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Characterization and pathogenicity of *Colletotrichum* spp. causing citrus anthracnose in Tunisia

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Abstract. In the winter and spring of 2014–2015, typical anthracnose symptoms were detected on different citrus varieties in Cap-Bon and Morneg-Tunis regions of Northern Tunisia. Surveys were conducted to determine the casual agents of these symptoms. A total of seven monosporic isolates were obtained from dark lesions on fruits, flowers, leaves and twigs of citrus from six orchards. One *Colletotrichum karstii* (from the *C. boninense* species complex) and six *C. gloeosporioides* isolates were identified through morphological analysis and sequencing of their ITS rDNA sequences. Pathogenicity tests with the seven isolates were performed on symptomless, detached citrus fruits (Valencia orange and Eureka lemon). All tested isolates caused anthracnose lesions after 1 week of incubation. Koch's postulates were fulfilled by re-isolation of pathogens from the inoculated fruits. This report elucidates the diversity of anthracnose pathogens in Tunisia. This is the first report of *C. karstii* from citrus in Tunisia.

Keywords. Citrus anthracnose, *Colletotrichum karstii*, *Colletotrichum gloeosporioides*, PCR.

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INTRODUCTION

The citrus industry is one of the most important fruit industries worldwide. Mediterranean countries are second only to China for citrus production, and are the largest exporter after South Africa (FAO, 2016). In Tunisia, citrus orchards cover 27,000 ha and include approx. 7 million trees. During the last decade, citrus production has steadily increased from 300,000 MT in 2007/08 as new orchards have begun production, reaching 560,000 MT in 2016/17. The most important crops are Maltese and Navel oranges [*Citrus sinensis* (L.) Osbeck], clementines (*Citrus* x *Clementina*), lemons (*C. limon* L.) and mandarins (*C. reticulata* Blanco) (Haas, 2017).

Knowledge of the pathogens affecting citrus crops is important. Previous investigations have demonstrated that these crops are affected by many fungal diseases such as Alternaria brown spot (Haddad *et al*., 2013), gummosis (Boughalleb-M'Hamdi *et al*., 2018) and anthracnose (Ben Hadj Daoud, 2013; Rhaiem and Taylor, 2016).

In the present study, we focus on citrus anthracnose. *Colletotrichum* is among the most economically important genera of plant pathogenic fungi worldwide (Sutton, 1992; Cai *et al*., 2009; Phoulivong, 2011). This genus has been designated the eighth most important group of plant pathogenic fungi, based on perceived scientific and economic importance (Dean *et al*., 2012). Several species of *Colletotrichum* cause diseases (anthracnoses) of a wide range of important crops (Sutton, 1992; Hyde *et al*., 2009a). Two species complexes, *C. acutatum* and *C. gloeosporioides* encompass the main causal agents of citrus diseases. Three important diseases of citrus caused by *Colletotrichum* spp. are anthracnose, post-bloom fruit drop and key lime anthracnose (Timmer *et al*., 2000; Lima *et al*., 2011; McGovern *et al*., 2012).

Colletotrichum gloeosporioides is reported as pathogen of the main cultivated citrus species worldwide (Huang *et al.,* 2013). *Colletotrichum gloeosporioides sensu stricto* occurs more frequently in the Old World, contrasting with the greater importance of *C. acutatum* in the Americas (Lima *et al*., 2011). Before multi-gene analysis, *C. acutatum* was identified as the only species responsible for post-bloom fruit drop (PFD) (Peres *et al.,* 2008) and key lime anthracnose (KLA) (Brown *et al*., 1996). Similarly, *C. gloeosporioides* was reported as the only *Colletotrichum* species to cause citrus fruit anthracnose (Timmer *et al*., 2000). PFD of citrus was first described in Belize (formerly British Honduras) in 1979, caused by *C. gloeosporioides* (Penz.) Sacc. (Denham, 1979; Fagan, 1979). This disease has been detected in Argentina, Brazil, Mexico and the United States (Schwarz *et al*., 1978; Orozco Santos and Gonzalez Garza, 1986; McMillan and Timmer, 1989; Kuramae-Izioka *et al*., 1997). PFD was widespread in the western hemisphere in the 1990s, including in Belize, Bermuda, Brazil, Costa Rica, Mexico and the United States (Florida), and has become less of a problem in Florida in recent years. However, PFD could re-emerge as a severe citrus disease (McGovern and Rouse, 1994; Timmer and Brown, 2000). In Florida, the causal agents of PFD and KLA were initially identified as strains of *C. gloeosporioides* (Agostini et *al*., 1992). Genetic characterization of *Colletotrichum* isolates from Brazil suggested that PFD can be caused by both *C. acutatum* and *C. gloeosporioides* (Kuramae-Izioka *et al*., 1997). A report from south Brazil identified *C. acutatum* as the cause of PFD (Theodoro *et al*., 2003). *Colletotrichum gloeosporioides* was reported as a causal agent of PFD on sweet orange (Lima *et al*., 2011; McGovern *et al*., 2012). An extensive investigation in citrus orchards of São Paulo state (Brazil) revealed only *C. abscissum* and *C. gloeosporioides sensu stricto* associated with PFD disease (Da Silva *et al*., 2016). Key lime anthracnose, a disease complex on leaves, flowers and fruits of Key lime, was initially reported to be caused by *C. acutatum* (Brown *et al*., 1996; Peres *et al*., 2008; MacKenzie *et al*., 2009), but later classified as *C. limetticola* (Damm *et al*., 2012a).

