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ANTHRACNOSE: RESEARCH ON ITS CAUSING AGENT IN THE AVOCADO-PRODUCING AREA OF MICHOACAN, MEXICO

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Anthracnose has become one of the most important threats for avocado (*Persea americana* Mill) in the avocado-producing belt of Michoacan, Mexico. Former studies had identified *Colletotrichum gloeosporioides* as the pathogen species causing this disease, with typical symptoms including circular or ellipsoidal dark, hidden lesions containing high amounts of salmon, orange, or pink compact masses of spores. In this research, 60 *Colletorichum* isolates from avocado fruit were collected in 22 areas of the avocado-producing area of Michoacán. These isolates were analyzed and characterized by using three different criteria: morphological comparisons, fungicide sensitivity, and molecular (DNA) approaches. Morphological studies, which included form and size of conidia, growth rate and color of colony, indicated presence of two pathogen species: *C. gloesporioides* and *C. acutatum*. Benomyl sensitivity tests proved positive for isolates formerly identified as *C. acutatum* by morphological analysis. Moreover, DNA analyses conducted for isolates identified as *C. gloesporioides* proved positive when a DNA sample was amplified with primer CgInt/ITS4 specific for this species, while those identified as *C. acuatum* proved positive when using primers CaInt-2/ITS4 specific for this species.

Key words: Avocado, anthracnose, *Colletotrichum*, *gloeosporioides*, *acutatum*

ANTRACNOSIS: UNA INVESTIGACIÓN SOBRE SU AGENTE CAUSAL EN LA FRANJA AGUACATERA DE MICHOACÁN, MÉXICO

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La enfermedad conocida como antracnosis se ha convertido en uno de los problemas de mayor importancia en el cultivo del aguacate (*Persea americana* Mill) en la franja aguacatera de Michoacán, México. Hasta ahora, se había señalado a *Colletotrichum gloeosporioides* como su agente causal. Se ha indicado que este fitopatógeno provoca diversos síntomas cuando infecta la cáscara y la pulpa del fruto; los síntomas típicos son: lesiones oscuras y hundidas, circulares o elipsoidales con grandes cantidades de esporas formando masas compactas de color salmón, naranja o rosada. En el presente trabajo de investigación se hizo un análisis de 60 aislados de Colletotrichum a partir de

frutos de aguacate colectados en 22 municipios de la franja aguacatera de Michoacán. Estos aislados fueron caracterizados utilizando tres diferentes criterios: los morfológicos, la sensibilidad a fungicidas y el análisis molecular en el nivel de ADN. Los resultados del análisis morfológico, incluyendo la forma y tamaño de los conidios, la velocidad de crecimiento y el color de la colonia, indicaron la presencia de dos especies: *C. gloeosporioides y C. acutatum*. Los aislados que morfológicamente se ubicaron como *C. acutatum* dieron positivo para esta especie al hacer las pruebas de sensibilidad a benomil. Adicionalmente, los análisis de ADN hecho para los aislados identificados como *C. gloeosporioides* dieron positivo cuando se amplificó una muestra de ADN con el iniciador CgInt/ITS4 específico para esta especie, mientras que los identificados como *C. acuatum* dieron positivo cuando se utilizaron los iniciadores CaInt-2/ITS4, específicos para esta especie.

Palabras clave: aguacate, antracnosis, *Colletotrichum*, *gloeosporioides*, *acutatum*

I. INTRODUCTION

Michoacán, Mexico is the world leading avocado producer, being avocado one of the most important crops due both to the amount of jobs and the economic returns generated yearly. In Michoacan, avocado is not exempt from being affected by *Colletrotrichum* (telemorph *Glomerella*), one of the most important plant pathogens affecting tropical and subtropical crops and which causes anthracnose (Bailey, 1992, Prusky, *et al*., 2000, Dodd, 1992.). *C. gloesporioides* has been reported on avocados in Michoacan, causing symptoms such as dark, sunken lesions on foliage, flower buds, and fruit; circular, dark-brown lesions of 0.5-mm or less in diameter on fruit surface, which are known as “varicela” (chicken pox); circular, dark-brown lesions of corky appearance on fruit known as “clavo” (nail); and wilting of young shoots. These symptoms all together are termed anthracnose. In New Zealand (Hartill, 1991) and Australia (Coates, 1995), *C. acutatum* has been found to be associated with avocado anthracnose. Due to the diversity of symptoms attributed to *C. gloesporioides* and morphological differences found among isolates of this pathogen, the aim of this research was to determine whether or not the above mentioned symptoms are exclusive to *C. gloesporioides*.

