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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *COLLETOTRICHUM GLOEOSPORIOIDES* FROM *CITRUS RETICULATA*

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INTRODUCTION

Colletotrichum spp. causes anthracnose disease and postharvest decay on many tropical, subtropical, and temperate fruits. On citrus, postbloom fruit drop is caused by *Colletotrichum acutatum* J.H. Simmonds. Infection results in necrotic brown lesions on petals and premature fruit drop. Postharvest anthracnose is caused by *C. gloeosporioides* (Penz.) Penz. & Sacc., and is a problem especially for citrus fruits harvested early that must be degreened with ethylene. In Serbia, *t*he occurrence of anthracnose on mandarin fruit (import from Greece), has been found during 2010. Typical symptoms include dark, sunken, and circular lesion that produce mucilaginous, orange conidial masses (Figure 1a). Economic losses caused by the disease are mainly attributed to lower fruit quality and marketability. Therefore, the objective of the present study was to identify the causing agent of mandarin anthracnose using morphological and molecular analysis.

MATERIAL AND METHODS

Isolation and pathogenicity

Pieces of the diseased tissues of mandarin fruits were sterilized (3% NaOCl), followed by several rinses with sterile distilled water, and placed on water agar (WA) in Petri plates at 25°C for 7 days. Pathogenicity test was conducted on symptomless, detached mandarin fruits. The fruits were surface sterilized (70% ethanol) and inoculated with 20 µl of the conidial suspensions (10^7 conidia/ml). Control fruit was inoculated with sterile distilled water.

Morphology

The isolates were cultured on potato dextrose agar (PDA) in darkness at 25°C. The appearance of the colonies, and the vegetative and reproductive structures were described after 10 days incubation. Appressoria were produced using a slide culture technique (Johnston and Jones, 1997).

DNA extraction , PCR amplification and ITS1-2 sequence analysis

Total genomic DNA was extracted from mycelium using the DNeasy Plant Mini Kit (QIAGEN, Germany). Species-specific primers for *C. gloeosporioides (*CgInt) and *C. acutatum* (CaInt2) were used in combination with the conserved primer ITS4 (White et al., 1990). PCR products were separated using electrophoresis in 1% agarose gels.

One representative isolate (MC-1), was chosen for DNA sequence analysis. Sequencing was performed by Macrogen Service (Seoul, Korea). Phylogenetic analysis using neighbor-joining was conducted with MEGA4 software (Tamura et al., 2007).

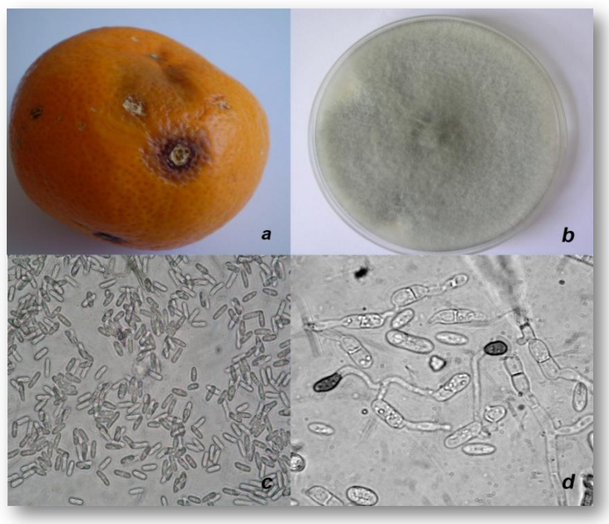

Figure 1. (a). Anthracnose symptom on mandarin fruit; (b). Cultural appearance of *C.__gloeosporioides*, (isolate MC-1); (c). Conidia of *C. gloeosporioides*, (isolate MC-1) (light microscope, 600x); (d). Appressoria of *C.gloeosporioides* (isolate MC-1) (light microscope, 1000x).

RESULTS

Pathogenicity

All tested isolates caused anthracnose lesions on mandarin fruit after 10 days of incubation. No lesion developed on control fruit. Koch's postulates were fulfilled by reisolation from inoculated mandarin fruits.

Morphology

Colonies of all mandarin isolates were dense aerial, initially white gray, becoming dark gray, as the cultures aged on PDA (Figure 1b). Conidia were hyaline, aseptate, and cylindrical with obtuse apices, 11.2-17.6 × 3.2-4.8 µm (Figure 1c). Appressoria produced directly from conidia were light to dark brown, smooth, simple, clavate or irregular, 6.9-12.8 × 5.5-8 µm (Figure 1d).

Molecular identification and ITS1-2 sequence analysis

The species-specific primer CgInt in conjunction with ITS4 primer amplified a 450 bp fragment from genomic DNA of all mandarin isolates, and the reference strain of *C. gloeosporioides* (CBS 516.97). Phylogenetic analysis based on ITS1-2 sequences resulted in a tree with three clusters (Figure 2).

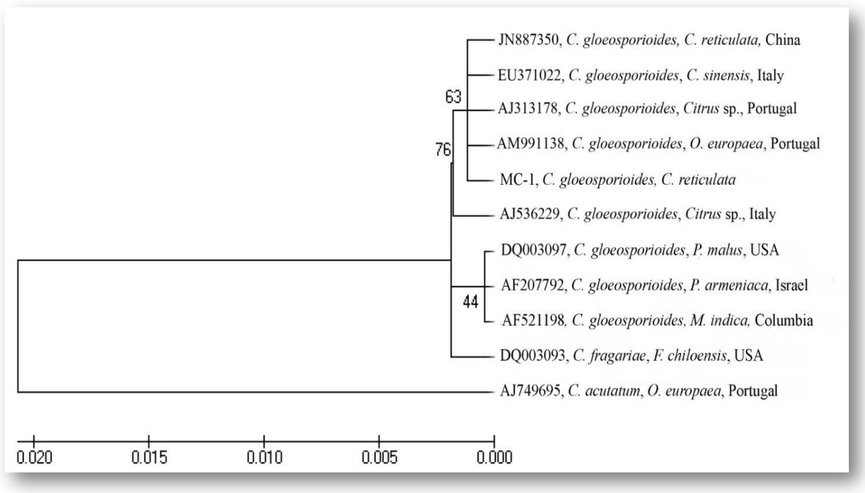

Figure 2. Phylogenetic tree of *C. gloeosporioides*, isolate MC-1 from mandarin, based on ITS rDNA sequences. The tree was generated using neighbor-joining analysis. Bootstrap values for 1000 replicates are shown on branches. Marks AJ, AF, AM, DQ, EU and JN are DNA sequence accession numbers from NCBI GenBank.

CONCLUSION

Morphological and molecular identification followed by nucleotide sequencing of the amplicons, and phylogenetic analysis of ITS rDNA sequences, demonstrated that the causal agent of mandarin anthracnose was *C. gloeosporioides*.

ACKNOWLEDGEMENT

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