Following adoption of the use of multi-gene phylogenetic analyses, the polyphasic protocols for studying the genus *Colletotrichum* significantly changed the classification and species concepts this genus (Cannon *et al*., 2012; Damm *et al*., 2012a; 2012b; 2013; 2014; Weir *et al*., 2012). In China, at least 13 species of *Colletotrichum* have been associated with citrus (Peng *et al*., 2012). Huang *et al.* (2013) reported the presence of four important species complexes of *Colletotrichum* in citrus in China, namely the complexes of *C. gloeosporioides*, *C. boninense* and *C. acutatum,* and a group including *C. truncatum*. The species in the *C. gloeosporioides* species complex comprised *C. gloeosporioides* and *C. fructicola*, the *C. boninense* complex included *C. karstii* and a new species *C. citricola*, and the *C. acutatum* complex included a new species, *C. citri*. The most important causal agent is *C. gloeosporioides*, and *C. gloeosporioides sensu lato* was also reported to cause pre-harvest symptoms such as wither-tip on twigs, tear-stain (Klotz and Fawcett, 1941; Benyahia *et al*., 2003) and stem-end rot on fruit (Kaur *et al*., 2007).

Colletotrichum acutatum sensu stricto, as member of *C. acutatum* species complex, was found on citrus for the first time in Europe by Guarnaccia *et al.* (2017). This fungus was isolated in many citrus growing areas surveyed during 2015 and 2016, including Spain, Italy, Portugal, Greece and Malta, and from several citrus species showing typical anthracnose symptoms on leaves fruits and twigs. *Colletotrichum novaezelandiae* (from the *C. boninense* species complex) was also first reported on citrus. Infections caused by *Colletotrichum* spp. Have strongly compromised citrus production in Mediterranean countries. In Portugal, since 2003, anthracnose symptoms were detected in lemon and orange orchards. Ramos *et al.* (2006) found isolates with characteristics that did not fit those commonly described for *C. acutatum* and *C. gloeosporioides*, raising the hypothesis of other species of *Colletotrichum* were involved on preharvest anthracnose of citrus. Ramos *et al.* (2016) demonstrated that many isolates collected from symptomat-

ic leaves, twigs and fruits of several citrus cultivars in Portugal were *C. gloeosporioides sensu stricto* predominated in flowers and fruits, and *C. karstii* was found in leaves and branches. In Italy, pre-harvest anthracnose symptoms appeared on orange fruits, caused by *C. gloeosporioides* and *C. karstii* (Aiello *et al.*, 2015). *Colletotrichum karstii*, a member of the *C. boninense* species complex, has been reported on many host plants with wide geographical distribution (Damm *et al.*, 2012b). In Europe, this species has been detected on tropical fruits, cotton and lupin plants (Damm *et al.*, 2012b; Ismail *et al.*, 2015). *Colletotrichum karstii* was previously found to be an important pathogen on *Orchidaceae* hosts (Yang *et al.*, 2011), and has also been isolated from citrus plants in South Africa, New Zealand (Damm *et al.*, 2012b) and China (Peng *et al.*, 2012). Peng *et al.* (2012) showed that this species causes citrus leaf anthracnose. Guarnaccia *et al.* (2017) reported the presence of three major species complexes in surveyed citrus regions in European countries namely, *C. gloeosporioides sensu stricto* and two new species (*C. helleniense* and *C. hystricis*) were identified in the *C. gloeosporioides* species complex. *Colletotrichum karstii*, *C. novaezelandiae* and two new species (*C. catinaense* and *C. limonicola*) in the *C. boninense* species complex, and *C. acutatum sensu stricto* was also identified as member of *C. acutatum* species complex.

