M.R.A. Barcelona.

Dang, J.K. & Daulta, B. S. (1892) Mango Malformation_ a review. Pesticides 16: 5-11

Díaz-Robledo, J. & Hermoso, J.M. (2009) Frutos Tropicales en la Costa Andaluza. Norma-Capitel S.L., Madrid, España

Dobinson, K.F., Patterson, N.A., White, G.J., Grant, S. (1998) DNA fingerprinting and vegetative compatibility analysis indicate multiple origins for *Verticillium dahliae* race 2 tomato isolates from Ontario, Canada. Mycological Research 102: 1089-1095

French, E.R. (1989) Taxonomía de las bacterias fitopatógenas. Fitopatología 24: 29-36

Ferrer, J. (1992) El mango en la Península Ibérica. Hortofruticultura 9: 62-63

Freeman, S. & Katan, J. (1988) Weakening effect on propagules of *Fusarium* by sublethal heating. Phytopathology 78: 1656

Freeman, S., Maymon, M., Biton, A., Levin, A.G., and Shtienberg, D (2014) Management of mango malformation disease based on a novel strategy of timing of fungicide applications combined with sanitation. Crop Protection 61: 84-91.

Freeman, S., Maymon, M., Pinkas, Y. (1999) Use of GUS transformants of *Fusarium subglutinans* for determining etiology of mango malformation disease. Phytopathology 89: 456-461

Gagnevin, L., & Pruvost, O. (2001) Epidemiology and control of mango bacterial black spot. Plant Disease 85: 928-935

Galán-Saúco, V. (2009) El cultivo del mango. 2º Ed. Mundi Prensa. Madrid

Gamliel-Atinsky, E., Freeman, S. Sztejnberg, A., Maymon, M., Ochoa, R., Belausov, E., Palevsky, E. (2009a) Interaction of the mite *Aceria mangiferae* with *Fusarium mangiferae*, the causal agent of mango malformation disease. Phytopathology 99: 152-159

Gamliel-Atinsky, E., Sztejnberg, A., Maymon, M., Shtienberg, D. Freeman, S. (2009b) Inoculum availability and conidial dispersal patterns of *Fusarium mangiferae* the casual agent of mango malformation disease. Phytopathology 99: 160-166

Gamliel-Atinsky, E., Sztejnberg, A., Maymon, M., Vintal, H., Shtienberg, D. Freeman, S. (2009c) Infection dynamics of *Fusarium mangiferae*, causal agent of mango malformation disease. Phytopathology 99: 775-778

Golzar, H. & Cother, E. (2008) First report of bacterial necrosis of mango caused by *Pseudomonas syringae* pv syringae in Australia. Australasian Plant Disease 3: 107-109

Gutiérrez-Barranquero, J.A. (2012) Unraveling the biology and control of bacterial apical necrosis (BAN) of mango. PhD Thesis

Gutiérrez-Barranquero, J.A., Cazorla F.M., Arrebola, E., Codina, J.C., Fernández-Galván, D., de Vicente, A. (2012a) La necrosis apical del mango en Canarias. ¿Una etiología alternativa? Boletín de la Sociedad Española de Fitopatología, 74: 43-50

Gutiérrez-Barranquero J.A., Arrebola, E., Bonilla, N., Sarmiento, D., Cazorla, F.M., de Vicente, A. (2012b) Environmentally friendly treatment alternatives to Bordeaux mixture for

controlling bacterial apical necrosis (BAN) of mango. Plant Pathology 61: 665-676

Haggag, W.M., El-Wahab, M.E.A. (2009) First report of *Fusarium sterilihyphosum* and *F. proliferatum* induced malformation disease of mango in Egypt. Journal of Plant Pathology 91, no. 1, p. 232

Hifni, H. A. A., El-Barkouki, M., El-Banna, G. S. (1978) Morphological and physical aspects of the floral malformation in mangoes. Egyptian Journal of Horticulture 5: 43-52

Ibar, L. (1986) El Mango, 149-162. In: Cultivo del Aguacate, Chirimoyo, Mango, Papaya. Aedos, Barcelona, España

Iqbal, Z., Dasti, A. A., Saleem, A. (2005) Selective growth media to study morphological and cultural characteristics of *Fusarium mangiferae*, the cause of mango malformation. In Malik et al. (Eds.) International Conference on Mango and Date Palm: Culture and Export. Faisalabad: University of Agriculture

Kerényi, Z., K. A. Zeller, L. Hornok, Leslie. J. F. (1999) Molecular standardization of mating type terminology in the *Gibberella fujikuroi* species complex. Applied and Environmental Microbiology 65: 4071-4076

Klittich, C.J.R., & Leslie. J.F. (1988) Nitrate reduction mutants of *Fusarium moniliforme* (*Gibberella fujikuroi*). Genetics 118: 417-423.

Kumar, J. & Beniwal, S.P.S. (1992) Mango Malformation. In: Kumar, J., Chaube, H.S., Singh, Mukhopadhyay AN (eds) Plant

diseases of international importance: diseases of fruit crops, vol 3 Prentice Hall. New Jersey, 357-393

Kumar, J., Singh, U.S., Beniwal, S.P.S. (1993) Mango Malformation: One Hundred Years of Research- Annual Review of Phytopathology 31: 217-232

Kvas, M., Steenkamp, E.T., Al Adawi A.O., Deadman, M.L., Al Jahwari, A.A., Marasas, W.F.O., Wingfield, B.D., Ploetz, R.C., Wingfield, M.J. (2008) *Fusarium mangiferae* associated with mango malformation in the Sultanate of Oman. European Journal of Plant Pathology 121: 195-199

Leslie, J.F., & Summerell, B.A. (2006) The Fusarium laboratory manual. Blackwell Publishing. USA

Lima C.S, Costa S.S, Campos M.A, Pfenning L.H, (2006). A new *Fusarium* species associated with mango malformation in Brazil. Fitopatologia Brasileira 31 (Suppl.) 191

Lima, C.S., Monteiro, J.H.A., Crespo, N.C., Costa, S.S., Leslie, J.F., Pfenning, L.H. (2009a) VCG and AFLP analyses identify the same groups in the causal agents of mango malformation in Brazil. European Journal of Plant Pathology 123: 17-26

Lima, C.S., Pfenning, L.H., Costa, S.S., Campos, M.A., Leslie, J.F. (2009b) A new lineage within the *Gibberella fujikuroi* species complex is the main causal agent of mango malformation in Brazil. Plant Pathology 58: 33-42

Lima, C.S., Pfenning, L.H., Costa, S.S., Abreu, L., Leslie, J.F. (2012) *Fusarium tupiense* sp. nov., a member of the *Gibberella fujikuroi* species complex that causes mango malformation in Brazil. Mycologia 104: 1408-1419

Litz, R.E. (1994) Mango, 33-34. In: Ploetz, R.C., Zentmyer, G.A., Nishijima, W.T., Rohrbach, K.R., Ohr, H.D., Eds. Compendium of Tropical Fruit Diseases. APS Press, St. Paul, Minnesota, USA

Marasas, W.F.O., Ploetz, R.C,. Wingfield, M.J., Wingfield, B.D. y Steenkamp, E.T. (2006) Mango Malformation Disease and the Associated *Fusarium* species. Symposium: *Fusarium*-Induced Diseases of Tropical Perennial Crops. Phytopathology 96: 667-672

Mohamed Nor, K.M.I., Salleh, B., Leslie, J.F. (2013) *Fusarium* species associated with mango malformation in Peninsular Malaysia. Phytopathology 161(9): 617-624

Möller, E.M., Chelkowski, J., Geiger, H.H., (1999) Species-specific PCR assays for the fungal pathogens *Fusarium moniliforme* and *Fusarium subglutinans* and their application to diagnose maize ear rot disease. Journal of Phytopathology 147: 497–508

Narasimhan, M. J. (1954) Malformation of panicles in mango incited by a species of *Eriophyes*. Current Science 23: 297-98

Narasimhan, M. J. (1959) Control of mango malformation disease. Curr. Sci., 28: 254-255

O'Donnell, K., & Cigelnik, E. (1997) Two different intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Molecular Phylogenetics and Evolution 7: 103-116

O'Donnell, K., Cigelnik, E., Nirenberg, H. I. (1998a) Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90: 465-493

O'Donnell, K., Nirenberg, H. I., Aoki, T., and Cigelnik, E. (2000) A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. Mycoscience 41: 61-78

O'Donnell, K., Sutton, D.A., Fothergill, A., McCarthy, D., Rinaldi, M.G., Brandt, M.E., Zhang, N., Geiser, D.M. (2008) Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. Journal of Clinical Microbiology 46: 2477-2490

O'Donnell, K., Humber, R.A., Geiser, D.M., Kang, S., Park, B., Robert, V.A., Crous, P.W., Johnston, P.R., Aoki, T., Rooney, A.P., Rehner, S.A. (2011) Phylogenetic diversity of insecticolous fusaria inferred from multilocus DNA sequence data and their molecular identification via FUSARIUM-ID and Fusarium MLST. Mycologia 104(2): 427-445.

Otero-Colina, G., Rodriguez-Alvarado, G., Fernández-Pavía, S., Maymon, M., Ploetz, R.C., Aoki, T., O´Donnell, K., Freeman, S. (2010) Identification and characterization of a novel etiological agent of mango malformation disease in Mexico *Fusarium mexicanum* sp. nov. Phytopathology 100: 1176-1184

Panizo, M.M., Reviakina, V., Montes, W. (2005) Mantenimiento y preservación de hongos en agua destilada y aceite mineral. Revista de la Sociedad Venezolana de Microbiología, 25: 35-40

Ploetz, R.C. (2001) Malformation: a unique and important disease of mango, *Mangifera indica* L. In B. A. Summerell, J. F. Leslie, D. Backhouse, W. L. Bryden and L. W. Burgess (eds), Fusarium: Paul E. Nelson Memorial Symposium (St Paul: APS Press), pp. 233-247

Ploetz, R.C. & N.F. Gregory (1993) Mango malformed in Florida: Distribution of *Fusarium subglutinans* in affected trees and relationship among strains within and among different orchards. Acta Horticulturae 341: 388-394.

