Original article

Morphological and molecular characterization of *Cladosporium tenuissimum* Cooke (Deuteromycotina: Hyphomycetes) on mango tree panicles: symptoms, pathogenicity and severity of the fungus

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Morphological and molecular characterization of *Cladosporium tenuissimum* Cooke (Deuteromycotina: Hyphomycetes) on mango tree panicles: symptoms, pathogenicity and severity of the fungus.

Abstract — Introduction. Mango producers of two important growing areas of the states of Guerrero and Michoacán, Mexico, reported extensive damage in mango trees caused by an abundant mycelial growth covering most diseased panicles of mango trees cv. Haden. **Materials and methods**. The fungus was isolated from panicles of mango. Pathogenicity was evaluated in the orchard by inoculating 20 inflorescences protected by cellophane paper bags. *In vitro* characterization was evaluated on monosporic cultures; conidial morphology and rate of growth were determined. A molecular characterization by DNA extraction was carried out to identify the microorganism. **Results**. *Cladosporium tenuissimum* caused necrosis of flowers, pedicels and small fruits on inoculated mango panicles cv. Haden in the long coast of Guerrero and Michoacan States of Mexico. The affected organs were covered with grey cottony mycelia and an olivaceous, green to grey sporulation. *In vitro*, young colonies were also olive green and velvety, turning dark green to grey with a whitish outer margin. The growth rate of colonies was 0.46 cm·day^{-1}. Conidia were subspherical, lime-shaped and fusiform, olivaceous in color with visible scars and denticle-like extensions. The mean size of conidia was (5.85 × 2.93) µm with variations of 3.2–23 µm × 1.98–5.47 µm. The molecular characteristics rendered *C. tenuissimum* after identification. Symptoms of inoculated or naturally infected tree organs were similar. A diagrammatic scale was developed for the evaluation of disease severity, which varied from 69% to 100% in infected panicles. The organs were susceptible from blooming to fruit set. **Discussion**. Further research is proposed to evaluate the geographical distribution of *C. tenuissimum* in the production areas of mango with the concomitant evaluations of strategies of control.

Mexico / *Mangifera indica* / plant diseases / *Cladosporium tenuissimum* / identification / symptoms

Caractérisation morphologique et moléculaire de *Cladosporium tenuissimum* Cooke (Deuteromycotina: Hyphomycètes) sur panicules de manguier : symptômes, pathogénicité et sévérité du champignon.

Résumé — Introduction. Les producteurs de mangues de deux importantes régions productrices du Mexique (États de Guerrero et de Michoacán) ont signalé la présence d'importants dommages en vergers, provoqués par une abondante croissance mycélienne couvrant la plupart des panicules malades de manguiers du cv. Haden. **Matériel et méthodes**. Le champignon a été isolé sur des panicules de manguier. La pathogénicité a été évaluée en verger en inoculant 20 inflorescences protégées par des sacs en papier de cellophane. La caractérisation *in vitro* a été évaluée sur des cultures monospores ; la morphologie et le taux de croissance des conidies ont été déterminés. Une caractérisation moléculaire par extraction d'ADN a été effectuée pour l'identification des micro-organismes. **Résultats**. *Cladosporium tenuissimum* Cooke a causé la nécrose des fleurs, des pédicèles et des petits fruits sur les panicules de manguiers du cv. Haden tout le long de la côte des états de Guerrero et de Michoacan. Les organes affectés ont été couverts de mycéliums cotonneux gris et d'une sporulation de vert olive à gris. Les jeunes colonies *in vitro* étaient également vert olive et veloutées, tournant au vert foncé et au gris avec une bordure blanchâtre. Le taux de croissance des colonies a été de 0,46 cm·jour^{-1}. Les conidies étaient plus où moins sphériques, en forme de lime ou fusiformes, de couleur olivacée avec des cicatrices bien visibles et des prolongements denticulés. Leur taille moyenne a été de (5,85 × 2,93) µm, avec des variations de 3,2–23 µm × 1,98–5,47 µm. La caractérisation moléculaire a permis d'identifier plus précisément *C. tenuissimum*. Les symptômes sur organes inoculés ou sur arbres naturellement infectés ont été semblables. Une échelle schématique a été développée pour évaluer la sévérité de la maladie qui a varié de 69 % à 100 % pour les panicules infectées. Les organes ont été sensibles à partir des stages de croissance et de floraison jusqu'à la formation des fruits. **Discussion**. Davantage de recherches sont suggérées pour évaluer la répartition géographique de *C. tenuissimum* dans les régions productrices de mangues au Mexique et pour évaluer simultanément des stratégies de contrôle.

