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Colletotrichum tropicale causal agent of anthracnose on noni plants (*Morinda citrifolia*) in Guerrero, Mexico

Ayvar-Serna S[1], Díaz-Nájera JF[1,2]*, Vargas-Hernández M[2], Plancarte-Galán PJ[1], Alvarado-Gómez OG[3], Tejeda-Reyes MA[2], Mena-Bahena A[1]

[1]Colegio Superior Agropecuario del Estado de Guerrero, Fitotecnia. Iguala Guerrero, 40000, Estado de Guerrero, México.
[2]Universidad Autónoma Chapingo, Programa de Postgrado en Protección Vegetal. Carretera México-Texcoco km. 38.5, Chapingo, 56230, Estado de México, México.
[3]Universidad Autónoma de Nuevo León, Facultad de Agronomía, Av. Universidad s/n, Cd. Universitaria San Nicolás de los Garza, 66455, Nuevo León, México

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Abstract

During sampling in March 2014 severe anthracnose symptoms were observed, mainly in foliage, on noni plants (*Morinda citrifolia*) in Cocula, State of Guerrero, Mexico. Fungal monosporic colonies were isolated from leaves with anthracnose symptoms. The morphological characteristics matched those of conidia of *Colletotrichum tropicale*. DNA analysis was performed on mycelium of the fungus by PCR amplification of ITS sequences. The identification and pathogenicity of isolates was confirmed by inoculation of the pathogen onto healthy plants free from disease. Control plants remained healthy, while those inoculated with the pathogen developed lesions and symptoms of rot anthracnose eight days after inoculation. The morphological, molecular characteristics and pathogenicity tests of the isolates confirmed that *C. tropicale* is the causal agent of anthracnose in *M. citrifolia*.

Key words – anthracnose – diagnosis – *Morinda citrifolia* – PCR

Introduction

Noni is the Hawaiian name given to the fruit of *Morinda citrifolia* L. (Rubiaceae). This species, which originated from Southeast Asia to the Australian region, is grown in Polynesia, India, Caribbean, Mexico, Central and South America. The Polynesians have used this plant for over 2000 years as a source of food and for medicinal purposes (Ulloa et al. 2012). There is evidence that noni has been used in traditional medicine in which the fruit helps to prevent and cure various diseases; these benefits include stimulation of the immune system, thus fighting against bacteria, viruses, parasites and fungal infections, preventing the formation of tumors including some malignant types (McClatchey 2002, Ulloa et al. 2012). Two clinical studies reported the relief of arthritis and diabetes associated with noni consumption; this is due to the beneficial effect that exists in certain compounds such as scopoletin, alkaloids and sterols as well as its antioxidant effects (Swetal & Krishanamurthy 2013). In Mexico, the leading producer of noni is the State of

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Corresponding Author: José Francisco Díaz Nájera – e-mail – apigro1988@hotmail.com

Nayarit, with 36.55 hectares of cultivated area and total production of 220.12 tons (SIAP 2018). The State of Guerrero has rapidly increased its demands for noni, and so have established new commercial plantations, principally in "La Costa Grande", although there are no updated statistics to prove the cultivated and production areas, or yield. In these plantations there is evidence that anthracnose exists, affecting branches, leaves, flowers and fruits; but in Mexico there is no reported knowledge on research of the phytosanitary status of noni plants, but in other parts of the world there are reports on the occurrence of *Colletotrichum* spp. in noni (McKenzie 1989, McKenzie & Jackson 1990a, b, Kumar et al. 2012, Hubballi et al. 2012). However, previous reports did not use molecular techniques to determine the species of *Colletotrichum* involved. For this reason, there was urgent interest in investigating the etiology of this disease.

Materials & Methods

Collection of samples, isolation, pathogenicity test, morphological and molecular identification