Ploetz, R. C & Freeman, S. (2009) Foliar, Floral, and soil borne diseases. The mango. Botany, production and uses. 2º Edition. Ed Litz, R. E. CAB International, USA

Prakash, O. & Srivastava, K.C. (1987) Mango Diseases and their Management - A World Review. Tomorrow's Printer, New Delhi

Prasad, A., Singh, H., Shukla, T. N. (1965) Present status of mango malformation disease. Indian Journal of Horticulture. 22: 254-265

Rodríguez-Alvarado, G., Fernández-Pavía, S.P., Ploetz, R.C., y Valenzuela-Vázquez, M. (2007) A *Fusarium sp.* different from *Fusarium oxysporum* and *F. mangiferae* is associated with mango malformation in Michoacán, Mexico. New Disease Reports 16: 37

Rodríguez-Alvarado, G., Fernández-Pavía, S.P., Ploetz, R.C., Valenzuela-Vázquez, M. (2008) A *Fusarium* sp. different from *Fusarium oxysporum* and *F. mangiferae* is associated with mango malformation in Michoacán, Mexico. Plant Pathology 57: 781

Samson, J.A., (1986) Tropical fruits. Second edition. Logman Scientific and Technical, Essex (UK)

Senghor, A.L., Sharma, k., Kumar, P.L., Bandyopadhyay, R. (2012) First report of mango malformation disease caused by *Fusarium tupiense* in Senegal. Plant Disease 96 (10):1582

Singh, L. B., Singh, S. M., Nirvan, R. S. (1961) Studies on mango malformation: Review, symptoms, extent, intensity and cause. Hort. Adv. 5: 197-207

Singh, R.N., Majumder, P.K., Sharma, D.K., Sinha, G.C., Bose, P.C. (1974) Effect of de-blossoming on the productivity of mango. Scientia Horticulturae., 2: 399-403

Singh, Z., & Dhillon, B. S. (1989) Hormonal changes associated with vegetative malformation of mango (*Mangifera indica* L.) Journal of Phytopathology 125:193-197

Singh, Z., Singh, L., Alora, C.L., Dhillon, B.S. (1994) Effects of cobalt, cadmium, and nickel as inhibitors of ethylene biosynthesis on floral malformation, yield, and fruit quality of mango. Journal of Plant Nutrition 17: 1659-1670

Srivastava, A. K., Smucker, A. J. M., McBurney, S. L. (1982) An improved mechanical method for multiple soil-plant root studies. Transactions of the American Society of Agricultural Engineers (ASAE) 25: 868-71

Steenkamp, E.T., Wingfield, B.D., Coutinho, T.A., Zeller, K.A., Wingfield, M.J., Marasas, W.F.O., Leslie, J.F. (2000) PCR-based identification of *MAT*-1 and *MAT*-2 in the *Gibberella fujikuroi* species complex. Applied and Environmental Microbiology 66: 4378-4382

Summanwar, A.S., Raichaudhari, S.P., Pathak, S.C., (1966) Association of the fungus *Fusarium moniliforme* Sheld. with the malformation in mango (*Mangifera indica* L.) Indian Phytopathology 19: 227-228

Summerell, B.A., Baharuddin Salleh, Leslie, J.F. (2003) An utilitarian approach to *Fusarium* identification. Plant Disease 87: 117-127

Summerell, B.A. & Leslie, J.F. (2011) Fifty years of *Fusarium*: how were 9 species ever enough?. Fungal Diversity 50: 135-144.

Swofford, D.L. (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods), version 4.0b 10. Sinauer Associates, Sunderland, MA.

Yun S-H., Arie T., Kaneko I., Yoder O.C., Turgeon B.G. (2000) Molecular organization of mating type loci in heterothallic, homothallic and asexual *Gibberella/Fusarium* species. Fungal Genetics and Biology 31 (1): 7–20.

Youssef, S.A., Maymon, M., Zveibil, A., Klein-Gueta, D., Sztejnberg, A., Shalaby, A.A., Freeman, S. (2007) Epidemiological aspects of mango malformation disease caused by *Fusarium mangiferae* and source of infection in seedlings cultivated in orchards in Egypt. Plant Pathology 56: 257–263

Zhan, R-L., Yang, S-J., Ho, H-H., Liu, F., Zhao, Y-L., Chang, J-M, He, Y-B. (2010) Mango malformation disease in south China caused by *Fusarium proliferatum*. Journal of Phytopathology 158: 721-725

Zheng, Q., & Ploetz, R.C. (2002) Genetic diversity in the mango malformation pathogen and development of a PCR assay. Plant Pathology 51: 208-216

Zwickl, D.J. (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence data sets under the maximum likelihood criterion. Ph.D. dissertation, The University of Texas, Austin.

ANNEX I

ANNEX I.

Stain and growing media used on this study are listed on this annex.

Fresh Carnation Leaf Agar (Iqbal *et al.,* 2005)

Fresh Carnation leaves (*Dianthus caryophyllus* L.)	20 g
Agar agar	15 g
Distilled water	1 l

Basal Medium

Sucrose	30 g
KH_2PO_4	1 g
$MgSO_4$	0.5 g
KCL	0.5 g
$FeSO_4$	0.01 g
Agar	20 g
Trace element solution	0.2 ml
Distilled water	1 l

Trace element solution

Citric acid	5 g
$ZnSO_4\ 7H_2O$	5 g
$Fe\ (NH_4)_2\ (SO_4)_2\ 6H_2O$	1 g
$CuSO_4\ 5H_2O$	0.25 g
$MnSO_4\ H_2O$	50 mg
H_3BO_4	50 mg
$NaMoO_4\ 2H_2O$	50 mg
Distilled water	95 ml

Minimal medium (MM) or Nitrate medium

Basal Medium plus	0.5 g $NaNO_3$

Chlorate minimal medium (MMC)

MM plus	15 g $KClO_3$
	1.6 g L-asparagine

Nitrite medium

Basal medium plus	0.5 g $NaNO_2$

Hypoxanthine medium

Basal medium plus	0.2 g hypoxanthine

Ammonium medium

Basal medium plus	1 g ammonium tartrate

Carrot agar (Leslie & Summerell, 2006)

Fresh carrots	400 g
Agar agar	20 g
Distilled water	1 l

Complete medium

Sucrose	30 g
$NaNO_3$	2 g
N-Z Amine	2.5 g
Yeast extract	1 g
Vitamin stock solution	10 ml
Distilled water	1 l

Vitamin stock solution

Inositol	4 g
Ca pantothenate	200 mg
Choline Cl	200 mg
Thiamine	100 mg
Pyridoxine	75 mg
Nicotinamide	75 mg
Ascorbic acid	50 mg
Riboflavin	30 mg
p-aminobenzoic acid	5 mg
Folic acid	5 mg
Biotin	5 mg
50:50 ethanol: H_2O	

Lactophenol Cotton Blue (LPCB) (Crespo *et al.*, 2006)

Cotton blue	0.5 g
Lactic acid	20 g
Phenol	20 g
Glycerin	40 g
Distilled water	20 ml

ANNEX II

ANNEX II.

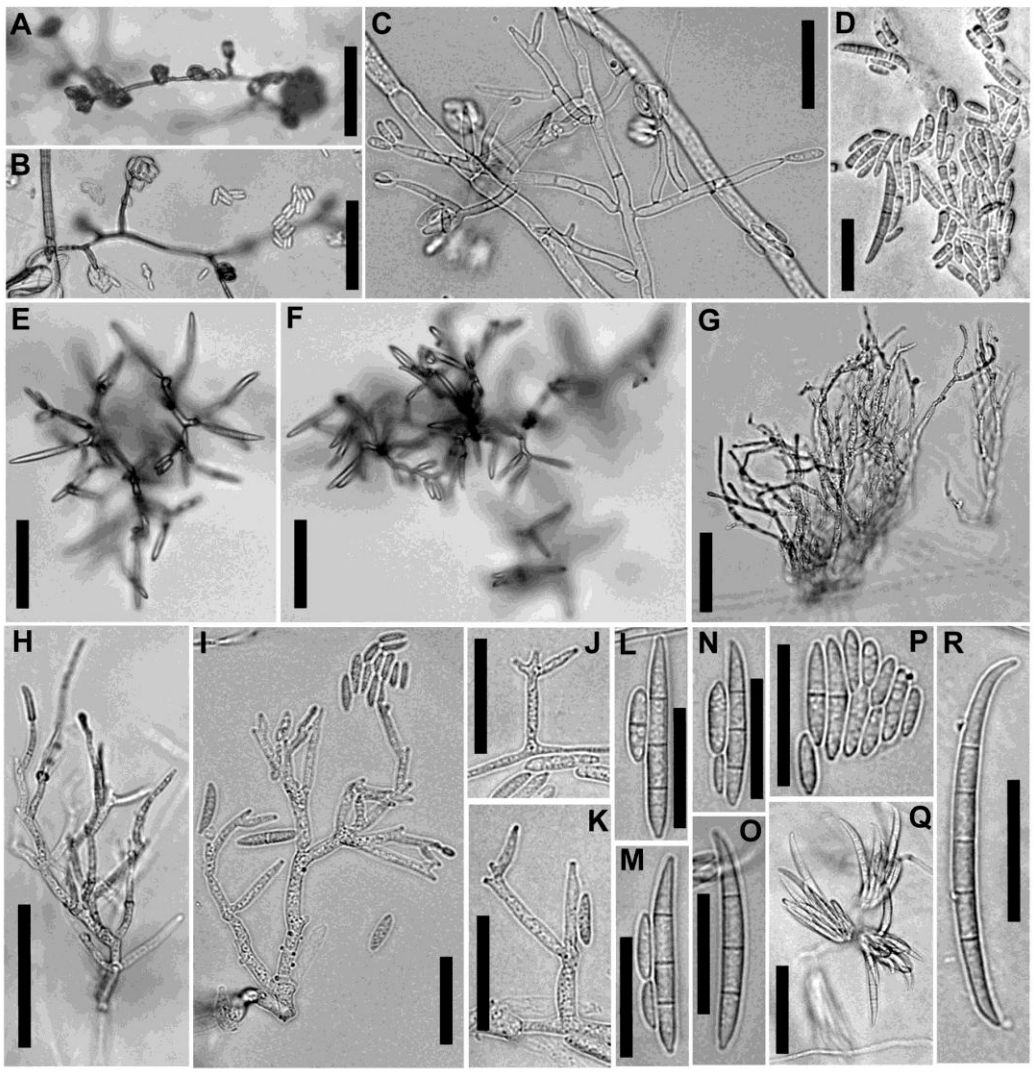
Table 8. Reference isolates of different *Fusarium* species used in phylogenetic analisys.

