FIRST RECORD OF *COLLETOTRICHUM ACUTATUM* ON LISIANTHUS (*EUSTOMA GRANDIFLORUM*)

Luis Cedeño[1], Armando Briceño[1], Gustavo Fermín[2], Ilka Domínguez[1], Henry Pino[1] and Kleyra Quintero[1]

[1]Instituto de Investigaciones Agropecuarias, Facultad de Ciencias Forestales y Ambientales y [2]Unidad de Biotecnología Vegetal-CIGEN, Facultad de Ciencias, Mérida 5101-A. Mérida, Venezuela. E-mail: cedenol@ula.ve

Recibido: 07 de abríl de 2007. Aceptado: 07 de septiembre de 2007.

ABSTRACT

Cedeño, L., Briceño, A. Fermín, G., Domínguez, I., Pino, H., and Quintero, K. 2007. First record of *Colletotrichum acutatum* on lisianthus (*Eustoma grandiflorum*). Fitopatol. Venez. 20:41-43

In March 2006, the fungus *Colletotrichum acutatum* was consistently isolated from stems of infected lisianthus (*Eustoma grandiflorum*) potted plants grown in an area of La Pedregosa, Mérida, Mérida State, Venezuela. Pathogenicity tests under greenhouse conditions and subsequent reisolations of the fungus from artificially infected lisianthus seedlings confirmed that *C. acutatum* was the causal agent of the disease investigated. The species identity was determined by traditional phytopathological procedures and confirmed by PCR-based molecular analysis. The typical disease symptoms appeared 5-6 days after experimental inoculation of sixteen lisianthus seedlings. This is the first time that lisianthus is reported as a natural host of *C. acutatum*. The fungus also produced anthracnose on fruits of strawberry var. Chandler inoculated with a conidial suspension.

Additional key words: Anthracnose, *Glomerella acutata*, PCR

RESUMEN

Cedeño, L., Briceño, A. Fermín, G., Domínguez, I, Pino, H. y Quintero, K. 2007. Primer registro de *Colletotrichum acutatum* en lisianthus (*Eustoma grandiflorum*). Fitopatol. Venez. 20:41-43

En marzo de 2006, el hongo *Colletotrichum acutatum* fue consistentemente aislado del tallo infectado de plantas de lisianthus (*Eustoma grandiflorum*) cultivadas en macetas en un área de La Pedregosa, Mérida, estado Mérida, Venezuela. Pruebas de patogenicidad bajo condiciones de invernadero y subsecuentes reaislamientos del hongo a partir de plántulas de lisianthus infectadas experimentalmente, confirmaron que *C. acutatum* fue el agente causal de la enfermedad investigada. La identidad de la especie se determinó mediante procedimientos fitopatológicos tradicionales y fue confirmada a través de análisis moleculares por PCR. Los síntomas típicos de la enfermedad aparecieron a los 5-6 días después de la inoculación artificial de dieciséis plántulas de lisianthus. Esta es la primera vez que se registra a lisianthus como hospedante natural de *C. acutatum*. El hongo estudiado también produjo antracnosis en frutos de fresa var. Chandler inoculados con una suspensión conidial.

Palabras clave adicionales: Antracnosis, *Glomerella acutata*, PCR.

INTRODUCTION

Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn. (=*Lisianthus russelianus* Hook.)] is a member of the family Gentianaceae that is grown as annual bedding plant for their attractive foliage and showy flowers (1). It produces flowers in a wide variety of forms and delicate colors from purple to white. The great versatility as a cut flower, potted plant, or bedding plant has prompted a dramatic increase in the demand of lisianthus. It has been recently introduced to Mérida State, Venezuela, for cut flower and potted plant production. In March 2006, a destructive stem canker disease (Fig. 1), caused by a *Colletotrichum* sp., was detected in a greenhouse devoted to potted plants of lisianthus in Mérida. The lesions were commonly observed near where the leaves are formed. The disease occurred on 10% of 3 months-old plants.

Occasionally, conidia from *C. acutatum* are morphologically indistinguishable of those from to *C. gloeosporioides*, which might lead to a misidentification of the species under analysis. Molecular biology techniques, however, allows for the differentiation of both species with high reliability.