II. MATERIALS AND METHODS

2.1 Area of study

The present study was conducted in the avocado-producing belt of Michoacan, Mexico located at lat. 19° 20’ N and between long. 101º 15’ and 102º 30’ W. This area is compressed within the so called Transversal Volcanic System.

2.2. Biological Material

Eight monoconidial *Colletotrichum* sp. isolates obtained from a strain bank at Facultad de Agrobiología (Universidad Michoacana de San Nicolás de Hidalgo at Uruapan) were chosen based on contrasting morphological characteristics among them shown on potato dextrose agar (PDA) medium, such as: mycelium color in the front and the back of Petri dishes, mycelium consistency (aerial or flat), color at center of colony, and both formation and color of conidial masses. Three of the isolates showed the following characteristics: gray at both the back and the front of dishes, dark-grey at center of colony, aerial cottony consistency of salmon color and rapid growth; all of these characteristics are typical of C*. gloeosporioides*. The characteristics of the other five isolates were: mycelium of a grayish color in the front but yellow and pale pink color in the back of the dish with an olive green center, conidial masses from salmon to orange color and rapid growth; all these characteristics have been described for *C. acutatum* (Sutton, 1992). The pathogen was isolated from fruit showing symptoms of anthracnose, "viruela" (nail), and "varicela" (chicken pox) collected in seven municipalities of Michoacan.

2.3.Conidia characterization

Monoconidial cultures were established on V8-agar medium (Slade, 1987) without calcium carbonate in order to induce spore formation. Size of conidia (length and diameter) was determined using an optical microscope (x) with 43x magnification. Shape of conidia was determined under a scanning electron microscope (x). A total of 100 conidia were sampled.

2.4. Benomyl sensitivity

Three concentrations (100, 600, and 1200 µg mL^{-1}) of Benomyl (Adaskaveg and Hartin, 1997) were prepared and 100 µL of each concentration were transferred onto filter paper disks (Dhingra and Sinclair, 1995). After drying, 50 µL of a $1x10^5$ conidia mL^{-1} solution was added to the disks; the latter were then equally spaced within a Petri dish containing PDA medium. Benomyl sensitivity was measured based on diameter growth of mycelia; measurements were done every 24 h and were ended once mycelium growth completely covered the Petri dish in the control (no fungicide) treatment. Data were subjected to analysis of variance by the procedure ANOVA of SAS (SAS Institute, 1988).

2.5. DNA isolation

Mycelium taken from each of eight isolates under study was grown in liquid medium (Ristaino *et al*., 1998). DNA was extracted by using the CTAB procedure (Henry, 1997) modified by Guillén *et al*. (2003). Nuclear ribosomal DNA (nrDNA) was amplified with conserved ITS1 and ITS4 nucleotides, including the ColF and ColR nucleotides; both ColF and ColR primers were specifically designed to

distinguish *Colletotrichum* isolates from other fungi (Cano *et al*., 2004). Differential DNAnr amplification was done with CgInt/ITS4 and CaInt-2/ITS4 primer combinations specific for *C. gloeosporioides* and *C. acutatum*, respectively under conditions described by Adaskaveg and Hartin (1998). DNA amplification reactions were performed in a Techne TC-412 PCR with the following program: 94 ºC for two minutes followed by 40 denaturizing cycles (94 ºC for 1 min; 50 ºC for 1 min; 72 ºC for 2 min). PCR products were separated by electrophoresis on 1.2% agarose gel and stained with ethidium bromide (0.4 g mL^{-1}); PCR products were observed under a UV transluminator.