Mexique / *Mangifera indica* / maladie des plantes / *Cladosporium tenuissimum* / identification / symptôme

1. Introduction

Mango (*Mangifera indica* L.) is an important fruit from the tropical and subtropical areas of Mexico because it is a leading exporter of fresh mangos, shipped mostly to the United States. Today mango production is estimated to be 194 540 t and valued at US$ 123 M. The highest mango production areas are in the states of Veracruz, Michoacán, Nayarit, Guerrero and Sinaloa [1]. Nevertheless, pests and diseases are important factors restricting mango production. The most important diseases registered until now in Mexico are: mango floral malformation (*Fusarium oxysporum* Schlecht and *F. subglutinans* Wollemweb & Reinking), anthracnose (*Colletotrichum gloeosporioides* Penz), stem-end rots (*Lasiodiplodia* sp., *Dothiorella* sp., *Pestalotiopsis* sp., *Nattrasia* sp. and *Cytosphaera* sp.), powdery mildew (*Oidium mangiferae*, Berthet), Texas root rot (*Phymatotrichopsis omnivorum* Shear), mango scab (*Elsinoe mangiferae* Bitancourt & Jenk.) and sooty mould (*Capnodium* sp., *Fumago* sp., *Tripospermum* sp.) [2–4].

Cladosporium tenuissimum has been reported as a pathogen of dry rot disease and blossom end of tomato fruits in India and Nigeria [5–7]; of leaf spot of banana in Almeria, a district of India [8]; of leaf blight and fruit rot in watermelons [9] and, more recently, it has been detected as a serious problem in various cultivars of cucumbers in Israel [10]. Some investigators have reported that certain species of *Cladosporium* are harmful, causing health problems in the indoor environment [11].

Mango producers of two important growing areas of the states of Guerrero and Michoacán, Mexico, reported extensive damage in mango trees caused by abundant mycelial growth covering most panicles of the cultivar Haden. At the beginning, this fungal growth was attributed to *O. mangiferae*, but symptoms were different to those reported for this fungus [12] and more similar to those presented by *C. tenuissimum*. Therefore, the objectives of our research were to confirm the presence of *C. tenuissimum* by characterizing the symptoms of the fungus attack on mango panicles, to determine its etiology and to evaluate the disease severity to estimate its regional significance.

2. Materials and methods

2.1. Area of study

Our study was carried out in two locations: La Unión (Guerrero state) and Lázaro Cárdenas (Michoacán state). Isolations were obtained from eleven mango orchards, all of them of approximately 15- to 20-year-old cv. Haden trees.

2.2. Symptoms and fungus isolation

Field symptoms were described from five randomly selected panicles per tree, in five trees, showing different levels of disease severity but with similar phenological stages. The fungus was isolated from diseased panicles. Isolation was carried out on petri plates containing potato dextrose agar (PDA) and subculturing was done from the margins of a 2-day-old growing colony. Monosporic cultures were obtained by the streak plate technique from pure sporulating colonies of approximately 15 days old.

Under aseptic conditions, with the aid of a sterile needle, a small portion of mycelium was placed in 10 mL of sterile distilled water. The conidial suspension was poured on plates and smeared across the surface of the PDA. After 24 h, an individual colony was placed again on PDA plates and incubated at (20 ± 2) °C for 15 days. Conidia were flooded with 10 mL of sterile distilled water, scrapped with a sterile glass rod and filtered through cotton wool. The concentration of the conidial suspension was adjusted to 10^5 spores·mL^{-1}.