MATERIALS AND METHODS

Pathogen isolation. Isolations were carried out on water-agar 2% acidified with 50% lactic acid (pH 4.5) using fragments (2 mm long) cut off the margin of lesions already present on the stems of lisianthus plants. Before isolation, tissues were submerged 1 min in a 0.5% sodium hypochlorite solution, rinsed three times with distilled sterile water (DSW) and dried on sterile tissue paper. Inoculated plates were incubated at room temperature (22 ± 2°C) wit a 12 hphotoperiod. From a mass culture monoconidial isolations were obtained, eight of which were selected for identification and pathogenicity tests and for PCR-based molecular analysis. Monoconidial cultures were obtained mixing 1 ìl of a conidial suspension (50 conidia/ìl) with 0.3 ml of DSW and spreading the mixture over PDA plates which were incubated inverted at room temperature and 12 h photoperiod. Spreading was performed using an L-shaped glass rod. Later on, emergent colonies were transferred to PDA bevel-edged tubes and kept cold (4°C). From every monoconidial culture 50 conidia fixed with formaldehyde 4% and stained with basic fuchsine 0.025% were examined and measured using a photonic microscope Zeiss, Axioplan model. Preliminary identification of the isolated microorganism was based on morphology and measurements of conidia and appressoria. To confirm the fungus identity a PCR-based molecular diagnostic assay was performed using a primer directed to the ribosome internal transcribed sequence, ITS4 (5'-TCCTCCGCTTATTAGTATGC-3') and a specific primer reverse for *C. acutatum* (CaInt2: 5'-GGGGAAGCCTCTCGCGG-3') or *C. gloeosporioides* (CgInt: 5'-GGCCTCCCGCCTCCGGCCGG-3'). Primers were originally designed in 1996 by Brown *et al.* (5,11).

DNA extraction. Monoconidial cultures suspensions were subcultivated for 5 d or more on PDA plates and incubated at room temperature and a 12 h photoperiod. Mycelium was collected, filtered and washed with SDW to eliminate remaining culture medium. DNA was extracted following the protocol described by Chris *et al.* (1994) (3): 0.5 g of mycelia was ground using liquid nitrogen until a fine dust was produced; 0.5 mL of extraction buffer (CTAB 2% p/v, Tris-Cl 100 mM. NaCl 1.4 M, EDTA 20 mM) wasadded and mixed by inversion. After an incubation at 55°C for 20 min, the mixture was centrifuged at 15000 g for 5 min and the aqueous fraction collected and extracted with one volume of chloroform:isoamylic alcohol (24:1, v:v).

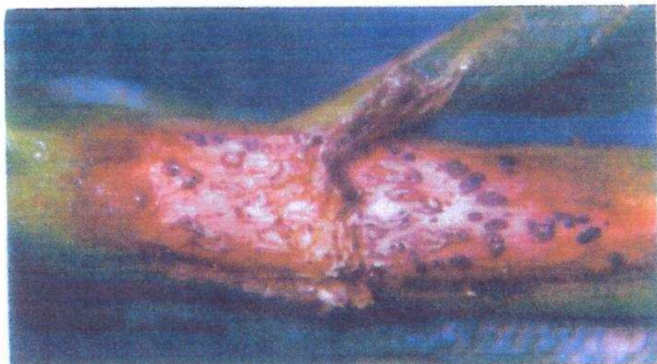

Fig. 1. Stem of lisianthus showing artificially induced lesions with acervuli of *Colletotrichum acutatum*.

Centrifugation and organic extraction were repeated once and the recovered aqueous phase mixed with 1/10 vol of an ammonium acetate 7.5 M solution plus 2 vol of absolute ethanol. Precipitation was allowed to proceed overnight, after which collection of DNA was performed centrifuging at 5000 g for 10 min. Pellet was washed once with 70% ethanol and DNA resuspended in 200 µl of DSW.

PCR analysis. For all amplification reactions 10-100 ng of DNA were used along with the ready-to-use GoTaq Green Master Mix (Promega, Madison, MI) which contains the GoTaq DNA polymerase, all dNTPs and $MgCl_2$ in an optimized buffer solution. Primer concentration in the 1X reaction mixture was 2 ng/ìl in a final reaction volume of 25 ìl. Amplification program was as follows: initial denaturation of 1 min at 94°C, and then 30 cycles of denaturation at 92°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 90 s. A final extension step at 72°C for 10 min ended the reaction. Amplification products were run in a 1.2% agarose gel, visualized under UV and digitally recorded.