During March 2014, noni leaves with different symptoms of anthracnose were collected from an established plot in the experimental field area of the Agricultural College (CSAEGro) in Cocula, State of Guerrero, Mexico at coordinates 18° 19' N, 99° 39' W (Díaz et al. 2018). Using a systematic W transect sampling method, 20 leaves were collected. The symptoms were lesions on leaves, represented by light brown to dark and sometimes irregular circular spots. On the leaves with symptoms, a 1 cm^2 section was made in the transition zone between healthy and necrotic tissue. The tissue fragments were sterilized with sodium hypochlorite (1.5% NaOCl) for 2 minutes, rinsed three times with sterile distilled water and allowed to surface dry on paper towels for 2 minutes. One hundred tissue samples were processed and five pieces were placed in each Petri dish containing potato dextrose agar (PDA) culture medium. The dishes were incubated at 24 °C in alternating light/dark. Actively growing fungal colonies were then transferred to new PDA medium in order to obtain monoconidial cultures (Crous et al. 2009). The isolated fungi were identified according to their conidia. Temporary slides of isolated pure fungal cultures were prepared in glycerol. The slides were observed using a light microscope (400×), and size and shape of conidia noted. Identification was carried out by comparing the morphological structures and following fungal keys of Barnett & Hunter (1998), Watanabe (2002), Rojas et al. (2010).

A conidial suspension of the commonly isolated fungus was prepared containing 8×10^5 conidia per mL^{-1}. This was sprayed at the rate of 2.5 mL on to healthy, 70-days-old, *M. citrifolia* plants, and on to healthy fruits, of about 100 g, until dripping point. A wetting agent, Tween 20®, was added at the rate of 2 mL per liter of sterile distilled water. The control consisted of three plants and five fruits that received only sterile distilled water. All plants were placed in a humidity chamber for seven days at 22 ± 2 °C and 100% relative humidity. Once the inoculated plants showed the same symptoms as those of the first tissues collected, re-isolation was carried out using the method described by Núñez et al. (2013).

DNA extraction was performed with 50 to 100 mg of pure isolate mycelium grown in PDA using the QIAGEN DNeasy Mini Kit according to the manufacturer's instructions (QIAGEN®, Hilden, Germany). The procedure was repeated four times for each isolate. Universal PCR reactions were performed using primers ITS-1fu 5'-tccgtaggtgaacctgcgg-3' and ITS-4 5'-tcctccgcttattgatatgc-3' (White et al. 1990), which amplify two internal transcribed spacers (ITS) and 5.8S ribosomal RNA gene, generating a product between 566 and 570 base pairs (bp). The reaction mixture used for PCR amplification was prepared containing: 1X Taq DNA polymerase buffer, 2 nM MgCl2, 200 mM dNTP, 20 pMol of each oligonucleotide primer and 1 unit of Taq polymerase enzyme (Promega, WI, USA) in a final volume of 25 µL. The thermal program used was at a temperature of 94 °C for 2 minutes followed by 35 cycles at 94 °C, 55 °C and 72 °C for 30, 30 and 60 seconds, respectively, and a final extension of 5 minutes at 72 °C. PCR reaction products were electrophoresed on 1.5% agarose gel, and the bands viewed using a UVP ultraviolet

light transilluminator. The PCR-amplified fragments were sequenced directly and then compared to similar sequences available in GenBank, National Center for Biotechnology Information (NCBI).

Results

During field sampling it was observed that the damage to *M. citrifolia* leaves was very severe when high humidity and precipitation, interspersed with periods of drought occurred. Fungal colonies developed on PDA, and were identified as *Colletotrichum tropicale*. This fungus produced unicellular hyaline conidia, subterete, with rounded ends and measuring 13–17.5 × 4.7–5.3 µm (Fig. 1A, B). On PDA abundant conidia were formed in concentric rings. The slimy conidial masses in the initial stages was brown-orange and finally brown (Fig. 1C, D, E).

Eight days after inoculation with *C. tropicale* conidia suspension all plants showed symptoms on leaves ranging from pale lesions to dark brown circular and irregular spots (Fig. 2, left), while the control plants remained free of disease (Fig. 2, right), in the inoculated fruits the symptoms of anthracnose were manifested, the control fruits remained healthy. *Colletotrichum tropicale* was re-isolated from symptomatic plants and fruits.

The obtained sequences (550 and 551 bp) showed 100% similarity to the ITS region, and their alignment coincided with sequences reported in GenBank for *C. tropicale*. The accessions KX364718.1, KX364719.1, CSAEGro-PAYDI 0.1 and CSAEGro-PAYDI 0.2 were deposited in GenBank.