2.3. Pathogenicity

Pathogenicity was evaluated in the orchard over 20 vegetative inflorescences protected by bracts. Panicles were disinfested by spraying sodium hypochlorite at 2% concentration and distilled water. Spore suspension was profusely sprayed (manual

sprayer, vol. 1 L) on each panicle. The inoculated panicles were enclosed in white cellophane paper bags (size 30 cm × 30 cm) for 48 h to induce infection symptoms. Bags had a thickness of 20 µm and were placed over the panicles by slipping the slit of the bag over them. The opening of the bag was pleated shut and a short piece of twist tie was used to secure the bag firmly. Control panicles were sprayed with water only and bagged. Symptoms were observed every 24 h. During bagging, field conditions varied in a range of (25 to 30) °C and (65 to 75)% relative humidity (RH). Once fungal mycelia were observed, subcultures were carried out following the traditional procedures on PDA plates.

2.4. *In vitro* characterization and identification

The growth of colonies ($cm \cdot day^{-1}$) was evaluated in 16 monosporic cultures every 24 h for 16 days. Rate of growth and colony color were also evaluated. Length and width were measured in 1406 conidia with the aid of the computing program Image Tool (version 3.0). Conidial dimension was estimated from digital images obtained with a photomicroscope (Carl Zeiss) at 40×. Fungal identification was carried out with the aid of taxonomic keys [13, 14].

2.5. Molecular characterization

From the isolate (MHI-Mich), DNA was extracted based on the method of Ahrens and Seemüller [15]. The DNA quality was evaluated by electrophoresis in agar gel at 0.8% and quantified in a Perkin Elmer spectrophotometer. The ITS1 and ITS2 regions of rRNA genes were amplified by PCR (Polymerase Chain Reaction) with combinations of the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC).

The reaction per sample for a final volume of 25 µL was: 12.145 µL ionized sterile water, 4 µL DNA problem adjusted to 20 ng, 2.75 µL buffer IX; 1.375 µL $MgCl_2$ to 1.5 mM, 2.2 µL dNTPS mixture to 0.2 mM, 1.1 µL primers (for each) to 10 pM and 0.33 µL Taq polymerase to 1.5 units.

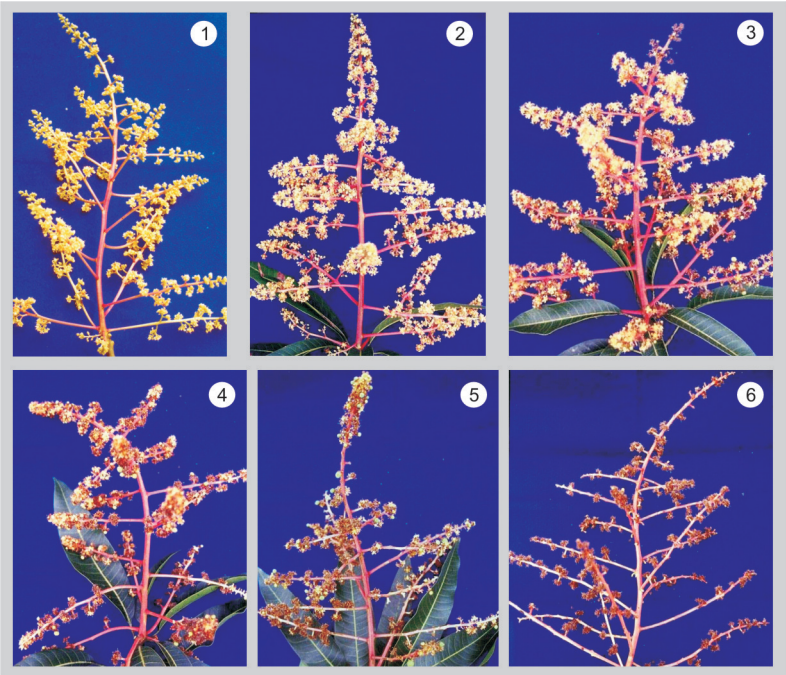

Figure 1.
Diagrammatic scale (from 1 to 6) to evaluate disease severity of *Cladosporium tenuissimum* on mango panicles cultivar Haden (Mexico) in function of the percentage of diseased flowers: 1 = 0%, 2 = 2–20%, 3 = 21–40%, 4 = 41–60%, 5 = 61–80%, 6 = 81–100%. (Color picture on www.fruits-journal.org.)