Identification of the amplified products. DNA from four amplification product bands of the tested isolates were column-purified using the Wizard Plus SV Gel and PCR Clean-Up System (Promega) and sent for sequencing (MWG Biotech, High Point, NC). Three of the four amplification products were sequenced using primer ITS4, and one with the primer CaInt2. Sequences were compared against all the sequences at the GenBank public database using the BLAST algorithm.

Pathogenicity test. In order to assess the fungus pathogenicity, six 20 days-old seedlings of lisianthus were inoculated by spraying the foliage with a conidial suspension (3.426 x 10^6 conidia.mL^{-1}) prepared from fresh cultures on PDA of 10 monoconidial isolates, sterile distilled water (100 ml/plate) and few drops of 50% Tween 20 solution (2 drops.100 mL^{-1}). Before inoculation, the seedlings, which averaged 23 cm of height, remained covered for 3 days with transparent plastic bags under greenhouse conditions. After inoculation the seedlings were again covered with the transparent plastic bags, placed in trays containing water, and incubated for 7 d at room temperature (22±2°C). Threeseedlings used as controls were sprayed with a similar suspension but without conidia, and incubated under the same conditions of the conidia-inoculated ones. Reisolationstests were performed from artificially infected seedlings to complete Koch's postulates.

RESULTS AND DISCUSSION

Pathogen isolation and identification. From infected tissue, a fungus belonging to the genus *Colletotrichum* was consistently isolated and their colonies on PDA were initially white but after 5-6 days of incubation at room temperature and 12 h photoperiod began to turn grey. Mycelial growth was evident in the form of concentric bands, and conidia emerged in the form of pink to salmon masses on PDA plates. On the opposite side of the plate a pink to salmon mycelium with irregular masses of darker color were observed. Conidia were hyaline, erect, predominantly fusiform and without septa. *In vitro* produced conidia dimensions ranged between 11.1-(12.2)-13.2 µm long X 3.0-(3.2)-3.4 µm wide, while those developed *in situ* ranged between 9.9-(12.1)-14.3 µm long X 2.9-(3.2)-3.5 µm wide. Appressoria were circular or bundled, of a clear or dark brown color measuring 7.5-(9.8)-12.1 µm. Morphology and dimensions of conidia and appressoria were similar to those previously described for *C. acutatum* Simmonds (4)(10). The species was first described by Simmonds in 1965 (9), and thirty six years later the characterization of its sexual form (*Glomerella acutata*) was reported by Guerber and Correll (2001) (6).

Molecular identification of the pathogen. Purification of *C. acutatum* DNA was simple and yielded good amounts of product with ease. Half gram of mycelium yielded enough DNA for any study involving molecular biology protocols. In the assays performed for the molecular identification of the species by PCR, DNA from monoconidial cultures was not degraded and was reliable for the amplification of specific products. (Fig. 2) Primers designed for the specific identification of *C. acutatum* allowed the amplification of a product very close to the 490 bp reported for this species (11). On the contrary, no product was observed when the same DNA was used for amplification assays with primers directed to *C. gloeosporiodes* (Fig. 3). In summary, all PCR tests using mococonidial culturescorroborated that the fungus analyzed belong to *C. acutatum*, and that the PCR-based assay is reliable for the discrimination between *C. gloeosporiodes*, *C. coccodes* and *C. acutatum* (7) (Fig. 3).

Sequencing of the amplified products and the high level of homology, when comparing the data using BLAST at the Gen Bank database, corroborated the identity of the fungus analyzed here with *C. acutatum* (*G. acutata*). The sequence

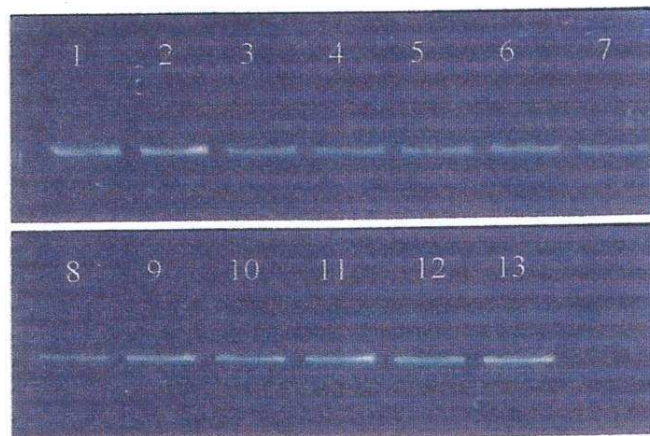

Fig. 2. Chromosomal DNA from thirteen different isolates of *Colletotrichum acutatum* run in a 0.8% agarose gel, during 30 min at 70 V using buffer TAE 1X.