III. RESULTS AND DISCUSSION

3.1. Conidia characterization

Based on their size and form, conidia were characterized into two groups. In one group, conidia were ovoid and cylindrical with obtuse apices, varying from 12.0 to 17.0 µm length and 2.5 to 5.4 µm diameter. These characteristics were typical of *C. gloeosporioides*. In the second group, conidia were fusiform, with size varying from 8.5 to 16.5 µm length and 2.5 to 4.0 µm diameter. These characteristics were typical of *C.acutatum*.

3.2.Benomyl sensitivity

The bioassay showed a statistically differential response in sensitivity to benomyl between *C. gloesporioides* and *C. acutatum* strains, being these results consistent with those obtained by morphological comparisons, which included size and from of conidia. Strains characterized as *C. gloesporioides* showed average sensitivity rates of 38.5%, 54.5%, and 56.5% for benomyl concentrations of 300, 600, and 1200 µg mL^{-}, respectively. In the same way, *C. acutatum* strains showed a linear response in sensitivity to benomyl concentrations, with average sensitivity rates of 22 %, 23.5 % y 25.5 % for of 300, 600 y 1200 µg mL^{-1} benomyl, respectively. In general, *C. acutatum* strains were respectively 16.5% and 31% less sensitive to benomyl concentrations of 300 and 1200 µg mL^{-} than *C. gloesporioides* strains. Differential response in benomyl sensitivity on both *C. gloesporioides* and *C. acutatum* strains can be seen in Figure 1.

3.3. Molecular analysis

Colletotrichum-specific primers (ColF/ColR) used on the eight isolates under study revealed a PCR product near 150 pb, being in agreement with sizes (130 a 150 pb) previously reported (Cano et al., 2004) for these primers. Further, DNA of the eight *Colletotrichum* isolates was targeted for PCR reactions with CgInt/ITS4 primers; the latter were generated to amplify a 500 pb fragment specific for *C. gloeosporioides* DNA (Adaskaveg y Hartin, 1997). It was found that a 500 pb amplification product was generated with CaInt-2/ITS4 primers on three *C.*

acutatum isolates. Similarly, DNA of *C. gloeosporioides* isolates amplified with CgInt/ITS4 primers generated a 500 pb PCR fragment. These amplification patters were similar to those reported by Adaskaveg and Hartin (1997) for the two *Colletotrichum* species.

IV. CONCLUSIONS

Morphological characteristics of conidia, benomyl sensitivity tests, and molecular analysis all together evidence presence of *C. acutatum* in close relationship with anthracnose symptoms of avocado previously attributed only to *C. gloeosporioides* in Michoacan, Mexico.

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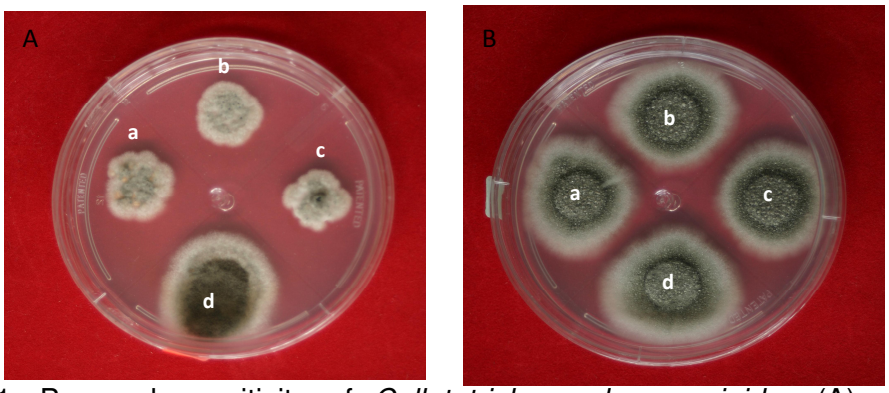

Figure 1. Benomyl sensitivity of *Colletotrichum gloeosporioides* (A) and *C. acutatum* (B) strains: a) 300 µg mL^{-1}, b) 600 µg mL^{-1}, c) 1200 µg mL^{-1}, and d) control.