The reaction of amplification was carried out in a Perkin Elmer thermocycler with the following program: initial denaturation at 95 °C for 2 min; 30 denaturation cycles, annealing, and extension of 95 °C for 1 min, 50 °C for 30 sec, and 72 °C for 2 min, respectively, and a final extension at 72 °C for 10 min. The product was purified with the Q1Aquick for PCR kit and sequenced in two directions (5' → 3' and 3' → 5') with the same ITS primers in an ABI 3700 sequencer. The sequences were analyzed with the Lasergene 2001 program, version 5, of DNASTAR, Inc. [16] and compared at the database of the NCBI (National Center for Biotechnology Information) gene bank [17].

2.6. Disease severity

A diagrammatic scale to evaluate disease severity was proposed on the progress of symptoms observed in the field (*figure 1*). The values of the scale were given by collecting 50 panicles showing different disease severities. For each inflorescence, six scales were given based on the frequency (percentage) of disease infection and healthy flowers: 1 = 0%, 2 = 2–20%, 3 = 21–40%, 4 = 41–60%, 5 = 61–80%, 6 = 81–100%. The values of this scale were applied at

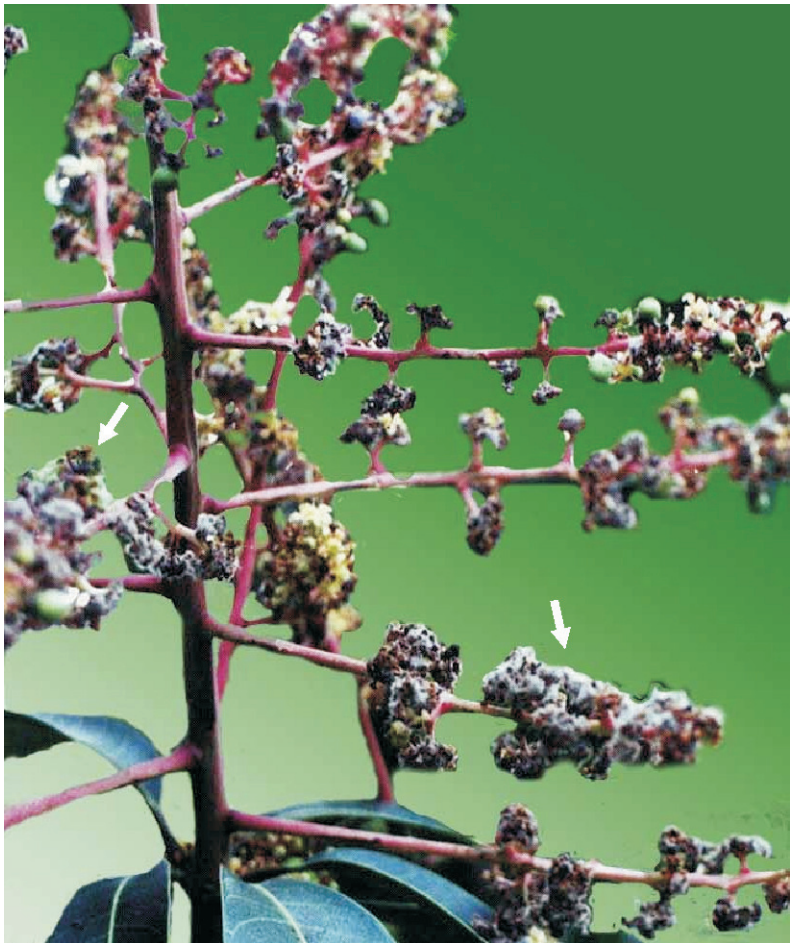

Figure 2.
Symptoms of *Cladosporium tenuissimum* in mango panicles cv. Haden. Abundant cottony mycelial growth and grey color showed over panicles and fruit set (Mexico). (Color picture on www.fruits-journal.org.)