Anthracnose diseases of citrus in Tunisia had not been well researched. Since early 2009, wither-tip symptoms of twigs, resembling anthracnose, were observed on cultivated citrus trees in Northern Tunisia. Using morphological and molecular characteristics and pathogenicity tests on isolates collected from withered twigs, Rhaiem and Taylor (2016) reported for the first time the presence of *Colletotrichum gloeosporioides* as the causal agent of anthracnose disease of citrus in Tunisia. This fungus was also identified on olive by Rhouma *et al.* (2010). Species of *C. acutatum sensu stricto* were found by Chattaoui *et al.* (2016) on olives fruits from Northern Tunisia, showing symptoms of anthracnose since November 2011. The *C. acutatum sensu stricto* group was recorded for the first time in the country as the causal agent of olive anthracnose. Because of the commercial yield losses in citrus orchards caused by *Colletotrichum* infections in Tunisia, further research was required to identify *Colletotrichum* spp. related to citrus anthracnose. In our surveys commencing in April 2014, severe anthracnose symptoms, such as the typical dieback and wither-tip of twigs and dark lesions on fruits, leaves and flowers, were observed on different citrus varieties in orchards of Cap-Bon and Morneg-Tunis (Northern Tunisia).

The objectives of the present investigation were to: (i) evaluate symptoms of anthracnose in surveyed citrus orchards; (ii) characterize the *Colletotrichum* spp. causing anthracnose on citrus in Tunisia, using morphological and molecular techniques; and (iii) evaluate the pathogenicity of the species on Valencia orange and Eureka lemon.

MATERIALS AND METHODS

Fungus isolations

Ten citrus fruit, twig and leaf samples with typical anthracnose symptoms were collected from orange varieties Thomson, Malti and Meski and clementine variety Cassar during spring 2014 and winter 2015. Small pieces of tissue (5 mm × 5 mm) were sterilized in sodium hypochlorite solution for 5 min, followed by several rinses with sterile distilled water (SDW), and then placed on Potato Dextrose Agar (PDA, BD DIFCO) in Petri dishes for one week at 27°C.

Monoconidial cultures were generated from each sample and stored on dry filter paper at -20°C, and were later identified morphologically. The conidia were taken from actively growing colonies and suspended in sterile water. Lengths and widths of 50 conidia from each isolate were measured under an ECLIPSE TE 2000-E microscope (×1,000 magnification) (Nikon) using differential interference contrast optics. Measurements were taken using a Nis-Elements AR (v 3.10) software (Zeiss) from images captured with a DXM1200F digital camera (Nikon). Q-value of conidia (the ratio of length to width in side view) was also calculated. The growth rate of all isolates was determined on PDA in 90 mm diam. Petri dishes, using three plates for each isolate, incubated at 25°C. Colony diameters were measured for the first 7 d after inoculation, and mean daily colony growth rates were calculated as described by Huang *et al.* (2013).

Molecular characterization and phylogeny of isolates

The identity of the isolates were examined by amplification and sequencing of ITS rDNA regions by a direct PCR approach, without DNA isolation (Iotti and Zambonelli, 2006; Mari *et al.*, 2012). Direct PCR was applied to cultures grown on PDA for 7 d at 25°C. Under a dissecting microscope, a few hyphae were taken from the aerial mycelium of each culture using a sterile needle, and were placed in a 0.2 mL capacity PCR tube containing 20 µL of sterile water. Tubes with hyphae were always maintained on ice until PCR amplification. ITS