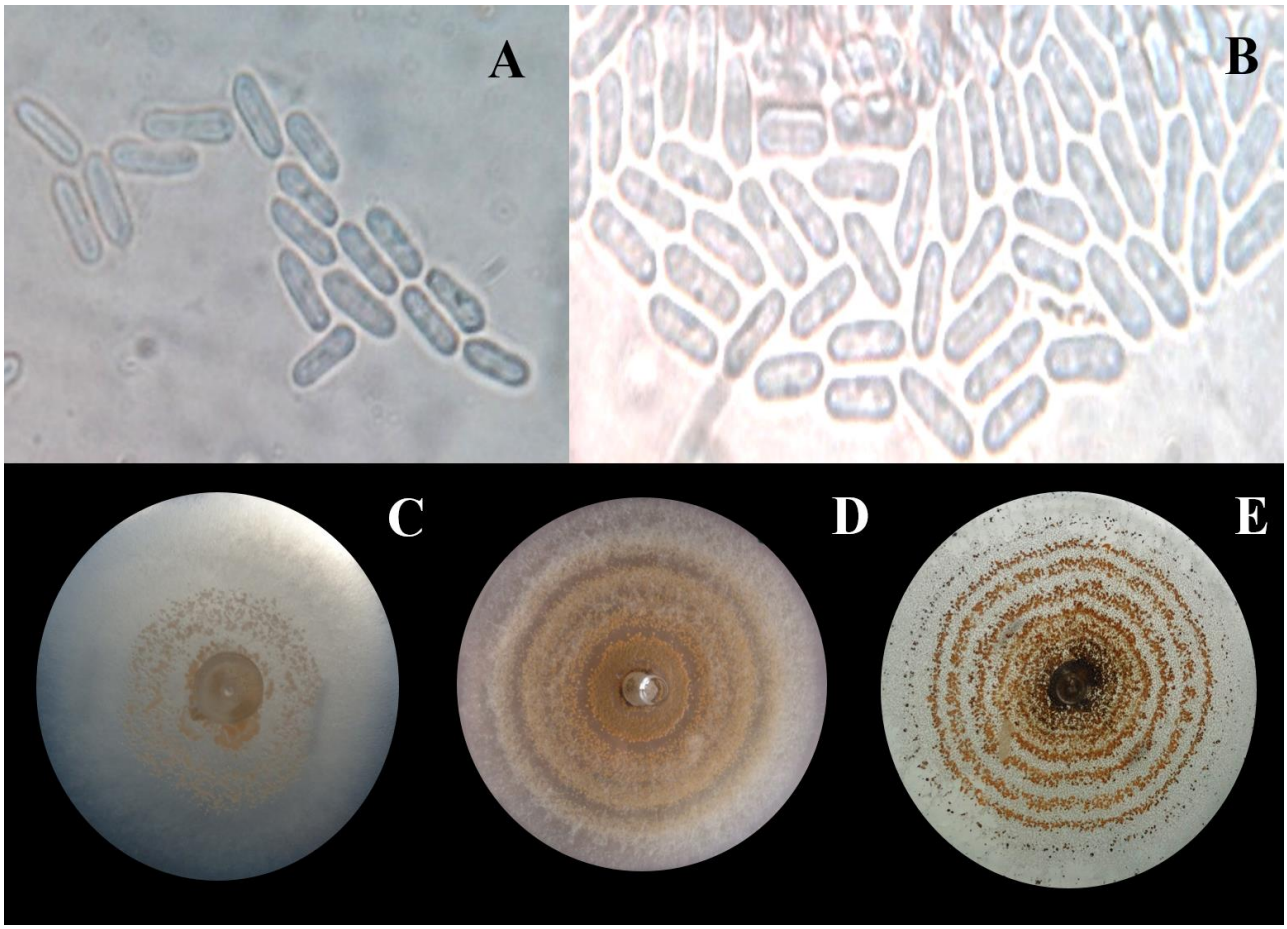

Fig. 1 – *Colletotrichum tropicale.* A, B Conidia. C, D, E Cultural characteristics on PDA.

Discussion

The morphological features of the isolated fungus corresponded to descriptions by Barnett & Hunter (1998), Watanabe (2002) for the genus *Colletotrichum*, and to Rojas et al. (2010) for *C. tropicale*. The symptoms were similar to those reported by Hubballi et al. (2012), Kumar et al.