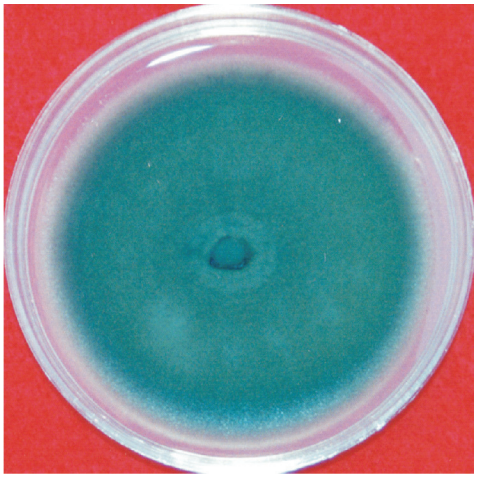

Figure 3.
A 22-day colony of *Cladosporium tenuissimum* on PDA, isolated from mango panicles cv. Haden, showing a grayish-green color. (Color picture on www.fruits-journal.org.)

different stages of the inflorescence: fully developed flower opening and 2-cm-diameter fruitlets in ten trees per orchard. Trees were selected based on the similitude of their floral phenology. A normal flux of natural blooming was evaluated for 1 year. Evaluations of severity were carried out every (8 to 10) days and, for each evaluation, the phenological stage was determined.

2.7. Statistical analysis

Mean separation by Tuckey's multiple test ($P \leq 0.05$) was calculated for disease severity.

3. Results

3.1. Symptoms and fungus isolation

In the field, the characteristic symptom shown in infected panicles was mycelial growth of the fungus, which was deep gray in color (*figure 2*). The mycelium completely covered individual flowers, pedicels and small fruits. The infected organs turned dark and dropped from the tree as the disease symptoms progressed. Sporulation over panicles was also abundant, becoming olive green to gray in color. Initially, symptoms were observed when panicles reached full bloom and the maximum colonization (mycelial growth and sporulation) took place when fruits were set.

3.2. Pathogenicity

Symptoms caused by this fungus were similar to those shown in inoculated inflorescences and those presenting natural infection. Eighty percent of the inoculated inflorescences showed wilting symptoms in flowers and petals with necrotic areas light brown in color, and, 8 days after inoculation, they were colonized by mycelia. It was observed that fruits of approximately 2 cm in diameter dropped from the trees. The typical lesion caused by *C. tenuissimum* was observed 15 days after inoculation. Control panicles did not show any infection symptoms.

3.3. *In vitro* characterization and identification

Monosporic colonies had an olive green color turning dark in color with time. During colony development, abundant sporulation was observed. Growth showed 1-, 4-, and 7.5-cm diameters for colonies of 2, 8, and 16 days old, respectively, with a growth rate of 4.6 cm·day^{-1} (*figure 3*). Generally, conidia were an olive-green color, variable in size and shapes; subspheric, fusiform, lime-shaped and ramo conidia (*figure 4*). Conidial scars and denticle-shaped projections were quite typical of the *Cladosporium* genus. The conidial average dimensions were (5.85 × 2.93) µm with variations of 3.2–23 µm × 1.98–5.47 µm.

3.4. Molecular characterization

The product of PCR:ITS was a band of 600 bp (base pairs). The two directions sequenced had 100% similarity. The isolate (MHI-Mich) sequence of 509 bp identified by morphology as *C. tenuissimum* was aligned with the sequence of the same species in the NCBI gene bank (AF393724), and the alignment was with the highest value of identity and 99.8 of similarity. The sequence was deposited in the gene bank and its access number was AY545639.