regions were amplified as described by Iotti and Zambonelli (2006), using the universal fungal primers ITS5 and ITS4 (White *et al.*, 1990). The amplifications were carried out with an initial denaturation at 94°C for 6 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 6 min. PCR products were purified using Nucleospin PCR cleanup kit (Macherey Nagel) and sequenced using both the amplification primers by the Eurofin Genomics sequencing service (https://www.eurofinsgenomics.eu/en/custom-dna-sequencing.aspx). The sequences were analysed using the BLASTn program with default parameters (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1990), and they were aligned using the ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Bayesian inference and maximum likelihood were applied to confirm the phylogenetic affiliation of isolates from this study. Two separate phylogenies were constructed for *C. boninense* and *C. gloeosporioides* species complexes. Sequences generated in this study were used in conjunction with those of Damm *et al.* (2012b) and Weir *et al.* (2012). One or more sequences for each *Colletotrichum* sp. were selected for tree construction, by giving priority to those from type specimens (NR GenBank accession prefix) when available. The best fit model of nucleotide substitution (TrNef + I) was selected by jModelTest 2.1.4 (Darriba *et al.*, 2012). Bayesian analysis was performed by two independent runs, with four chains each, and were carried out for 5 million generations, sampling every 100 generations. A 50% majority-rule consensus tree was generated after exclusion of the first 250 trees. Maximum likelihood (ML) analysis was performed in Raxml GUI 1.1.3 (Silvestro *et al.*, 2012). The ML search included 100 random addition replicates and 1,000 nonparametric bootstrap replicates.

Pathogenicity tests

Seven *Colletotrichum* isolates were grown on PDA for 1 week at 27°C, and were used in pathogenicity tests to compare their virulence and evaluate the susceptibility of citrus varieties Valencia orange and Eureka lemon. Ten fruits of each variety were inoculated with each isolate. The fruits were washed, disinfected by immersion for 10 min in sodium hypochlorite solution , rinsed twice in SDW, and were each inoculated by placing a 10 µL droplet of spore suspension (10^5 conidia mL^{-1}) on the surface. Inocula were obtained from cultures grown on PDA for 10 d at 25°C. Conidia were washed from the dishes, passed through four layers of cheesecloth, and diluted in sterile distilled water to 10^5 conidia mL^{-1}. Fruits were previously injured using a sterile tip (wounds of 2 mm diam.). Control fruits were inoculated with SDW. Fruits were each labelled with a permanent marker, and the inoculation points were circled on each fruit. The fruits were then incubated in plastic containers at 25°C and 100% relative humidity, and were examined for lesion development 7 d after inoculation (Aiello *et al.*, 2015). After 14 d, spores from infected fruits were aseptically transferred and sub-cultured onto PDA plates, which were incubated at 25°C. The resulting cultures were checked for colony and spore morphology to confirm Koch's postulates.

Statistical analyses

Data of conidium size, colony growth rate and isolate pathogenicity were compared using one way ANOVA, and mean values among treatments were compared by the Tukey's *post hoc* test ($P < 0.05$).

RESULTS

Surveys, and Colletotrichum *species isolation*

Surveyed orchards located in major citrus production areas in Northern Tunisia showed typical symptoms of anthracnose. These consisted of typical dieback and wither-tip of twigs showing orange conidium masses, which were observed on different citrus varieties (Figure 1). On fruits, symptoms were small, light to dark irregular and sunken lesions (Figure 2). On leaves, circular, light tan, flat areas with purple margins, containing black acervuli, were observed (Figure 3). Seven *Colletotrichum* spp. isolates were obtained from surveyed orchards of citrus growing areas from Northern Tunisia. Four isolates were from orange Malti, one was from orange Meski, one was from orange Thomson and one was from clementine Cassar. Three isolates were from fruits, two were from leaves, one was from a flower and one was from a twig (Table 1).