(2012) who inoculated *M. citrifolia* with *C. gloeosporioides* and *Colletotrichum* spp. in fruits, typical necrotic anthracnose lesions was presented. Recently, several authors have pointed out the incidence of *C. tropicale* in various crops. On comparing the sequences obtained in this investigation with sequences available in GenBank, we found similarities of 99% and 100%, with previously reported sequences of *C. tropicale* KC512125.1 obtained from *Anona muricata* in Colombia Álvarez et al. (2014), García & Manzano (2017) reported the anthracnose incidence in pre-harvest in fruits of *Annona cherimola* caused by *C. tropicale* in Cuba (Accession Nos. LT853592, LT853593 and LT853594). Araújo et al. (2018) reported *C. tropicale* (MF289371.1) causing anthracnose on fruits of carnauba palm in Brazil. Weir et al. (2012) report that within the complex of *C. gloeosporioides*, 22 subspecies are grouped, which include among others *C. tropicale*, according to the authors the subspecies are defined genetically on the basis of phylogeny, in addition brief morphological descriptions are provided for the species where there is no modern description available.

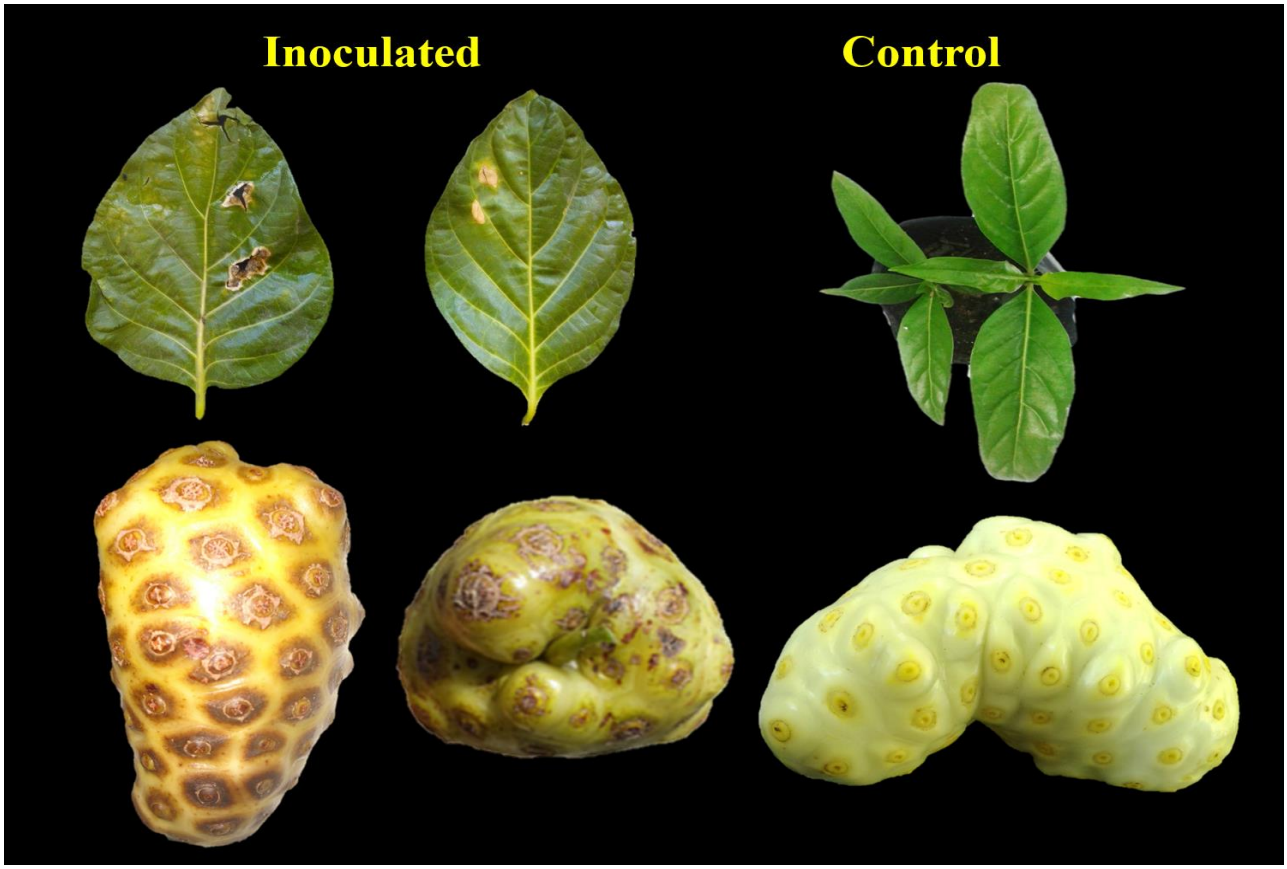

Fig. 2 – Pathogenicity test with *Colletotrichum tropicale* on *Morinda citrifolia*. Control leaves and fruit (right), inoculated leaves and fruit (left).

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