Species	Isolate[a]	Origin	Host
F. acutatum	NRRL 13308	India	unknown
F. circinatum	NRRL 25331	CA (USA)	*Pinus radiata*
F. concentricum	NRRL 25181	Costa Rica	*Musa sapientum*
F. fujikuroi	NRRL 13566	Taiwan	*Oryza sativa*
F. mangiferae	MRC 7560	Mexico	*Mangifera indica*
F. mangiferae	NRRL 25226	India	*Mangifera indica*
F. mexicanum	NRRL 47473	Mexico	*Mangifera indica*
F. mexicanum	NRRL 53147	Mexico	*Mangifera indica*
F. nygamai	NRRL 13448	Australia	*Sorghum bicolor*
F. oxysporum	NRRL 20433		
F. oxysporum	NRRL 22902	ID/(USA)	*Pseudotsuga menziesii*
F. phyllophilum	NRRL 13617	Italy	*Dracaena deremensis*
F. proliferatum	NRRL 22944	Germany	*Cattleya* hybrid
F. pseudocircinatum	NRRL 53573	Mexico	*Mangifera indica*
F. sacchari	NRRL 13999	India	*Saccharum officinarum*
Fusarium sp.	NRRL25195	Venezuela	wood
F. sterilihyphosum	CML 283	Brazil	*Mangifera indica*
F. sterilihyphosum	NRRL 22623	South Africa	*Mangifera indica*
F. subglutinans	NRRL 22016	IL (USA)	*Zea mays*
F. tupiense	CML 262	Brazil	*Mangifera indica*
F. tupiense	CML 345	Brazil	*Mangifera indica*
F. tupiense	CML 389	Brazil	*Mangifera indica*
F. udum	NRRL 22949	Germany	*Zea mays*
F. verticillioides	NRRL 22172	Germany	*Zea mays*

[a]Accession prefixes: CML = Coleção Micológica de Lavras (Universidade Federal de Lavras, Brazil); NRRL =Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA.

ANNEX III

ANNEX III.



Fusarium tupiense from Spain

A-D: on SNA in the dark;E-R: on SNA under black light

A, B, I-R from UMAF F1190, C, E from UMAF F1194, D, F from UMAF F0933, G, H from UMAF F0917.

Scale Bars; A, B, E-H, Q: 50 um, C, D, I-P, R: 25 um

ANNEX IV

La malformación del mango, una nueva enfermedad en España

M. Crespo, F.M. Cazorla, A. de Vicente (Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", Universidad de Málaga (IHSM-UMA-CSIC), Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Málaga).
E. Arrebola, J.M. Hermoso, E. Guirado, J. A. Torés (IHSM La Mayora-UMA-CSIC. Estación Experimental La Mayora, Consejo Superior de Investigaciones Científicas, Málaga).
S. Freeman (Department of Plant Pathology and Weed Research , ARO, The Volcani Center, Israel).

La malformación del mango es una de las enfermedades más importantes que afectan a este cultivo en todo el mundo causando importantes pérdidas económicas. Este trabajo pone de manifiesto de manera concluyente la presencia por primera vez de la malformación del mango en España y la determinación de su agente causal.

INTRODUCCIÓN

En la actualidad, el mango es el tercer fruto tropical en términos de producción y exportación a nivel mundial, inmediatamente después del plátano y la piña tropical. La distribución del cultivo del mango es amplia, abarcando países tropicales y subtropicales tanto del hemisferio Norte como del hemisferio Sur. En la península Ibérica, se estima que existen 3.500 ha plantadas, y el ritmo de plantación es elevado, 350-400 ha por año.

El mango (*Mangifera indica* L.) es un árbol originario de la región indobirmana, laderas del Himalaya y Sri Lanka, donde aún existen poblaciones silvestres y ha sido cultivado desde la antigüedad en la India (Galán- Saúco, 2009). La dispersión del mango fue muy rápida por el subcontinente de la India y el archipiélago malayo con la apertura del comercio entre Asia y Europa. Introducido en África Oriental por viajeros persas, los españoles lo introdujeron desde Filipinas a los puertos comerciales del continente americano hacia el siglo XVII. Los portugueses llevaron el mango al sur de África en el siglo XVI y a Brasil en 1700. Hacia la segunda mitad del siglo XVIII aproximadamente el mango fue introducido en la península Ibérica a través de las Islas Canarias. A finales del siglo pasado, el mango comenzó a extenderse comercialmente por Andalucía oriental, fundamentalmente en las costas de Málaga y Granada.

El mango es la especie de mayor importancia de la familia de las Anacardiáceas tanto por su amplia distribución geográfica como por el valor económico de su fruto (Galán-Saúco, 2009). Es un árbol siempre verde, sus hojas de color verde oscuro, configuran una copa redondeada y densa. Su robusto tronco, de corteza gruesa y áspera, junto con sus ramas alcanzan normalmente los 25 metros de altura pudiendo alcanzar en los trópicos hasta los 40 metros, aunque en áreas subtropicales como la región mediterránea su porte es mucho menor. Las hojas son alternas, dispuestas en verticilos, algo coriáceas y lacadas en superficie, de forma variable entre elíptica y lanceolada exhiben colores que van desde el amarillento al rojo vino junto con el verde oscuro. Usualmente el árbol florece por primera vez cuando alcanza aproximadamente los diez años, aunque las plantas reproducidas vegetativamente comienzan a florecer durante el primer año y a dar fruto a los cuatro o cinco. La inflorescencia del árbol es un tirso compuesto (Coetzer *y col.*, 1995) formado por un eje principal que porta varios ejes secundarios ramificados en ejes terciarios. La planta es monoica pero presenta en la misma panícula flores masculinas y hermafroditas (polígama). En plantaciones, el árbol del mango no se suele propagar directamente desde semilla para evitar el riesgo de que aparezcan variedades de baja calidad. La propagación se suele realizar mediante varios métodos de injerto, desde el tradicional sistema de injerto por aproximación utilizado en la India desde tiempo inmemorial hasta diversos tipos de injerto de yema o púa.

Debido a los requerimientos climáticos del mango, su cultivo en Andalucía queda restringido a la zona de costa, principalmente de Málaga y Granada en las que se cultivan principalmente las variedades Osteen, Keitt y Tommy Atkins. Estos cultivos se localizan en fincas de dimensiones y topografía variables (pudiéndose encontrar árboles pequeños en laderas de elevada pendiente). Los requerimientos hídricos de este cultivo son menores en comparación con otros frutales cultivados en la costa Andaluza como son el chirimoyo o el aguacate.

Entre las enfermedades que afectan al cultivo del mango en la zona de la Axarquía (Málaga), cabe destacar el oídio, una enfermedad de difusión mundial causada por el hongo *Oidium mangiferae.* Los síntomas de esta enfermedad aparecen en hojas, inflorescencias y frutos jóvenes. En los casos de ataques graves puede causar pérdidas de hasta el 90% (Galán-Saúco, 2009). Otra enfermedad que afecta al cultivo del mango en la Axarquía es la necrosis apical bacteriana, producida por *Pseudomonas syringae* pv. *syringae*. (Cazorla *y col.*, 1997; 1998). Esta bacteria afecta sobre todo a yemas terminales tanto vegetativas como florales, siendo un factor determinante para el desarrollo de la enfermedad las condiciones de baja temperatura y alta humedad. Una segunda enfermedad de origen fúngico que afecta al cultivo del mango en esta zona es la malformación del mango, una de las enfermedades más importantes que afectan a este cultivo a nivel mundial, causando importantes pérdidas económicas; y recientemente introducida en España, como se describe en este artículo.

La malformación del mango

Foto 1. Árbol de mango con síntomas de malformación vegetativa.

La malformación fue observada por primera vez en la India a finales del siglo XIX (Marasas *y col.,* 2006). Esta enfermedad se encuentra en la mayoría de los países productores del mundo tales como Egipto, Sudáfrica, Sudán, Bangladesh, Israel, Brasil, Méjico, EE UU, Malasia y Pakistán. La malformación afecta a brotes vegetativos y/o florales, dándose la primera sobre todo en plantas jóvenes y en plantas de vivero. En la malformación vegetativa la pérdida de dominancia apical conduce a que las yemas vegetativas axilares o apicales produzcan brotes deformes como muestran las Fotos 1 y 2, donde además se observa la reducción de los entrenudos y de la lámina foliar (Kumar *y col.*, 1993). Las hojas pueden enroscarse hacia abajo en dirección al tallo que las sostiene y generalmente son quebradizas.

En la malformación floral (síntoma más característico de la enfermedad) que muestran las Fotos 4 y 5 en comparación a las panículas de mango sanas que se observan en la Foto 3; las inflorescencias presentan una reducción en la longitud del eje primario y secundario, los cuales son más gruesos que los ejes normales, además pueden ser muy ramificados, presentando un aspecto de racimo. En las flores puede ocurrir un aborto temprano o a veces un cambio de sexo con desplazamiento de flores hermafroditas a masculinas (Kumar *y col.*, 1993). Las inflorescencias afectadas, generalmente no producen fruto y cuando lo hacen los pierden prematuramente, con las consiguientes pérdidas económicas. Estas inflorescencias continúan su crecimiento hasta el final de la temporada de floración, se marchitan y aparecen como masas compactas de color negruzco que persisten hasta el año siguiente. Las inflorescencias marchitas constituyen una fuente importante de infección puesto que al secarse se fragmentan y caen sobre yemas subyacentes aumentando la probabilidad de contagio de la enfermedad a yemas sanas (Gamliel-Atinsky *y col.,* 2009).

Foto 2. Árbol de mango con síntomas avanzados de malformación vegetativa.

Etiología de la malformación del mango en España

Varias especies de *Fusarium* han sido asociadas con la enfermedad incluyendo *Fusarium mangiferae* descrita en India, Israel, Florida (EE UU), Egipto, Sudáfrica y Omán; *Fusarium sterilihyphosum* en Sudáfrica y Brasil; *Fusarium proliferatum* en Malasia y China; y recientemente, *Fusarium mexicanum* en México (Britz *y col.,* 2002; Freeman *y col.*, 1999; Marasas *y col.,* 2006; Otero-Colina *y col.,* 2010).

La aparición de los primeros síntomas de la malformación del mango en la costa andaluza, una enfermedad desconocida hasta el momento en España, es difícil de determinar. En la primavera de

Foto 3. Panículas florales de árbol de mango sanas.

Foto 4. Panículas florales de árbol de mango con síntomas de malformación floral.

Foto 5. Panículas florales de árbol de mango con síntomas de malformación floral y vegetativa.