Morphological and cultural characteristics of isolated fungi

The seven isolates were initially characterized by morphological analysis of mycelium and conidia. Colonies of most of the isolates (six) closely resembled those typically formed by *C. gloeosporioides*, with dense, raised, cottony mycelium, which, after 7 d, turned from white or pale white (for isolate C21) and pink to orange (for isolates C2, C9, C31 and C32) (Figure 4). Conidia

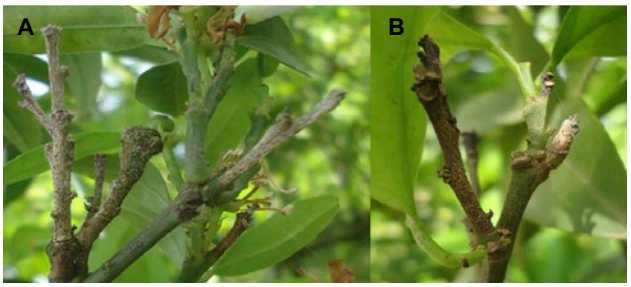

Figure 1. Symptoms on citrus twigs caused by *Colletotrichum* spp. A) wither-tip of twigs of clementine Cassar; B) orange conidiu masses of *Colletotrichum* spp. on twigs of clementine Cassar.

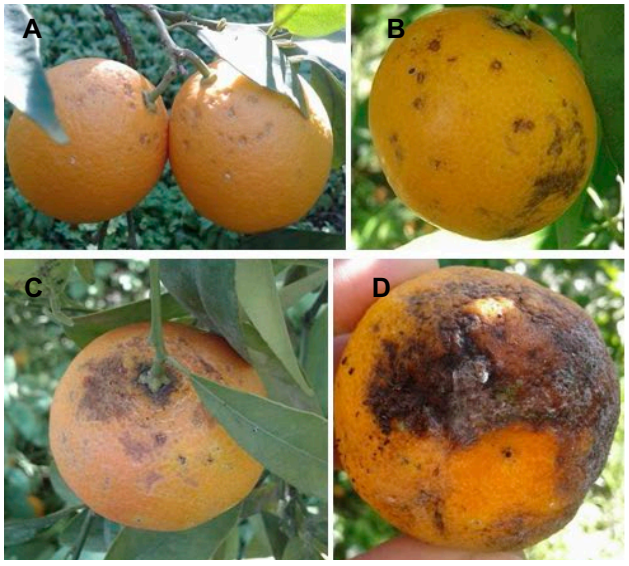

Figure 2. Symptoms on citrus fruits caused by *Colletotrichum* spp. A) Brown and dark lesions on orange, and B) on clementine. C) Stem-end rot on clementine. D) Typical anthracnose on fallen clementine fruit.

Table1. Characteristics of *Colletotrichum* species used in this study.

Isolate	Date of isolation	Location	Host citrus variety	Host tissue	GenBank[a]
C35	11/02/2015	Morneg	Malti	Fruit	MF581072
C2	14/04/2014	Hammamet	Meski	Flower	MF581073
C5	14/04/2014	Hammamet	Cassar	Leave	MF581074
C9	23/04/2014	BouArgoub	Malti	Leave	MF581075
C21	11/02/2015	Morneg	Malti	Fruit	MF581076
C31	11/02/2015	Morneg	Thomson	Twig	MF581077
C32	11/02/2015	Morneg	Malti	Fruit	MF581078

[a] Accession number of ITS sequences.

Figure 3. Anthracnose symptoms on leaves of clementine (A, B and C).

were consistently cylindrical with rounded or obtuse ends, and measured 7 to 21 × 2 to 5 µm (average = 10.9 × 3.7 µm) (Table 2). Some cultures developed black acervuli and bright orange spore masses, which were produced in the colony centres (Figure 4), as typical for *C. gloeosporioides*. In contrast, colonies of isolate C35 were similar to those produced by *C. karstii,* with moderately dense white-orange early mycelium, which, after 7 d, turned pink/orange (Figure 5). Conidia were cylindrical, hyaline and with round apices, and measured 11 to 16 × 4 to 6 µm (average = 13.1 × 4.7 µm) (Table 2). Statistical analyses of conidium dimension data revealed significant differences in length, width and Q-values. The conidia of C35 isolate were longer and their Q-value was greater than those of the other six isolates (Table 2). Statistically significant differences of conidium dimensions were also found among these six isolates.

The mean daily growth colony rate of the isolates, measured at 25°C, ranged from 4.1 to 6.9 mm (Table 2). Colonies of C35 grew more rapidly than the other isolates, and differences in colony growth rates were found between the isolates (Table 2).