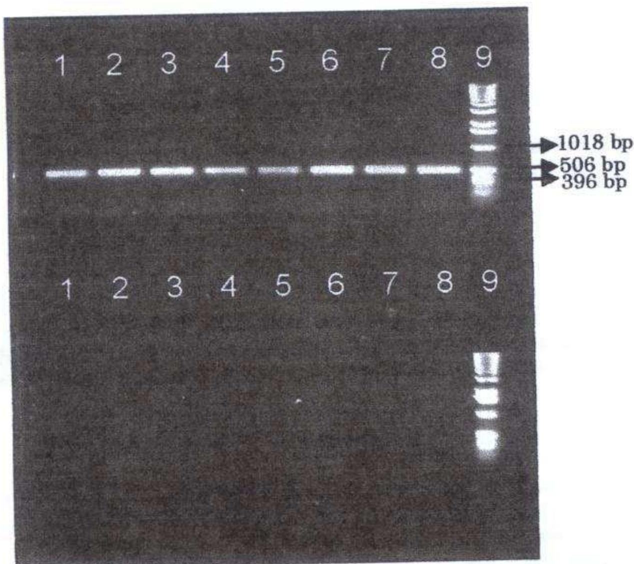

Fig. 3. PCR-based identification of *Colletotrichum acutatum*. Amplification products were run in a 1,2% agarose gel for 45 min, at 70V using buffer TAE 1X. In the first row amplification products from eight different monoconidial isolates using specific primers for *C. acutatum* (ITS4-CaInt2) are shown. In the row below, the same DNA was used for amplification experiments but with primers specific for *C. gloeosporioides* (ITS4-CgInt). Molecular markers correspond to the 1 Kb DNA Ladder from Invitrogen.

we obtained showed no similarity with that of *C. gloeosporiodes* (*G. cingulata*). The length of the sequence analyzed here was 491 bp, from which only two nucleotides were different from the reported sequence of the CA456 isolate of *C. acutatum*, corroborating thus the identity of ours (Fig. 4).

Pathogenicity tests. Assays of artificial inoculation were positive and allowed the reproducibility of symptoms which were identical to those observed on lisianthus plants naturally infected. In one third of the artificially inoculated plants signs and symptoms of infection were evident 5-6 days post infection (dpi); 11 dpi all infected plants showed canker-like lesions invaded by acervuli with salmon-colored immersed conidia. When the lesions strangulated the stem, the upper tissues of lisianthus plants wilted and rapidly died. Control plantlets remained asymptomatic during all the experiment. In the symptomatic plants lesions were up to 24 mm long, and acervuli ranged between 246.5-(390.7)-534.9 µm. Yellowish-grey lesions appeared on leaves where acervuli were also present.

The analyzed pathogen produced anthracnose on artificially-inoculated strawberry 'Chandler' fruits. Signs and symptoms characteristic of the infection were obvious 7 dpi, while control fruits remained healthy during the observation period.