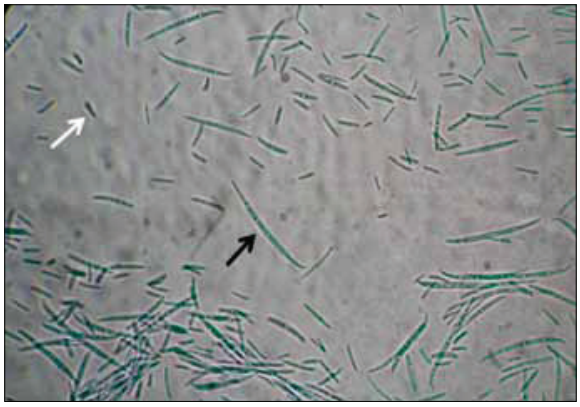


Foto 6. Microconidios (flecha blanca) y macroconidios (flecha negra) típicos de las especies de *Fusarium* en medio FCLA (20X).

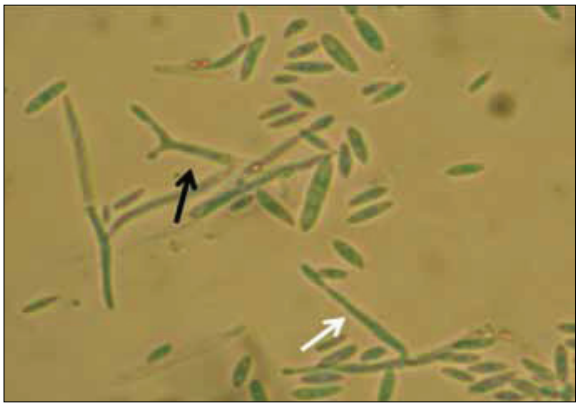


Foto 7. Monofiálides (flecha blanca) y polifiálides (flecha negra) de *Fusarium mangiferae* creciendo en PDA.

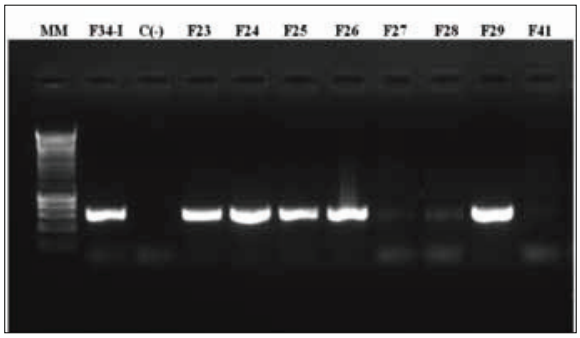


Foto 8. Amplificación por PCR de DNA de aislados de Fusarium sp. utilizando los cebadores específicos descritos para *Fusarium mangiferae*. Calle 1: control positivo, cepa de F. mangiferae 34-I. Calle 2: control negativo. Calles 3-9: aislados de Fusarium F23, F24, F25, F26, F27, F28, F29 y F41 respectivamente.

2006 se realizaron las primeras tomas de muestras en plantaciones comerciales de mango de la Axarquía con síntomas sospechosos, para determinar la presencia de esta enfermedad. Ante la creciente preocupación de los agricultores y técnicos que observan síntomas sospechosos de malformación en las plantaciones de mango, se desarrollan prospecciones de forma sistemática en 2009, 2010 y 2011 para confirmar la presencia de la malformación del mango en la Axarquía y determinar el agente causal de esta enfermedad. Dichas prospecciones se realizaron en fincas comerciales de mango abarcando diferentes términos municipales esta comarca (Velez Málaga, Benajarafe, Benamargosa, Almayate, Frigiliana, Benamocarra y Cútar). Los árboles muestreados pertenecen en su mayoría a las variedades cultivadas con mayor frecuencia en esta zona entre las que se encuentran Osteen, Keitt, Kent y Tommy Atkins; aunque también se tomaron en menor medida muestras de otras variedades minoritarias. Los muestreos se llevaron a cabo en un período de tiempo comprendido entre la segunda quincena de abril y finales de junio del mismo año; es decir, cuando el mango está en floración y presenta los síntomas más evidentes. Para la recogida de muestras fue necesario tomar medidas higiénicas preventivas para evitar la posible propagación del patógeno dentro de la misma finca o incluso a fincas adyacentes durante el muestreo, ya que pueden quedar las esporas adheridas a la ropa o al calzado. Para minimizar el riesgo de dispersión durante las prospecciones se utilizaron monos de trabajo desechables y guantes de látex; así como productos como la lejía y el alcohol de 70º para la desinfección de las herramientas de poda y de la suela del calzado.

A partir de las panículas florales o las yemas vegetativas que presentaban síntomas de malformación se han obtenido, hasta el momento, un total de 99 aislados fúngicos. Estos aislados monospóricos se cultivaron en medio patata dextrosa agar (PDA), y se identificaron en base a características morfológicas del hongo (Leslie y Summerell, 2006), como pertenecientes al género *Fusarium* mediante la observación al microscopio óptico de los conidios fusiformes típicos, que dan nombre al género como se observa en la Foto 6.

Para la identificación a nivel de especie de los aislados de *Fusarium*, se realizó un abordaje doble analizando en profundidad características morfológicas de los conidios y su modo de formación

y disposición en la célula conidiógena, así como un diagnóstico molecular basado en la técnica de la reacción en cadena de la polimerasa (PCR). Un examen detallado al microscopio óptico de los aislados monospóricos cultivados en PDA y agar hoja de clavel fresco (FCLA) reveló que una parte de éstos presentaba características morfológicas que concordaban con aquellas descritas para *F. mangiferae* como son la forma y el número de septos o tabiques de los conidios, y como muestra la Foto 7, el tipo de célula que los producen (mono y polifiálides), (Britz *y col.,* 2002).

La identificación definitiva de una parte de estos aislados como *F. mangiferae* se llevó a cabo mediante un análisis de PCR empleando unos cebadores específicos para esta especie que amplifican un fragmento de DNA de 608 pb (Zheng y Ploetz, 2002). El resultado fue la amplificación en aproximadamente la mitad de los aislados fúngicos del fragmento esperado de 608 pb que se visualizó en un gel de agarosa como muestra la Foto 8. Sin embargo, otra serie de aislados no mostraron esta reacción específica, aquellos aislados que no amplificaron en la PCR con los cebadores anteriormente mencionados quedaron diagnosticados por el momento como *Fusarium* sp. Estos resultados ponen de manifiesto la presencia de al menos dos especies de *Fusarium* que aparecen asociadas a los síntomas de malformación en la Axarquía, *F. mangiferae,* (Crespo *y col.,* 2012) y otra u otras especies aún pendientes de un diagnóstico específico concluyente.

Para confirmar la patogenicidad de las especies de *Fusarium* aisladas, asociadas a los síntomas de malformación floral y vegetativa en plantas de mango, se llevaron a cabo ensayos de inoculación artificial empleando árboles de mango sanos de la variedad Keitt de dos años. Se seleccionaron para este ensayo 3 aislados identificados como *F. mangiferae* y 5 aislados identificados como *Fusarium* sp. Como control positivo se utilizó una cepa control de *F. mangiferae*. Estos ensayos se llevaron

Foto 9. Síntomas de malformación floral en árboles de mango inoculados artificialmente.

a cabo en una cámara confinada para cultivo de plantas en la Estación Experimental "La Mayora", Consejo Superior de Investigaciones Científicas (CSIC) en Algarrobo–Costa en dos períodos de tiempo diferentes, marzo y noviembre de 2010. En ambos ensayos se obtuvieron los mismos resultados reproduciéndose en condiciones controladas síntomas similares a los observados en campo, como muestra la foto 9, para un total de 7 de los 8 aislados inoculados, tanto de *F. mangiferae* como de *Fusarium* sp.

En resumen, se ha confirmado la presencia de la malformación en las plantaciones de mango en la Axarquía, alcanzando un considerable grado de dispersión; y afectando a fincas de varios términos municipales y a diferentes variedades de cultivo. Se ha determinado la presencia en alrededor de la mitad de las muestras del principal agente causal de la enfermedad a nivel mundial, *F. mangiferae*. Además se ha puesto de manifiesto la presencia de al menos una segunda especie de *Fusarium* aún por identificar a nivel específico y que también está asociada a la malformación del mango en España. Es sabido que una vía muy importante de infección y propagación del hongo patógeno es a través del material de injerto, por lo que se deben extremar las medidas fitosanitarias en la producción de árboles de mango en viveros y en el manejo de las fincas afectadas.

Para evitar la dispersión del patógeno una vez se ha detectado su presencia en la finca, se recomienda cortar las ramas afectadas por debajo de la madera de dos años de edad. Las ramas cortadas se depositarán sobre un plástico del tipo invernadero extendido en el suelo. Sin que los montones alcancen el metro de altura se cubrirán con el mismo plástico donde quedarán expuestas al sol durante los meses de verano, alcanzando temperaturas superiores a los 36ºC. Transcurridos los meses de estío se retirarán las ramas dejándose que pudran sobre el suelo, y el plástico se guardará para el año siguiente. Para la desinfección de las herramientas utilizadas en las tareas de saneamiento se recomienda sumergirlas en lejía comercial diluida al 50% con agua, preferiblemente agua desionizada. Esta operación debe repetirse después de cada corte (Cazorla *y col*., 2009).

Por último, resaltar la importancia de no utilizar varetas o púas para el injerto, procedentes de fincas con síntomas de esta enfermedad o sospechosas de poseerla, puesto que ésta vía es la principal y más peligrosa, fuente de transmisión.

Agradecimientos: Este proyecto ha sido financiado por ayudas CICE-Junta de Andalucía, Proyecto de Excelencia P07-AGR-02471, cofinanciado con fondos FEDER (UE). Asimismo ha recibido ayudas de un convenio con SAT-2803 TROPS, Reyes Gutiérrez S.L. y Viveros Brokaw S.L. Los autores desean agradecer a Jose Mª Farré, Jorge González (CSIC) y David Sarmiento (TROPS) por su inestimable ayuda y su cooperación incondicional durante el desarrollo de este trabajo.

BIBLIOGRAFÍA

Britz, H., Steenkamp, E.T, Coutinho, T.A. 2002 *Two new species of* Fusarium *section Liseola associated with mango malformation.* Mycología 94: 722-730.

Cazorla, F.M., Torés, J.A., Olalla, L., Duran, V.E., De Vicente, A. 1997 *La necrosis apical del mango: una enfermedad causada por* Pseudomonas syringae *pv* syringae. PHYTOMA 86: 22-30.

Cazorla, F.M., Torés, J.A., Olalla, L., Pérez-García, A., Farré, J.M. y De Vicente, A. 1998 *Bacterial apical necrosis of mango in Southern Spain: a disease caused by* Pseudomonas syringae *pv* syringae. Phytopathology 88: 614-620.