PCR amplification and sequence analysis

Sequencing of the ITS regions of the seven isolates was performed in order to allow species identification. PCRs generated amplicons of 599 bp (isolates C2, C5, C9, C21, C31, and C32) and 618 bp (C35). BLASTn search against GenBank revealed that the ITS sequences of seven isolates were identical to those of *C. gloeosporioides* (for isolates C2, C5, C9, C21, C31, C32) and *C. karstii* (isolate C35). Sequence similarity ranged between 99.8 and 100% among *C. gloeosporioides* isolates (one variable position in ITS1) and 91.1 and 91.3% between *C. gloeosporioides* and *C. karstii* isolates (37 to 38 variable positions in ITS1 and six in ITS2). Phylogenetic reconstruction of the *C. boninense* and *C. gloeosporioides* species complexes placed the

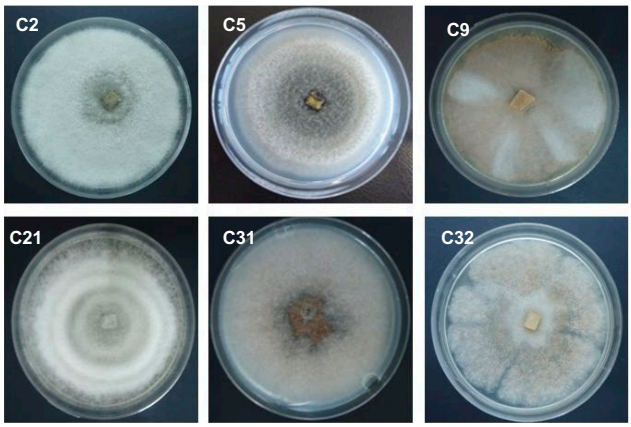

Figure 4. Morphology of *Colletotrichum gloeosporioides* isolates C2, C5, C9, C21, C31 and C32 on PDA plates incubated at 25 °C for 7 d.

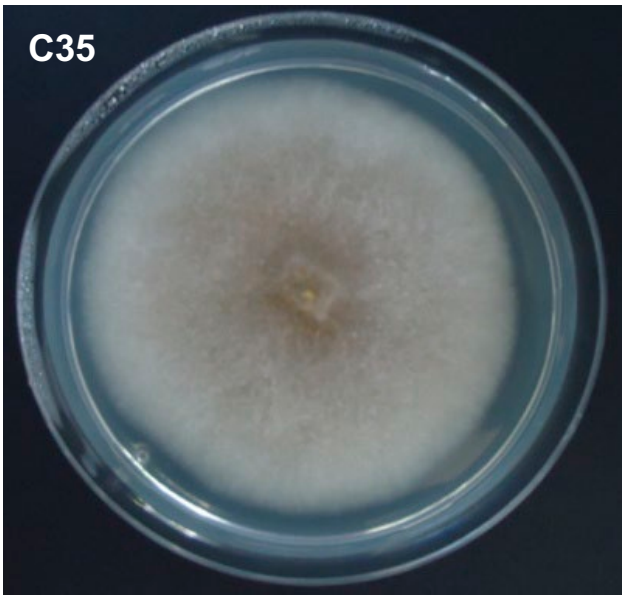

Figure 5. Morphology of *Colletotrichum karstii* (*C. boninense* complex) isolate (C35) on PDA plates incubated at 25 °C for 7 d.

isolates from this study into the clade *C. gloeosporioides sensu stricto* (isolates C2, C5, C9, C21, C31, C32) and the clade *C. karstii* (isolate C35) (Figure 6). Unlike other clades in the genus, both of these clades were well supported, by posterior probabilities and by ML bootstrap values. Two distinct subclades were also evident within the *C. gloeosporioides* clade, and respective Tunisian isolates were present in both. No correlations were found between ITS types and the metadata of the respective isolates (e.g. host tissue and variety, collection site).

Pathogenicity tests

The seven studied isolates induced symptoms on Valencia orange and Eureka lemon, which were identical to those observed in citrus orchards. One week post-inoculation, brown sunken and dark lesions appeared surrounding the inoculation points. After 14 d, the lesions contained dense mycelium, orange conidium masses and black acervuli (Figure 7). No lesions developed on fruits inoculated with water.

The data obtained from this assay were analysed to compare virulence of the studied isolates, taking into consideration the different susceptibilities of orange and lemon. Pathogen virulence and host species susceptibil-

Table 2. Conidium dimensions and growth rates of seven *Colletotrichum* isolates grown on PDA at 25°C. Isolates C2, C5, C9, C21, C31 and C32 are *C. gloeosporioides* and isolate C35 is *C. karstii*.