3.5. Disease severity

The ranges of the scale proposed in the section material and methods were adequate to stratify the more representative intervals of severity related to the behavior of this disease in the field. Disease severity was statistically different among the eleven orchards sampled ($P \leq 0.05$) and scale values were between five and six (*table I*). Percentage of disease severity varied from 69% to 100%.

4. Discussion

Symptoms of *C. tenuissimum* were always reproduced after inoculation, clearly demonstrating the pathogenicity of the fungal strain to mango panicles. The pathogenicity was confirmed by completing Koch's postulates when the repeated inoculation and subsequent isolation of *C. tenuissimum* from mango panicles were done successfully. In addition, repeated subculturing of the fungal strain on PDA medium during the inoculation assay always yielded the same fungus with identical morphological characteristics, confirming the identification of *C. tenuissimum*. Further verification of *C. tenuissimum* based on colony morphology, growth rate and molecular tools was carried out at the Institute of Ecology, Xalapa

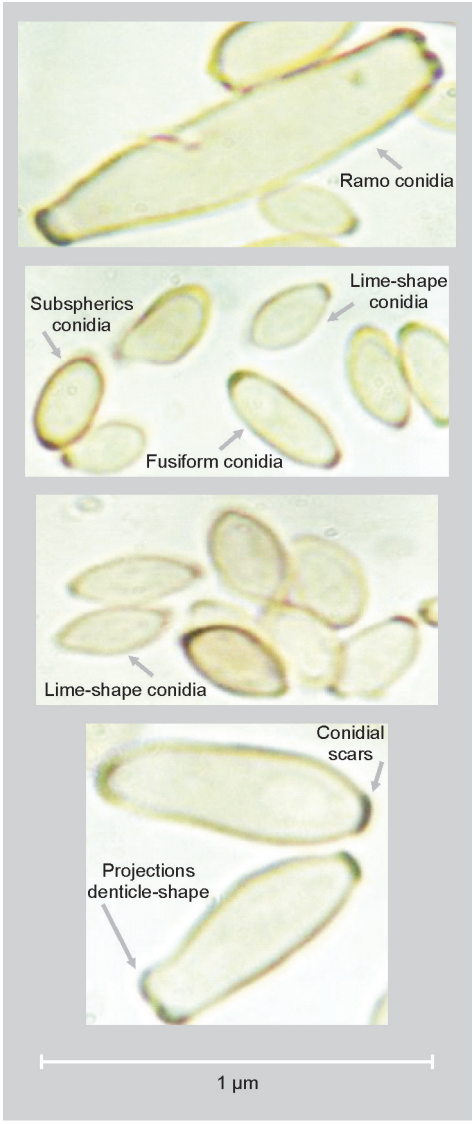

Figure 4.
Conidia of *Cladosporium tenuissimum* from monosporic cultures on PDA, isolated from mango panicles cv. Haden. Note the variability of shapes and forms. (Color picture on www.fruits-journal.org.)

Table I.
Experimental orchards and disease severity of *Cladosporium tenuissimum* of mango cv. Haden in the states of Guerrero and Michoacán, Mexico.

State	Growing area	Orchard	Scale values[1]	Disease severity (%)
Guerrero	La Unión	Guaricho	5	69 c
		Sorcua	6	100 a
		Coacoyulito	6	100 a
		Coyuquilla	6	100 a
		La Salada 1	6	87 b
		La Salada 2	6	90 b
Michoacán	Lázaro Cárdenas	Acalpican 1	6	100 a
		Acalpican 2	6	100 a
		Solera de Agua 1	6	89 b
		Solera de Agua 2	6	88 b
		Solera de Agua 3	6	100 a

[1] For each inflorescence, six scales were given based on the frequency (percentage) of disease infection and healthy flowers: 1 = 0%, 2 = 2–20%, 3 = 21–40%, 4 = 41–60%, 5 = 61–80%, 6 = 81–100%.

a, b, c: Means followed by the same letter are not significantly different by Tukey's test at ($P \leq 0.05$).

campus, Veracruz, Mexico, by Dr. Gabriela Herrera.