Cazorla, F.M., Farré, J.M., González, J., Guirado, E., Hermoso, F J.M., Torés, J.A., De Vicente, A. 2009 *Malformación floral y vegetativa* (Fusarium mangiferae). *Nueva enfermedad del mango en el sur peninsular.* Folleto divulgativo editado por Caja Rural de Granada, 14 pp.

Coetzer, L.A., Oothuyse, S.A., Wishart, D.L. y Robbertse, P.J. 1995. *Influence of pruning on the flower sex ratio in some mango cultivars.* S. Afr. Mango Growers' Association Yearbook, 15: 27-30.

Crespo, M., C Cazorla, F.M., Hermoso, J.M., Guirado, E., Maymon, M., Tores, J.A., Freeman, S., De Vicente, A. 2012 *First report of mango malformation disease caused by* Fusarium mangiferae *in Spain.* Plant Disease 96 (2): 286

Freeman, S., Maimon, M., Pinkas, Y. 1999 *Use of GUS transformants of* Fusarium subglutinans *for determining etiology of mango malformation disease.* Phytopathology 89 (6): 456-461.

Galán-Saúco, V. 2009 *El cultivo del mango.* 2º Ed. Mundi Prensa. Madrid.

Gamliel-Atinsky, E., Sztejnberg, A., Maymon, M., Vintal, H., Shtienberg, D. y Freeman, S. 2009 *Infection dynamics of* Fusarium mangiferae, *causal agent of mango malformation disease.* Phytopathology, 99 (6):775-81.

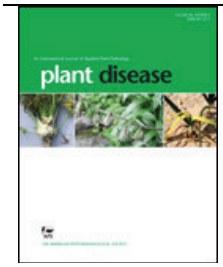
Kumar, J., Singh, U.S., y Beniwal, S.P.S. 1993 *Mango Malformation: One Hundred Years of Research.* Annual Review of Phytopathology. 31: 217-232.

Leslie, J.F., y Summerell, B.A. 2006 *The* Fusarium *laboratory manual.* Blackwell publishing. USA.

Marasas W.F.O, Ploetz R.C, Wingfield M.J, Wingfield B.D, Steenkamp E.T. 2006. *Mango malformation disease and the associated* Fusarium *species.* Phytopathology 96: 667–72.

Otero-Colina, G; Rodríguez-Alvarado, G; Fernández-Pavía, S; Maymon, M; Ploetz, R.C.; Aoki, T; O´Donnell, K; Freeman, S. 2010. *Identification and characterization of a novel etiological agent of mango malformation disease in Mexico,* Fusarium mexicanum *sp. nov.* Phytopathology 100 (11): 1176-1184.

Zheng, Q., y Ploetz, R.C. 2002. *Genetic diversity in the mango malformation pathogen and development of a PCR assay.* Plant Pathology. 51:208-216.



plant disease

Editor-in-Chief: R. Michael Davis
Published by The American Phytopathological Society

ISSN: 0191-2917

February 2012, Volume 96, Number 2
Page 286
http://dx.doi.org/10.1094/PDIS-07-11-0599

Disease Notes

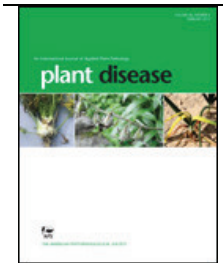
First Report of Mango Malformation Disease Caused by *Fusarium mangiferae* in Spain

M. Crespo and **F. M. Cazorla**, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", Universidad de Málaga (IHSM-UMA-CSIC), Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Campus Teatinos, 29071 Málaga, Spain; **J. M. Hermoso** and **E. Guirado**, IHSM La Mayora-UMA-CSIC, Estación Experimental La Mayora, Consejo Superior de Investigaciones Científicas, Algarrobo-costa 29750 Málaga, Spain; **M. Maymon**, Department of Plant Pathology and Weed Research, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel; **J. A. Torés**, IHSM La Mayora-UMA-CSIC, Estación Experimental La Mayora, Consejo Superior de Investigaciones Científicas, Algarrobo-costa 29750 Málaga, Spain; **S. Freeman**, Department of Plant Pathology and Weed Research, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel; and **A. de Vicente**, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", Universidad de Málaga (IHSM-UMA-CSIC), Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Campus Teatinos, 29071 Málaga, Spain. This work has been supported by grants from Proyecto de Excelencia (P07-AGR-02471), cofinanced by FEDER (EU), and "SAT-2803 TROPS"

Mango (*Mangifera indica* L.) malformation disease (MMD) is one of the most important diseases affecting this crop worldwide, which causes severe economic losses because of the reduction of productivity. Symptoms of MMD in Spain were observed for the first time in April of 2006 in three mango orchards in the Axarquia Region (southern Spain). Symptoms included an abnormal development of vegetative shoots with shortened internodes and dwarfed leaves and hypertrophied short and thickened panicles. In the years of 2006, 2009, and 2010, isolates of *Fusarium* were obtained from vegetative shoots and floral tissue of symptomatic mango trees from 21 different orchards of cvs. Keitt, Kent, Osteen, Tommy Atkins, and a variety of minor commercial cultivars, all showing typical symptoms of MMD. Different *Fusarium*-like strains were isolated from infected tissues. Colonies from single-spored isolates possessed dark purple-to-salmon-colored mycelium when grown on potato dextrose agar medium. On fresh carnation leaf agar medium, mycelium contained aerial conidiophores possessing three- to five-celled macroconidia and abundant microconidia in false heads from mono- and polyphialides; while cream-orange-colored sporodochia were produced on the surface of the medium, typical for *Fusarium mangiferae*. The identification of 37 isolates was confirmed as *F. mangiferae* by species-specific PCR analysis with the primer pair 1-3 F/R that amplified a 608-bp DNA fragment from all Spanish isolates as well as a representative Israeli control strain, Fus 34, also designated as MRC7560 (2). Pathogenicity using four representative isolates, UMAF F02, UMAF F10, UMAF F17, and UMAF F38 of *F. mangiferae* from Spain as well as isolate MRC7560, was

tested on 2-year-old healthy mango seedlings cv. Keitt by inoculating 15 buds from three different trees with a 20-µl conidial suspension (5×10^7 conidia per ml) per isolate (1). This experiment was conducted twice with two independent sets of plants and at different times (March and November 2010). Typical mango malformation symptoms were detected after bud break in March 2011, 5 and 12 months after inoculation. Symptoms were observed for 60% of the inoculated buds with the four *F. mangiferae* Spanish isolates and 75% with the MRC7560 control strain, but not with water-inoculated control plants. Recovered isolates from the infected floral and vegetative malformed buds were identical morphologically to those inoculated, and the specific 608-bp fragment described for *F. mangiferae* was amplified with specific-PCR, thus fulfilling Koch's postulates. To our knowledge, this is the first report of mango malformation disease caused by *F. mangiferae* in Spain and Europe.

References: (1) S. Freeman et al. Phytopathology 89:456, 1999. (2) Q. I. Zheng and R. C. Ploetz. Plant Pathol. 51:208, 2002.



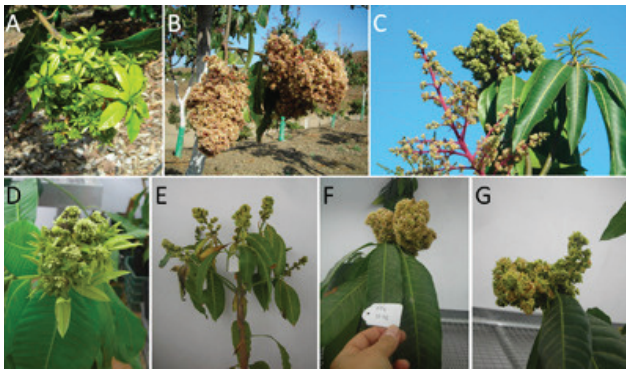
plant disease

Editor-in-Chief: R. Michael Davis
Published by The American Phytopathological Society

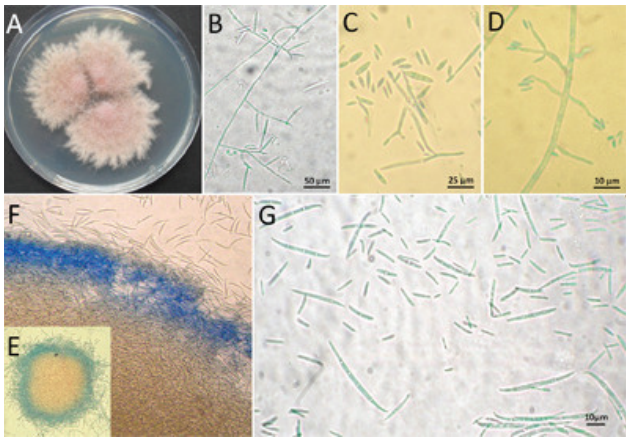
ISSN: 0191-2917

February 2012, Volume 96, Number 2
Page 286
http://dx.doi.org/10.1094/PDIS-07-11-0599

Supplemental Material



Symptoms of mango malformation disease caused by *Fusarium mangiferae* on mango trees from the south of Spain. Commercial orchards: **A,** vegetative malformation and **B to C,** floral malformation. Pathogenicity test on mango trees artificially inoculated with *F. mangiferae* (isolates UMAF F02, UMAF F10, UMAF F17, and UMAF F38): **D,** vegetative and floral malformation and **E to G,** floral malformation.



Macro- and microscopic characteristics of *Fusarium mangiferae* isolates (UMAF F02, UMAF F10, UMAF F17, and UMAF F38) from Spain. **A,** Seven-day-old colony of *F. mangiferae* on potato dextrose agar. **B,** Branched conidiophores bearing polyphialides. **C,** Mono-and poliphyalides. **D,** Microconidia in false heads. **E to F,** sporodochia. **G,** macroconidia.

Eur J Plant Pathol (2014) 139:247–253
DOI 10.1007/s10658-014-0398-5

Characterization of *Fusarium mangiferae* isolates from mango malformation disease in Southern Spain

M. Crespo · E. Arrebola · F. M. Cazorla · M. Maymon · S. Freeman · J. A. Torés · A. de Vicente

Accepted: 10 February 2014 / Published online: 27 February 2014

Abstract During the last years, *Fusarium* strains have been isolated from shoots and inflorescences of mango trees affected with floral and vegetative malformation in different orchards from the Axarquía region (south of Spain), highlighting the identification of *Fusarium mangiferae*. With the aim of elucidate epidemiological aspects and design more efficient control strategies, population diversity among the strains of *F. mangiferae* associated with MMD in Spain was determined by ap-PCR, RAPD-PCR, vegetative compatibility groups (VCGs) and mating type analyses. Three different VCGs were found among the *Fusarium mangiferae* Spanish isolates, two of them showing similar ap–PCR and RAPD profiles. PCR with primers specific for the mating type (*MAT*) alleles resulted in amplification of the *MAT-2* allele fragment among the majority of the isolates, there being only two isolates *MAT-1*. This population diversity suggests at least three possible independent introductions of the pathogen into the Axarquía region.