C. acutatum was consistently isolated from the lesions experimentally reproduced both on lisianthus plantlets andstrawberry fruits, demonstrating that the fungal species here studied is the causal agent of the disease that decimated lisianthus plants in La Pedregosa, Mérida. This is the first time that *E. grandiflorum* is reported as a natural host of *C. acutatum*. Additionally, *C. acutatum* isolates can potentially cause anthracnose on strawberry 'Chandler' fruits. *C. acutatum* was reported for the first time causing anthracnose on fruits of different varieties of strawberries in Venezuela in 1997 (2), becoming a very important limiting factor in its production since then. Apparently, *C. acutatum*

```
Query     1  GCCTCCCCTCCCGGCGCCGGCCCCCACCACGGGGACGGGGCGCCCGCCGGAGGAAACCAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 105  GCCTCCCCTCCCGGCGCCGGCCCCCACCACGGGGACGGGGCGCCCGCCGGAGGAAACCAA  164

Query    61  ACTCTATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTTTAACAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 165  ACTCTATTTACACGACGTCTCTTCTGAGTGGCACAAGCAAATAATTAAAACTTTTAACAA  224

Query   121  CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 225  CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT  284

Query   181  TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCATTCTGGC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 285  TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCATTCTGGC  344

Query   241  GAGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCAC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject 345  GAGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCCAC  404
                              ↓
Query   301  GGCACACGTGGGCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAA  360
             ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Subject 405  GGCACACGTGGGCCCTTGAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAA  464
                                ↓
Query   361  CTAACGTCTCGCACTGGGACCCGGAGGGACTCTTGCCGTAAAACCCCCAATTCTTTACA  420
             ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Subject 465  CTAACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCAATTCTTTACA  524

Query   421  GGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAA  460
             ||||||||||||||||||||||||||||||||||||||||
Subject 525  GGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAA  564
```

Fig. 4. Schematic representation of the best result obtained using the BLAST algorithm with the sequence obtained in this work. The query was the sequence assembled from the amplification product of the pathogen DNA, and the subject was the best match at the GenBank that corresponds to *Colletotrichum acutatum* document AJ749674.1. Identity value was of 99%, and the only two differences found are indicated with a solid black arrow.

entered the country in strawberry stolons imported from California (2) by the Chesnut Hill Company, and were subsequently planted in La Mucuy, Mérida. Finally, *C. acutatum* can infect several other plants that are also hosts for other species of *Colletotrichum* (8).

LITERATURE CITED

1. Alfieri, Jr., Langdon, K.R., Kimbrough, J.W., El-Gholl, N.E. and Wehlburg, C. 1994. Bulletin N° 14. Diseases and Disorders of Plants in Florida. Florida Department of Agriculture and Consumer Services. 1114 pp.

2. Cedeño, L. y Carrero, C. 1997. Antracnosis causada por *Colletotrichum acutatum* en frutos de fresa en Mérida, Venezuela. Interciencia 22: 315-319.

3. Chirs H., Wilkinson D., Zgafas K. and R. Harrison. 1994. A simplified method for the preparation of fungal genomic DNA for PCR an RAPD analysis. BioTecniques 16 (1): 48-49.

4. Dyko, B. J. and Mordue, J. E. M. 1979. *Colletotrichum acutatum*. Descriptions of pathogenic fungi and bacteria N° 630. CMI/AAB. Kew, Surrey, England.

5. Freeman, S., Shabi, E. and Katan, T. 2000. Characterization of *Colletotrichum* causing anthracnose of anemone (*Anemone coronaria* L.). American Society for Microbiology 66: 5267-2272.

6. Guerber, J. C. and Correll, J. C. 2001 Characterization of *Glomerella acutata*, the teleomorph of *Colletotrichum acutatum*. Mycologia 93: 216-229.

7. Melanie L., Ivey L., Nava-Diaz C. and S. Miller. 2004. Identification and management of *Colletotrichum acutatum* on immature Bell peppers. Plant Disease 88: 1198-120.

8. Peres, N. A., Timmer, L. W., Adaskaveg, J. E. and Correll, J. C. 2005. Lifestyles of *Colletotrichum acutatum*. Plant Disease 89: 784-796.

9. Simmonds, J. H. 1965. A study of *Colletotrichum* species causing ripe fruit rots in Queensland. Queensland Journal of Agricultural Sciences 22: 437-459.

10. Sutton, B. C. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. Pp. 1-27. *in* J.A. Bailey and M.J. Jeger(eds.): *Colletotrichum*, Biology Pathology and Control. CAB-International, Wallingford, Oxon Oxio 8DE, United Kingdom. 388 pp.

11. Talhinhas, P., Sreenivasaprasad, S., Neves-Martins, J. and Oliveira, H. 2005. Molecular and phenotypic analysis reveal association of diverse *Colletotrichum acutatum* groups and low level of *C. gloeosporioides* with olive anthracnose. American Society for Microbiology 71:2987-2998.