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NEW OR UNUSUAL DISEASE REPORTS

## First report of *Colletotrichum gloeosporioides* on citrus in Algeria

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**Summary.** Wither-tip symptoms of twigs were observed on cultivated sweet orange and lemon trees in Algeria. The causal agent was identified as *Colletotrichum gloeosporioides* on the basis of cultural and morphological features. The identification of the fungus was confirmed by sequencing of the internal transcribed spacer region of rDNA. Pathogenicity tests revealed that *C. gloeosporioides* isolated from withered twigs caused typical anthracnose symptoms on orange leaves. This is the first report on *Colletotrichum gloeosporioides* as causal agent of anthracnose in Citrus trees in Algeria.

**Keys words:** Anthracnose, pathogenicity, ITS.

### Introduction

Citrus are crops of major importance in many areas of the world, particularly in the Mediterranean region. In Algeria the annual production of citrus fruit has ranged from 281,000 to 568,000 tons in the last decade (FAO, 2015). Citrus orchards in Algeria comprise various species of citrus, such as oranges [*Citrus sinensis* (L.) Osbeck], clementines (*Citrus clementina* Hort. ex Tan.), lemons [*Citrus limon* (L.) Burm.] and grapefruits (*Citrus paradisi* Macf.). The cultivar range of the group of oranges is the widest, with a predominance of Washington Navel and Thomson Navel varieties (50% of the area covered) (Kerboua, 2002).

Among the diseases known to affect citrus production worldwide, three are caused by *Colletotrichum* spp.: postbloom fruit drop caused by *C. acutatum*, which affects flowers of all citrus species and induces drop of fruitlets, Lime anthracnose, also caused by *C. acutatum*, which attacks juvenile tissues of Mexican lime [*Citrus aurantifolia* (Christm.) Swing.], and

anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Timmer, 2000). The latter is also reported to cause wither-tip of twigs and tear stain and stem-end rot of fruits (Benyahia *et al.*, 2003; Huang *et al.*, 2013; Rhaïem and Taylor, 2016). However, none of these three diseases have been previously reported in Algeria.

During winter 2015, farmers reported anthracnose-like symptoms in citrus orchards in the province of Mostaganem, western Algeria. We investigated the fungal disease in 5 citrus orchards located in the Bouguirat region, Province of Mostaganem. We observed anthracnose symptoms on orange trees in all sites and obtained 30 fungal isolates of the same fungus from symptomatic leaves and twigs. We proved this fungus was the causal agent of orange anthracnose by performing Koch's postulates.

### Material and methods

#### Isolates and morphology

Thirty fungal isolates were obtained from 5 locations in Bouguirat, Mostaganem province. Each iso-

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late corresponds to a different tree. Pieces of 5 × 5 mm were taken from the margin of diseased tissue (leaves, twigs) presenting necrosis. They were surface-sterilized in 1% sodium hypochlorite for 30 s, rinsed three times in sterile distilled water and dried on sterile filter paper. Samples were plated on potato dextrose agar (PDA) and incubated at 25°C, in the dark, for 7 days.

The growing tips of hyphae developing from the leaf disks were then transferred aseptically onto fresh PDA. Size and shape of conidia were recorded from the colonies grown on PDA plates at room temperature (23–25°C). Monospore isolates were prepared from a representative culture of each sampling site, three of them were isolates from leaves and two were isolates from twigs. Pure cultures were stored at 4 °C.

### Pathogenicity tests

Koch's postulates were tested by inoculating detached leaves. Three orange leaves (cv. Thomson Navel) were surface sterilized for 10s in 96% alcohol, washed in sterile water and dried on sterile towel paper. Conidia were collected in sterile distilled water from 7-day-old cultures on PDA medium and adjusted to a concentration of 1×10<sup>6</sup> conidia mL<sup>-1</sup>. Tween 20 (final concentration 0.01%) was added to the spore suspension as a surfactant agent.

The surface-sterilized leaves were placed in a plastic box containing sterile tissue paper soaked with sterile distilled water to maintain 95% relative humidity (Montri *et al.*, 2009). They were inoculated by using the wound/drop method and non-wound/drop inoculation method (Than *et al.*, 2008; Montri *et al.*, 2009; Rhaiem and Taylor, 2016). Five drops of 6 µL were placed onto the leaf surface following wounding with a sterile needle or without wounding. Control leaves were inoculated with sterile distilled water plus 0.01% Tween 20. The inoculated leaves were incubated in containers at room temperature on the bench under natural light for 7 days. Twelve isolates were used, and the tests were repeated twice.