Keywords VCGs · ap-PCR · *Fusarium mangiferae* · Population structure

Electronic supplementary material The online version of this article (doi:10.1007/s10658-014-0398-5) contains supplementary material, which is available to authorized users.

M. Crespo · F. M. Cazorla · A. de Vicente (✉)
Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", Universidad de Málaga, Consejo Superior de Investigaciones Científicas, Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Campus Teatinos, 29071 Málaga, Spain
e-mail: adevicente@uma.es

E. Arrebola · J. A. Torés
Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", Universidad de Málaga, Consejo Superior de Investigaciones Científicas, Estación Experimental "La Mayora", Algarrobo-Costa, 29750 Málaga, Spain

M. Maymon · S. Freeman
Department of Plant Pathology and Weed Research, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

Mango (*Mangifera indica*) is cultivated throughout a wide range of frost-free climates and is one of the world's most important fruit crops (Litz 1998). One of the most serious diseases affecting mango worldwide is malformation (Ploetz 2001), which causes severe economic losses due to the reduction of productivity. *Fusarium mangiferae* occurs in most of the production areas of the world, and is firmly established as one of the causal agents of mango malformation disease (MMD) after completion of Koch's postulates and detailed cytological examinations of infected tissue (Freeman et al. 1999; Ploetz 2001; Iqbal et al. 2010). Genetic diversity was previously determined among *F. mangiferae* isolates from different origins where Zheng and Ploetz (2002) identified six different VCGs for *F. mangiferae*, and found additional heterogeneity for RAPD bands within some of the *F. mangiferae* VCGs.

Symptoms of MMD in Spain were observed for the first time in 2006 in the Axarquía Region (southern Spain), and *F. mangiferae* was determined as one of its causal agent (Crespo et al. 2012). Surveys were conducted during the years 2009 to 2012 in 35 different

orchards showing MMD symptoms, where *Fusarium* spp. were isolated. *F. mangiferae* was isolated and identified conclusively from nine of these different orchards at four different locations in the Axarquía region (Table 1).

Isolates were purified as single conidial subcultures and grown on Potato Dextrose Agar (PDA) and Fresh Carnation Leaf Agar (FCLA). The majority of these isolates possessed dark purple-to-salmon-coloured mycelium when grown on potato dextrose agar medium. On fresh carnation leaf agar medium, mycelium contained aerial conidiophores possessing three- to five-celled macroconidia and abundant microconidia in false heads from mono- and polyphialides; while cream-orange-coloured sporodochia were produced on the surface of the medium, typical for *F. mangiferae* (Iqbal et al. 2005; Leslie and Summerell 2006). From a collection of 43 isolates obtained from symptomatic mango plants in Spain, 38 *Fusarium* spp. isolates were diagnosed as *F. mangiferae* by a specific PCR assay with the primer pair 1–3F/R that amplified a 608-bp DNA fragment (Zheng and Ploetz 2002), as well as the reference strains MRC7560 and EM50B, in contrast with the other discarded isolates (Table 1; Fig. 1). One of these isolates was misidentified as *F. mangiferae* (UMAF F0923), due to its atypical morphology, and for not causing disease symptoms in mango inoculation assays was discarded from the collection.

Pathogenicity tests were carried out using seven representative isolates (UMAF F0910, UMAF F0920, UMAF F0924, UMAF F0925, UMAF F0938, UMAF F1174, and UMAF F1192) of *F. mangiferae* isolated from Spain, as well as the reference isolate MRC7560 from Israel (Table 1). Pathogenicity assays were performed on 1–2 year-old healthy mango seedlings cv. Keitt by inoculating five buds per isolate above mentioned with a 20-μl conidial suspension (5×10^7 conidia per ml) (Freeman et al. 1999). These experiments were conducted during the winter months of 2010 and 2011 and typical MMD symptoms were detected after bud break in March 2011 and 2012 respectively. Recovered isolates from the infected floral and vegetative malformed buds were identical morphologically to those inoculated, and the specific 608-bp fragment described for *F. mangiferae* was also amplified with specific-PCR in all of these isolates.

To determine the population variability of the Spanish *F. mangiferae* isolates, ap-PCR was performed on DNA extracted from 43 Spanish isolates of *Fusarium* spp., and six representative isolates from different *Fusarium* species causing MMD worldwide as reference: *F. mangiferae* from Israel (MRC7560), Egypt (EM50B) and Florida (CG-1-4); *F. sterilihyphosum* from South Africa (MRC2802); *F. mexicanum* from Mexico (GOC521); and *F. tupiense* from Brazil (NRRL 53984) (Table 1). Fungal DNA was extracted using DNeasy Plant Mini Kit following the manufacturer's instructions (Qiagen, USA). Analysis by ap-PCR of these isolates were conducted with three repeat motif primers- GACACGACACGACAC, CAGCAGCAGC AGCAG and GACAGACAGACAGACA, designated as $(GACAC)_3$, $(CAG)_5$ $(GACA)_4$, respectively. All PCR reactions were performed as previously described (Otero-Colina et al. 2010). Among the 49 tested isolates, identical banding patterns were observed in all of the 40 *F. mangiferae* isolates with primers $(GACAC)_3$ and $(CAG)_5$, a pattern which was clearly different from that of representative isolates of *F. sterilihyphosum*, *F. mexicanum* and *F. tupiense*, and the other Spanish isolates of *Fusarium* sp. (Supplementary Fig. 1). Different banding patterns were also observed for primer $(GACA)_4$ when comparing *F. mangiferae* isolates to those of the other tested *Fusarium* species isolates. However, additional differences were detected among some of the *F. mangiferae* isolates. Therefore, 37 *F. mangiferae* Spanish isolates, and three *F. mangiferae* control strains (MRC7560, EM50B and CG-1-4) (Table 1), were tested for intraspecific genetic diversity using the repeat motif primers $(GACA)_4$, and a ten base RAPD primer OPF13 (GGCTGCAGAA). With primer $(GACA)_4$, *F. mangiferae* Spanish isolates showed two different banding patterns (Fig. 2). Isolates UMAF F0924, UMAF F0926, UMAF F0939 and UMAF F1192, collected in the same orchard but in different years, and the reference isolates MRC7560 and EM50B from Israel and Egypt, showed an identical profile (genotype 2) (Fig. 2). Likewise, a second group containing a different profile was observed with the remaining 33 Spanish isolates (genotype 1) (Table 1; Fig. 2). The banding pattern of *F. mangiferae* isolate CG-1-4 (USA) was unique and different to the rest of the isolates included in this experiment (Fig. 2). Similar results were also obtained when using RAPD primer OPF13. No differences in the banding patterns were detected when using the $(CAG)_5$ primer. The ap-PCR and RAPD assays were performed at least twice for each isolate with each primer to ensure that amplification patterns were reproducible.

Table 1 Isolates of *Fusarium* species from mango (*Mangifera indica*) used in this study

Species	Isolate[a]	Origin/(reference- orchard)	Date	Cultivar[b]	Malformation[c]	VCG[d]	ap-PCR genotype[e]
Fusarium sp.	UMAF F0916	Spain (Vélez-Málaga-Lara)	2009	Osteen	Vegetative	nd	nd
Fusarium sp.	UMAF F0923[f]	Spain (Cútar-Botín)	2009	Tommy Atkins	Floral	nd	nd
Fusarium sp.	UMAF F0927	Spain (Vélez-Málaga-Tejares)	2009	Keitt	Floral	nd	nd
Fusarium sp.	UMAF F0931	Spain (Vélez-Málaga-Cortijo)	2009	Keitt	Vegetative	nd	nd
Fusarium sp.	UMAF F1045	Spain (Vélez-Málaga-Arroyo)	2010	Keitt	Vegetative	nd	nd
Fusarium sp.	UMAF F1058	Spain (Algarrobo-Melgares)	2010	Osteen	Floral	nd	nd
F. mangiferae	UMAF F0908	Spain (Benamargosa-Huerta)	2009	Keitt	Floral	VCG 7	1
F. mangiferae	UMAF F0909	Spain (Benamargosa-Huerta)	2009	Keitt	Vegetative	VCG 7	1
F. mangiferae	UMAF F0910	Spain (Benamargosa-Huerta)	2009	Kent	Floral	VCG 7	1
F. mangiferae	UMAF F0911	Spain (Benamargosa-Huerta)	2009	Keitt	Floral	VCG 7	1
F. mangiferae	UMAF F0912	Spain (Benamargosa-Huerta)	2009	Osteen	Floral	VCG 7	1
F. mangiferae	UMAF F0918	Spain(Benajarafe-Pintao)	2009	Osteen	Floral	nd	1
F. mangiferae	UMAF F0919	Spain(Benajarafe-Pintao)	2009	Osteen	Floral	VCG 7	1
F. mangiferae	UMAF F0920	Spain(Benajarafe-Pintao)	2009	Osteen	Floral	VCG 7	1
F. mangiferae	UMAF F0921	Spain(Benajarafe-Pintao)	2009	Osteen	Floral	nd	1
F. mangiferae	UMAF F0922	Spain(Benajarafe-Pintao)	2009	Osteen	Floral	nd	1
F. mangiferae	UMAF F0924	Spain (Vélez-Málaga-Potril)	2009	Keitt	Floral	VCG 8	2
F. mangiferae	UMAF F0925	Spain (Cútar-Botín)	2009	Dusheri	Floral	VCG 7	1
F. mangiferae	UMAF F0926	Spain (Vélez-Málaga-Potril)	2009	Keitt	Floral	VCG 8	2
F. mangiferae	UMAF F0929	Spain (Vélez-Málaga-Cabrera)	2009	Osteen	Floral	VCG 7	1
F. mangiferae	UMAF F0936	Spain (Benamargosa-Barranco)	2009	Keitt	Floral	VCG 7	1
F. mangiferae	UMAF F0937	Spain (Benamargosa-Barranco)	2009	Keitt	Floral	VCG 7	1
F. mangiferae	UMAF F0938	Spain (Benamargosa-Barranco II)	2009	Keitt	Floral	VCG 7	1
F. mangiferae	UMAF F0939	Spain (Vélez-Málaga-Potril)	2009	Keitt	Floral	VCG 8	2
F. mangiferae	UMAF F0940	Spain (Cútar-Botín)	2009	Otts	Floral	VCG 7	1
F. mangiferae	UMAF F1049	Spain (Benamargosa-Barranco)	2010	Osteen	Floral	VCG 7	1
F. mangiferae	UMAF F1064	Spain (Benamargosa-Arcas)	2010	Keitt	Floral	VCG 7	1
F. mangiferae	UMAF F1065	Spain (Benamargosa-Arcas)	2010	Keitt	Floral	VCG 7	1
F. mangiferae	UMAF F1174	Spain (Benamargosa-Huerta)	2011	Tommy Atkins	Floral	VCG 7	1
F. mangiferae	UMAF F1175	Spain (Benamargosa-Huerta)	2011	Tommy Atkins	Floral	VCG 7	1
F. mangiferae	UMAF F1176	Spain (Benamargosa-Huerta)	2011	Tommy Atkins	Floral	VCG 7	1
F. mangiferae	UMAF F1192	Spain (Vélez-Málaga-Potril)	2011	Keitt	Floral	VCG 8	2
F. mangiferae	UMAF F12108	Spain (Benamargosa-Huerta)	2012	Osteen	Floral	nd	1
F. mangiferae	UMAF F12109	Spain (Benamargosa-Huerta)	2012	Keitt	Vegetative	nd	1
F. mangiferae	UMAF F12110	Spain (Benamargosa-Huerta)	2012	Keitt	Vegetative	VCG 7	1
F. mangiferae	UMAF F12123[g]	Spain(Benajarafe-Pintao)	2012	Osteen	Floral	VCG 7	1
F. mangiferae	UMAF F12124	Spain(Benajarafe-Pintao)	2012	Osteen	Floral	VCG 7	1
F. mangiferae	UMAF F12125	Spain(Cútar-Botín)	2012	Tommy Atkins	Floral	VCG 5	1
F. mangiferae	UMAF F12126[g]	Spain(Cútar-Botín)	2012	Dusheri	Vegetative	VCG 5	1
F. mangiferae	UMAF F12127	Spain(Cútar-Botín)	2012	Dusheri	Vegetative	VCG 5	1
F. mangiferae	UMAF F12132	Spain (Vélez-Málaga-Lorca)	2012	Osteen	Floral	VCG 7	1
F. mangiferae	UMAF F12133	Spain (Vélez-Málaga-Lorca)	2012	Osteen	Floral	VCG 7	1
F. mangiferae	UMAF F12134	Spain (Vélez-Málaga-Lorca)	2012	Osteen	Floral	VCG 7	1
F. mangiferae	EM42C	Egypt/(Zheng and Ploetz 2002)	–	–	–	VCG 1	nd
F. mangiferae	X4707	Egypt/(Zheng and Ploetz 2002)	–	–	–	VCG 1	nd