The present characterization under similar conditions (medium and temperature) was similar to that reported in other studies [10, 18, 19]. However, symptoms caused by strains of *C. tenuissimum* vary according to the type of tissue attack (plant organ or animal), which explains the symptoms we observed in mango panicles compared with reports of other plant organs such as leaf spots in banana [8], leaf blight of watermelon [9] and blossom end of tomato [5, 6]. *C. tenuissimum* has been isolated from 40 different plant species. It can be found in soils and air and it is considered as a cosmopolitan fungus mainly in tropical areas. This microorganism is also reported to be a hyper-parasite of telia, uredia and uredospores of *Cronartium* spp., *Puccinia* spp. and *Melampsora larici-populina* (Kleb.), respectively [13, 18]. *Cladosporium* sp. has been isolated from mango trees cv. Keitt in the state of Florida [20]. Several *Cladosporium* species are reported to be pathogenic microorganisms of various crops. Documented cases include *C. fulvum* (Cooke) of tomato [21, 22], C. *carygenum* (Ellis & Lang) of pecan [23, 24], *C. alli-cepae* [(Ranojevic) M.B. Ellis] of onion and leek [25] and *C. variabile* (Cooke) of spinach [26], among others. In our study, field evaluations in the states of Guerrero and Michoacán showed that the totality of the orchards sampled presented infection by *C. tenuissimum*. Taking into account the high disease severity shown in our investigation, it is relevant to know the geographical distribution and significance of this fungus in other production areas of mango in Mexico with the aim of developing future strategies for the control of this disease.

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Caracterización morfológica y molecular de *Cladosporium tenuissimum* Cooke (Deuteromycotina: Hyphomycetes) en panículas de árboles de mango: síntomas, patogenicidad y severidad.

Resumen — Introducción. Productores de mango de dos áreas importantes del estado de Guerreo y Michoacán, México, reportaron un daño extensivo en árboles de mango causado por un crecimiento micelial abundante que cubría la mayoría de las panículas enfermas de árboles de mango cv. Haden. **Material y métodos**. El hongo fue aislado de panículas de mango. La patogenicidad fue evaluada en huertos de mango mediante la inoculación de 20 inflorescencias cubiertas con bolsas de papel celofán. La caracterización *in vitro* fue evaluada en cultivos monospóricos; se determinó la morfología del conidio y la tasa de crecimiento. Para la identificación del microorganismo, se llevó a cabo una caracterización molecular mediante la extracción de DNA. **Resultados**. *Cladosporium tenuissimum* causó necrosis en las flores, pedicelos y frutos pequeños en las panículas inoculadas de mango cv. Haden a lo largo de la costa de los estados de Guerrero y Michoacán, México. Los órganos afectados se cubrieron con un micelio algodonoso gris y una esporulación olivácea verde gris. En *in vitro*, las colonias jóvenes fueron también verde olivo aterciopelado, cambiando de verde oscuro a gris con un margen blanquecino al exterior. La tasa de crecimiento fue de 0.46 cm·día^{-1}. Conidios semiesféricos con forma de lima y fusiformes, color oliváceo con cicatrices visibles y extensiones tipo denticular. El tamaño promedio de los conidios fue de (5.85 × 2.93) µm con variaciones de 3.2–23 µm × 1.98–5.47 µm. Las características moleculares representaron a *C. tenuissimum* después de la identificación. Los síntomas de los órganos de los árboles inoculados artificial y naturalmente infectados fueron similares. Se desarrolló una escala diagramática para la evaluación de la severidad de la enfermedad la cual varió desde 69% a 100% en las panículas infectadas. Los órganos fueron susceptibles desde la floración hasta la fructificación. **Discusión**. Se propone una investigación a futuro que determine la distribución geográfica de *C. tenuissimum* entre las áreas de producción de mango con evaluaciones simultáneas de estrategias de control.

México / *Mangifera indica* / enfermedades de las plantas / *Cladosporium tenuissimum* / identificación / síntomas