### DNA extraction

Discs from the growing margin of single spore colonies on PDA were used to inoculate 200 mL potato dextrose broth (PDB, Difco) in 250 mL flasks. After incubation at 25°C for 7 days, mycelia mats were harvested by filtering through Whatman paper n° 1

and lyophilized. Genomic DNA was extracted with 2% hexadecyltrimethyl-ammonium bromide (CTAB) extraction buffer (1.4 M NaCl, 0.1 M Tris-HCl pH: 8.0, 20 mM EDTA, 0.2%-mercaptoethanol) using the method of Weising *et al.* (1991). DNA concentration was measured in a spectrophotometer at 260 nm.

### Polymerase chain reaction and sequence analysis

The ITS gene region was amplified by PCR using the universal ITS1 and ITS4 primers (White *et al.*, 1990), in a 25 µL reaction volume containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, 25 mM Mg Cl<sub>2</sub>, 0.2 mM dNTPs, 2 µg mL<sup>-1</sup> fungal DNA and 1 Unit Taq polymerase (Invitrogen). Primer concentrations were 0.2 µM for ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC), respectively. Amplifications were performed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) with an initial denaturation step of 1 min at 94°C followed by 32 cycles of 30 sec at 94°C, 1 min at annealing temperature (50°C), 1 min at 72°C, and an extension step of 10 min at 72°C.

Amplification products were separated electrophoretically in a 1.5% agarose gel in 40 mM Tris-acetate, 1 mM EDTA pH 8.0 (TAE) buffer. The gels were stained with ethidium bromide (0.1 µg mL<sup>-1</sup>) and visualized under UV light. Size marker was 1 Kb ladder from Invitrogen.

PCR products of the ITS regions were sequenced by Sanger sequencing with sequenase kit (ThermoFisher Scientific) after exo-SAP treatment, using universal ITS1 and ITS4 primers at the Genomics facility at Auzeville, France (GeT-PlaGe) as described by the manufacturer.

Sequences were aligned with ClustalW and proved to be identical for the 5 monospore isolates, with only one nucleotide differing.

## Results and Discussion

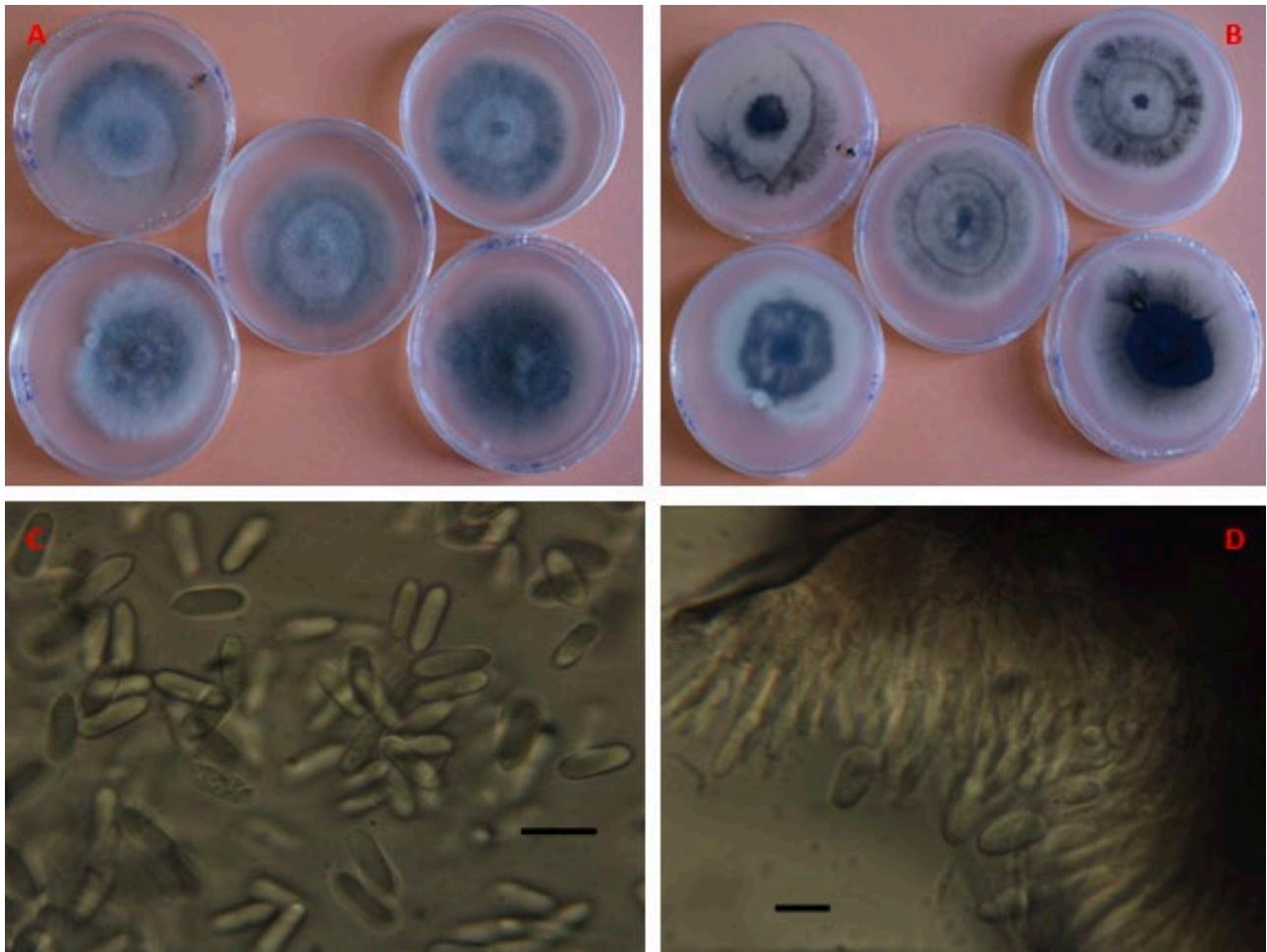
In the examined mixed citrus orchards, sweet orange varieties Thomson Navel and Washington Navel and lemon trees were affected by anthracnose disease, whereas clementines did not show any symptoms. Affected twigs were desiccated and defoliated several centimeters from their tips (Figure 1a). The leaves showed large brown, confluent necrotic patches. Black fructifications of the fungus were observed on the surface of the lesions on leaves (Figure 1b, c) and twigs.