Table 1 (continued)

Species	Isolate[a]	Origin/(reference- orchard)	Date	Cultivar[b]	Malformation[c]	VCG[d]	ap-PCR genotype[e]
F. mangiferae	EM50B	Egypt/(Zheng and Ploetz 2002)	–	–	–	VCG 2	2
F. mangiferae	EM43C	Egypt/(Zheng and Ploetz 2002)	–	–	–	VCG 2	nd
F. mangiferae	EM73C	Egypt/(Zheng and Ploetz 2002)	–	–	–	VCG 3	nd
F. mangiferae	EM44F	Egypt/(Zheng and Ploetz 2002)	–	–	–	VCG 3	nd
F. mangiferae	EM22B	Egypt/(Zheng and Ploetz 2002)	–	–	–	VCG 4	nd
F. mangiferae	EM32E	Egypt/(Zheng and Ploetz 2002)	–	–	–	VCG 4	nd
F. mangiferae	X3875-5	South Africa/(Zheng and Ploetz 2002)	–	–	–	VCG 5	nd
F. mangiferae	X3875-2	South Africa/(Zheng and Ploetz 2002)	–	–	–	VCG 5	nd
F. mangiferae	CG-1-4	USA/(Zheng and Ploetz 2002)	–	–	–	VCG 6	3
F. mangiferae	CG-2-7	USA/(Zheng and Ploetz 2002)	–	–	–	VCG 6	nd
F. mangiferae	MRC7560	Israel/(Volcani Center Collection)	–	Kent	Floral	VCG 2	2
F. mexicanum	GOC521 (NRRL 53580)	Mexico/(Otero-Colina et al. 2010)	–	Haden	Floral	–	nd
F. sterilihyphosum	MRC2802	South Africa/(Britz et al. 2002)	–	–	–	–	nd
F. tupiense	CML262 (NRRL 53984)	Brazil/(Lima et al. 2009)	–	–	–	–	nd

[a] *UMAF* University of Málaga, Spain; *MRC* Medical Research Council, Tygerberg, South Africa; *GOC* Gabriel Otero-Colina; *NRRL* (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois USA) designations as identified according to multilocus sequencing of IGS rDNA, histone H3, β-tubulin, *EF-1α*, and calmodulin genes; *CML* Coleção Micológica de Lavras, Universidade Federal de Lavras, Brazil

[b] Infected mango cultivar

[c] Malformation symptoms: either vegetative or floral malformation

[d] Vegetative compatibility groups 7 and 8 were determined in this study for the first time. Those from 1 to 6 were determined previously by Zheng and Ploetz (2002). *nd* not determined

[e] Genotypes were designated according to band patterns using three different repeat motif and one RAPD primer, containing identical band patterns. *nd* not determined

[f] Isolate UMAF F0923 diagnosed as *F. mangiferae* according to PCR, but non pathogenic according to Koch's postulates and showed morphological characteristics distinct from *F. mangiferae*

[g] Isolate's mating type identified as MAT-1 according to MAT gene amplification

Furthermore, we identified mating-type idiomorphs (*MAT-1* or *MAT-2*) for 37 *F. mangiferae* Spanish isolates (Kerényi et al. 1999; Steenkamp et al. 2000). PCR amplification of *MAT-1* and *MAT-2* were analyzed using the respective primer pairs GFmat1a and GFmat1b (forward 5′-GTTCATCAAAGGGCAAGCG-3′; reverse 5′-TAAGCGCCCTCTTAACGCCTTC-3′) and GFmat2c and GFmat2d (forward 5′-AGCGTCATTATTCGATCAAG-3′; reverse 5′-CTACGTTGAGAGCTGTACA-3′) (Steenkamp et al. 2000). Among the 37 Spanish isolates, two were identified as *MAT-1* (UMAF F12123 and UMAF F12126, Table 1), and 33 as *MAT-2*; however, isolates UMAF F12133 and UMAF F12134 did not amplified with the primer pairs described above.

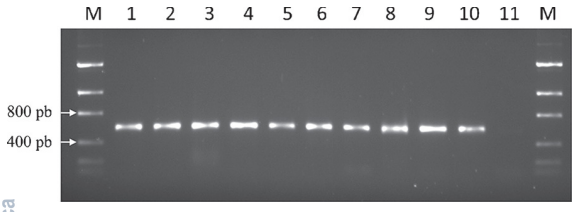


Fig. 1 PCR amplification of a 608-pb DNA fragment from *Fusarium* spp. isolates from mango malformation in Spain with the 1-3F/R primer pair. Lanes 1 to 7, *Fusarium mangiferae* Spanish isolates (F0910, F1174, F0938, F0924, F0926, F0939, F1192); lane 8, 9, and 10, reference isolates MRC7560, EM50B and CG-1-4 from Israel, Egypt and Florida, USA, respectively; lane 11 isolate UMAF F0916 of *Fusarium* sp. from Spain. M: Marker lanes are 100 pb ladders

Vegetative Compatibility Groups (VCGs) were evaluated with nearly all (34) of *F. mangiferae* Spanish isolates (Table 1, Fig. 3) and a collection of 12 reference isolates of *F. mangiferae* representative of the six VCGs previously described (Zheng and Ploetz 2002); also

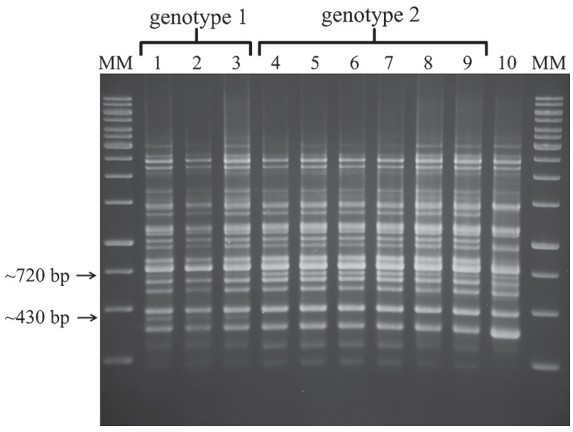


Fig. 2 Band patterns generated from ap-PCR analysis with $(GACA)_4$ repeat motif. Lanes 1 to 3, *F. mangiferae* representative Spanish isolates genotype 1 (F0910, F1174, F0938); lanes 4 to 7, *F. mangiferae* Spanish isolates genotype 2 (F0924, F0926, F0939, F1192); lane 8 isolate MRC7560 from Israel; lane 9 isolate EM50B from Egypt and line 10 isolate CG-1-4 from Florida. Outer marker lanes are 1Kb. Coincident band profile (genotype 1) was observed in the rest of the 30 *F. mangiferae* Spanish isolates (Table 1)

three additional control isolates were included; *F. mangiferae* (MRC7560) from Israel, *F. sterilihyphosum* (MRC2802) from South Africa, and *F. mexicanum* (GOC521) from Mexico (Table 1). For this purpose, nitrate-nonutilizing (*nit*) auxotrophic mutants were generated for all of the isolates mentioned above with the exception of the 12 *F. mangiferae*