Character	C2	C5	C9	C21	C31	C32	C35
Length of conidia (µm)							
Mean ± SE[a]	9.6[b] ± 0.2 CD[c]	9.1 ± 0.2 D	10.9 ± 0.2 B	10.3 ± 0.2 BC	11.1 ± 0.2 B	8.9 ± 0.2 D	13.1 ± 0.2 A
Range	7.4-13.8	7.4-11.0	8.3-21.1	8.3-15.7	9.4-14.6	7.4-10.5	10.8-16.1
Width of conidia (µm)							
Mean ± SE	3.7 ± 0.1 D	3.9 ± 0.1 CD	4.1 ± 0.1 BC	4.1 ± 0.1 BC	4.2 ± 0.1 B	3.8 ± 0.1 D	4.7 ± 0.1 A
Range	2.6-5.3	2.9-5.0	2.4-4.9	2.9-4.9	3.4-5.0	2.7-4.6	4.0-5.9
Q-value of conidia							
Mean ± SE	2.6 ± 0.1A	2.4 ± 0.1B	2.7 ± 0.1A	2.6 ± 0.1AB	2.7 ± 0.1A	2.4 ± 0.1B	2.8 ± 0.1A
Growth rate (mm)							
Mean ± SE	4.6 ± 0.5B	4.3 ± 0.5 B	4.5 ± 0.5 B	4.1 ± 0.5 B	5.1 ± 0.5 B	4.3 ± 0.5 B	6.9 ± 0.5 A

[a]SE = standard errors of means.
[b]Each value represents the mean of 50 measurements.
[c]Values derived from the average of 50 measurements. Different letters accompanying means in each row indicate statistically significant differences ($P < 0.05$) according to Tukey's *post hoc* test.