**Figure 1.** Anthracnose symptoms observed on sweet orange cv. Thomson Navel (A); leaf lesions associated with black fructifications (acervuli) on orange leaf (B) and on lemon leaf (C).

Fungal cultures were obtained from diseased leaves and twigs and purified as single spore isolates. After 7–10 days of growth on PDA medium, colonies exhibited a fluffy mycelium, initially white gray, with abundant bright orange conidiomata (acervuli) (Figure 2a, d). Mean radial growth rate was 11.3 mm per day at 25°C in the dark, during 7 days of culture.

Conidia, borne on conidiophores, were cylindrical and unicellular with a mean length of 12.87  $\mu$ m, ranging from 10.4  $\mu$ m to 16.2  $\mu$ m, and mean width of 4.3  $\mu$ m, ranging from 3.12  $\mu$ m to 5.2  $\mu$ m, and were rounded at the ends (Figures 2c, d). The mycelium of growing culture was hyaline, septate and branched. Setae (dark-pigmented, unbranched, thick-walled sterile hyphae usually pointed at the tip) were



**Figure 2.** Cultural and microscopic characters of *Colletotrichum gloeosporioides* isolated from orange leaves after 7 days on PDA. A, culture on PDA (upper surface); B, culture on PDA (reverse surface); C, conidia ( $\times 600$ ); D, acervulus and conidiophores bearing conidia. Bars= 20  $\mu\text{m}$ .

abundant. According to morphological and cultural characteristics on PDA medium, the isolates were tentatively identified as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc (Sutton 1980; 1992).

Sequences of the rDNA ITS region confirmed this identification as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (*Ascomycota*: *Sordariomycetes*: *Glomerellaceae*) with 99 to 100% sequence identity to the sequence of the ex-type isolate (Genbank accession number JX010152.1, culture accession number ICMP 17821). The sequence was identical for the five isolates with the exception of a single nucleotide polymorphism (T instead of C) at position 57, which distinguished the two twig isolates from the three leaf iso-

lates. The sequences of a leaf and a twig isolate were deposited in Genbank with the accession numbers MH014782 and MH015008, respectively. According to Gautam (2014) *C. gloeosporioides* primarily invades injured or weakened tissues of plants. In agreement with these reports, all tested isolates caused anthracnose lesions on orange leaves only when inoculated by the wound/drop method. Dark brown lesions appeared at the inoculation sites after 48–72 hours of incubation (Figure 3). Leaves inoculated without prior wounding and control leaves did not develop any symptoms. Koch's postulates were fulfilled by re-isolating the fungus from lesions of inoculated leaves, thus proving that these isolates are the anthracnose



**Figure 3.** Necrotic lesions caused by *Colletotrichum gloeosporioides* on artificially inoculated orange leaves (injured/drop method) after 48 hours of incubation. Control leaf on the left.

infectious agent in this crop. Our experiments show that the parasite requires wounds to penetrate the leaves, hence we consider that injuries caused by biotic and abiotic factors allow development of citrus anthracnose.

*Colletotrichum gloeosporioides* was previously reported to cause wither-tip of twigs and tear stain on citrus fruits in Morocco (Benyahia *et al.*, 2003) and Tunisia (Rhaiem and Taylor, 2016) but the disease has not previously been reported from Algeria. This is the first report of *C. gloeosporioides* infecting citrus in Algeria.

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