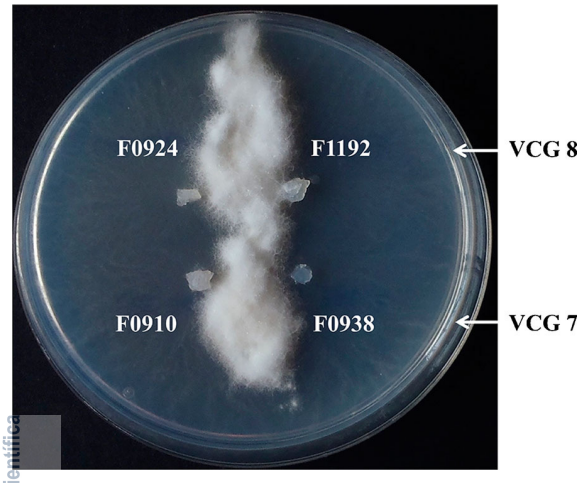


Fig. 3 Complementation test among four different *nit* mutants of four *Fusarium mangiferae* Spanish isolates growing on minimal medium containing $NaN0_3$ as the sole nitrogen source. Isolates F0924 and F1192 belonging to VCG 8 and isolates F0910 and F0938 belonging to VCG 7. Robust mycelial growth indicates complementation between isolates

reference isolates, kindly provided by Dr. R. Ploetz (University of Florida, USA), and utilized as described previously (Puhalla 1985; Correll et al. 1987). The initial level of potassium chlorate in the minimal medium for producing *nit* mutants (MM + $KCLO_3$) was 1.5 %, but when isolates failed to form mutants on this media, chlorate concentration increased to 3 %. Initially, 1.6 g l^{-1} of L-asparagine was added as an alternative nitrogen source, but in later experiments L-asparagine was replaced by 1.4 g l^{-1} of L-threonine. The change from L-asparagine to L-threonine resulted in an increase in the percentage of recovered NitM mutants (Klittich and Leslie 1988). VCGs were determined through the complementation of nitrate non-utilizing (*nit*) mutants as a visual indicator of heterokaryon formation (Fig. 3). Complementation tests were made on minimal medium by mycelial pairings from different phenotypic classes, *nit1*/NitM in Petri dishes (90× 15 mm). Inoculated plates were incubated at 25ºC (Leslie and Summerell 2006). Heterokaryon formation was evaluated after 3 to 7 days. All vegetative compatibility tests were conducted at least twice. Three VCGs were found among the 34 assayed Spanish isolates, VCG 7 grouping the majority of isolates,, VCG 8 grouping isolates UMAF F0924, UMAF F0926, UMAF F0936 and UMAF F1192, which originated from the same orchard and were collected in two different years, and also separated according to their unique ap-PCR profile (genotype 2) (Table 1, Figs. 2 and 3); and a third VCG grouping three Spanish isolates (UMAF F12125, UMAF 12126 and UMAF 12127) with isolates X3875-5and X3875-2 from South Africa in VCG 5, previously described by Zheng and Ploetz (2002). None of the 34 representative Spanish isolates complemented with the tested *F. mangiferae* reference isolates from Egypt, USA and Israel; nor with the isolates of *F. sterilihyphosum* or *F. mexicanum* included as negative controls. Therefore, they constituted two new described VCGs for *F. mangiferae*, designated here as VCG 7 and VCG 8. Nevertheless, *F. mangiferae* isolate MRC7560 was included with *F. mangiferae* isolates EM50B and EM43C in VCG 2, according to Zheng and Ploetz (2002). Although these isolates showed close genetic similarity according to the ap- PCR markers with the Spanish isolates UMAF F0924, UMAF F0926, UMAF F0939 and UMAF F1192 (VCG 8), they were located in a distinct VCG (VCG2) (Fig. 2). In the same manner, isolates UMAF F12125, UMAF F12126 and UMAF F12127 shared a similar ap-PCR profile with

the 30 *F. mangiferae* Spanish isolates of VCG 7 (genotype 1), but were located in a different VCG, VCG 5 (Table 1). This result suggests that the Spanish population of *F. mangiferae* is different from the populations of *F. mangiferae* tested from Egypt, USA and Israel. In recent studies, diversity among *F. mangiferae* isolates from different areas has also been revealed (Newman et al. 2012). Our study indicates at least three possible introductions of the pathogen into the Axarquía region. According to this work, in the south of Spain, three populations of *F. mangiferae* exist, one more widespread, consisting of a majority of the isolates; and other two minor populations located in restricted orchards. The two different *F. mangiferae* ap-PCR genotypes observed could not be the result of a mutation from a unique Spanish population, because isolates of both genotypes belong to different VCGs; and therefore, they clearly constitute separated populations that may have been introduced in Spain from different sources.

All of the *F. mangiferae* isolates analyzed in the present work were collected from a single and restricted geographic area, which had recently suffered the first incidence of MMD. It appears that exchange of nuclear material through sexual or parasexual recombination among isolates of the same VCG has still not occurred in the south of Spain where MMD occurs, and that these three populations probably reproduce clonally. Conidia of the pathogen are dispersed by wind and may disseminate over distances of up to 35 m over a limited time period according to recent epidemiological studies of the disease (Gamliel-Atinsky et al. 2009), and survival of conidia on the soil surface or when buried is limited (Youssef et al. 2007). The pathogen is frequently spread by grafting and in infected nursery stock, and spread on a small scale is clearly evident in nurseries (Prakash and Srivastava 1987). Thus, dissemination across large distances is most likely to occur via propagation material (Lima et al. 2009). Spatial distribution of the *F. mangiferae* genotypes in the Axarquía region also strengthens the hypothesis that, in this area, spread of the pathogen is most likely via propagation material; thus, stronger sanitary measurements should be consider involving movement of propagation plant material into the country to avoid new introductions of primary inoculums, and through the nurseries stocks to prevent the spread of the pathogen in the region.

Acknowledgments This work was granted by CICE-Junta de Andalucía, Proyecto de Excelencia P07-AGR-02471, co-funded by FEDER funds (EU). This work was also financed by a grant from SAT-2803 TROPS, Reyes Gutiérrez S. L. and Viveros Brokaw S. L. The authors particularly acknowledge for their contribution in the sampling works to J. M. Hermoso, E. Guirado, J. González, (IHSM La Mayora-UMA-CSIC) and D. Sarmiento, (SAT-2803 TROPS). Also we wish to thanks Dr. Ploetz (University of Florida, USA) for gifted the VCGs reference *F. mangiferae* isolates. M. Crespo also was supported by a grant from University of Málaga for a short stay in the Volcani Center, Bet Dagan, Israel.

References

Britz, H., Steenkamp, E. T., Coutinho, T. A., Wingfield, B. D., Marasas, W. F. O., & Wingfield, M. J. (2002). Two new species of *Fusarium* section *Liseola* associated with mango malformation. *Mycologia, 94*, 722–730.

Correll, J. C., Klittich, C. J. R., & Leslie, J. F. (1987). Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology, 77*, 1640–1646.

Crespo, M., Cazorla, F. M., Hermoso, J. M., Guirado, E., Maymon, M., Torés, J. A., Freeman, S., & de Vicente, A. (2012). First report of mango malformation disease caused by *Fusarium mangiferae* in Spain. *Plant Disease, 96*, 286–287.

Freeman, S., Maymon, M., & Pinkas, Y. (1999). Use of GUS transformants of *Fusarium subglutinans* for determining etiology of mango malformation disease. *Phytopathology, 89*, 456–461.

Gamliel-Atinsky, E., Sztejnberg, A., Maymon, M., Vintal, H., Shtienberg, D., & Freeman, S. (2009). Infection dynamics of *Fusarium mangiferae*, causal agent of mango malformation disease. *Phytopathology, 99*, 775–778.

Iqbal, Z., Dasti, A. A., & Saleem, A. (2005). Selective growth media to study morphological and cultural characteristics of *Fusarium mangiferae*, the cause of mango malformation. In Malik et al. (Eds.), *International Conference on Mango and Date Palm: Culture and Export*. Faisalabad: University of Agriculture.

Iqbal, Z., Hameed, S., Anjum, M. A., Dasti, A. A., & Saleem, A. (2010). Cytology of infection of *Fusarium mangiferae* Britz in different malformed reproductive parts of mango. *European Journal of Plant Pathology, 127*, 391–398.

Kerényi, Z., Zeller, K., Hornok, L., & Leslie, J. F. (1999). Molecular standardization of mating type terminology in the *Gibberella fujikuroi* species complex. *Applied and Environmental Microbiology, 65*, 4071–4076.

Klittich, C. J. R., & Leslie, J. F. (1988). Nitrate reduction mutants of *Fusarium moniliforme* (*Gibberella fujikuroi*). *Genetics, 118*, 417–423.

Leslie, J. F., & Summerell, B. A. (2006). *The Fusarium laboratory manual*. Ames: Blackwel Publishing.

Lima, C. S., Pfenning, L. H., Costa, S. S., Campos, M. A., & Leslie, J. F. (2009). A new *Fusarium* lineage within the *Gibberella fujikuroi* species complex is the main causal agent of mango malformation disease in Brazil. *Plant Pathology, 58*, 33–42.

Litz, R. E. (Ed.). (1998). *The mango botany production and uses* (p. 587). Wallingford: CABI.

Newman, Z., Freeman, S., Biton, I., Sa'ada, D., Paz, T., Maymon, M., & Lavi, U. (2012). Molecular diagnosis of mango malformation disease and phylogeny of *Fusarium mangiferae*. *Phytoparasitica, 40*(3), 287–297.

Otero-Colina, G., Rodríguez-Alvarado, G., Fernández-Pavía, S. P., Maymon, M., Ploetz, R. C., Aoki, T., O'Donnell, K., & Freeman, S. (2010). Identification and characterization of a novel etiological agent of mango malformation disease in Mexico, *Fusarium mexicanum* sp. nov. *Phytopathology, 100*, 1176–1184.

Ploetz, R. C. (2001). Malformation: A unique and important disease of mango. *Mangifera indica* L. In B. A. Summerell, J. F. Leslie, D. Backhouse, W. L. Bryden, & L. W. Burgess (Eds.), *Fusarium: Paul E. Nelson memorial symposium* (pp. 233–247). St Paul: APS Press.

Prakash, O., & Srivastava, K. C. (1987). *Mango diseases and their management—A world review* (p. 175). New Delhi: Today and Tomorrow Printer and Publishers.

Puhalla, J. E. (1985). Classification of strains of *Fusarium oxysporum* in the basis of vegetative compatibility. *Canadian Journal of Botany, 63*, 179–183.

Steenkamp, E. T., Wingfield, B. D., Coutinho, T. A., Zeller, K. A., Wingfield, M. J., Marasas, W. F. O., & Leslie, J. F. (2000). PCR-based identification of *MAT-1* and *MAT-2* in the *Gibberella fujikuroi* species complex. *Applied and Environmental Microbiology, 66*, 4378–4382.

Youssef, S. A., Maymon, M., Zveibil, A., Klein-Gueta, D., Sztejnberg, A., Shalaby, A. A., & Freeman, S. (2007). Epidemiological aspects of mango malformation disease caused by *Fusarium mangiferae* and source of infection in seedlings cultivated in orchards in Egypt. *Plant Pathology, 56*, 257–263.

Zheng, Q., & Ploetz, R. (2002). Genetic diversity in the mango malformation pathogen and development of a PCR assay. *Plant Pathology, 51*, 208–216.