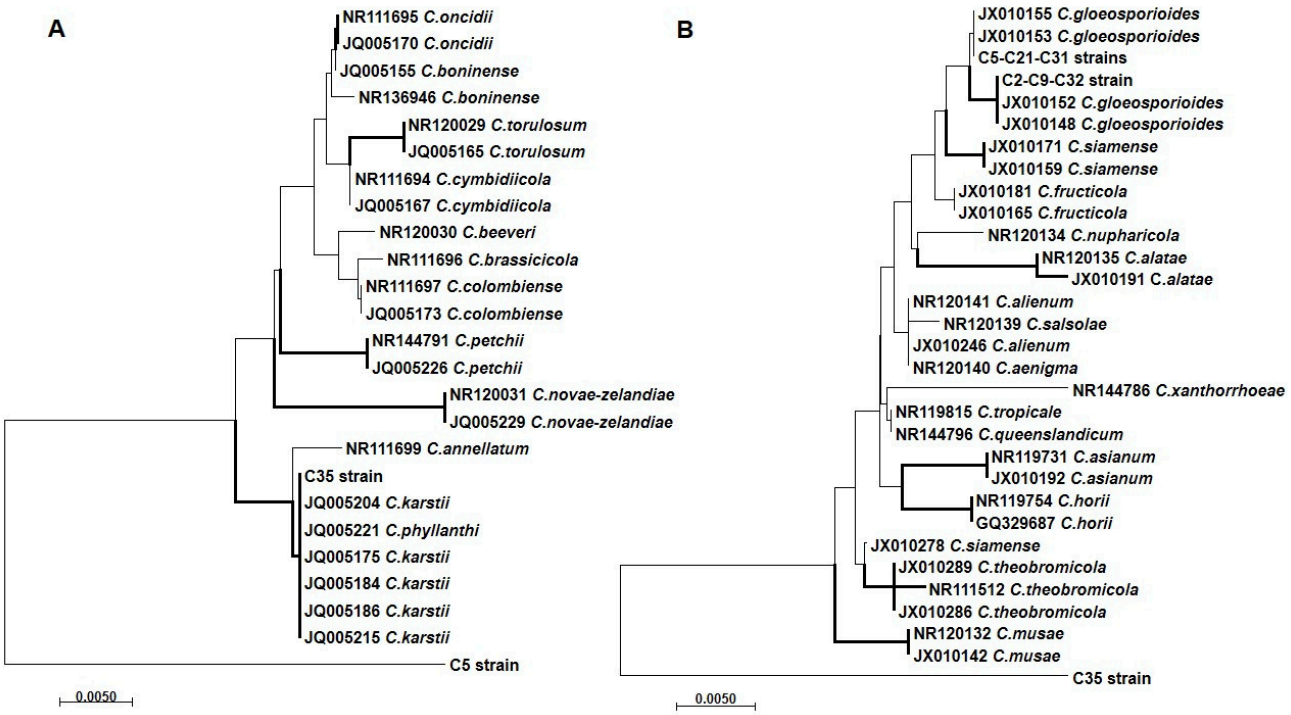

Figure 6. Maximum likelihood trees of *Boninense* (A) and *Gloeosporioides* (B) species complexes, based on the ITS1-5.8S-ITS2 region. Topologies resulting from maximum likelihood and Bayesian inference were congruent and the most likely trees are showed. Thickened branches are supported by likelihood bootstrap values of >70% and by Bayesian posterior probabilities of >95%. Taxa are labelled by their GenBank code (NR codes identify the type vouchers) or isolate code. Sequences from GenBank were selected based on results after Dumm *et al.* (2012) and Weir *et al.* (2012). Sequences of isolates C5 and C35 were differentially used as outgroups. The tree was edited using FigTree v 1.4.3.

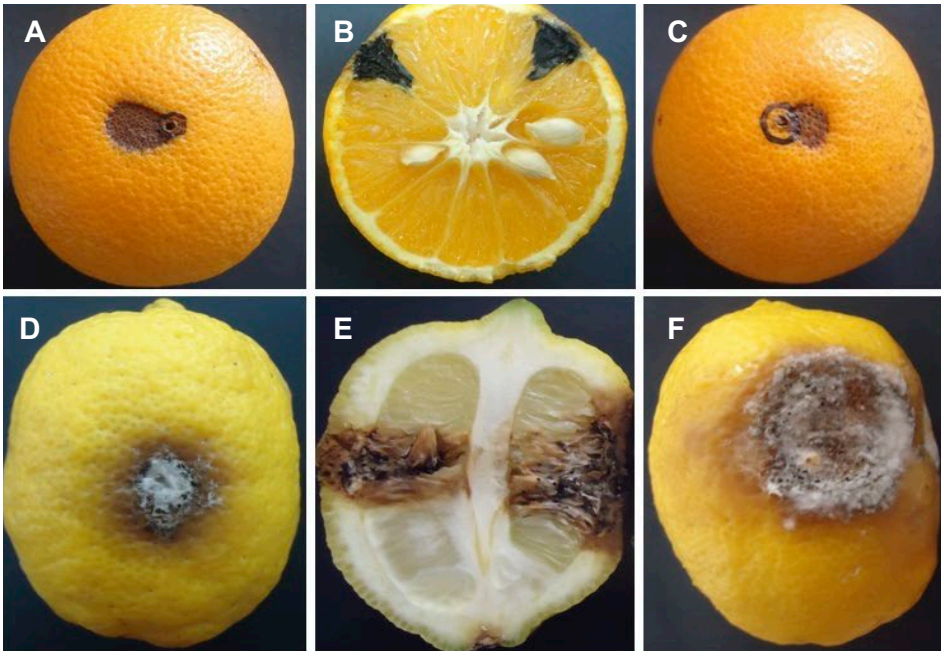

Figure 7. Lesions caused by *Colletotrichum karstii* (isolate C35) on artificially inoculated fruits of Valencia sweet orange (A and B) and on fruits of Eureka lemon (F). Lesions caused by *C. gloeosporioides* (isolate C31) on artificially inoculated fruit of Valencia sweet orange (C) and on fruits of Eureka lemon (D and E).

Table 3. Mean lesion diameter caused by *Colletotrichum* isolates on Valencia sweet orange and Eureka lemon.

Citrus variety	Isolate						
	C2	C5	C9	C21	C31	C32	C35
Valencia orange							
Inoculated fruit	5.4[a] ± 0.1[b] AB	0 ± 0.1 C	6.7 ± 0.1AB	5.3 ± 0.1 AB	8.2 ± 0.1 A	3.4 ± 0.1 BC	8.0 ± 0.1 A
Control fruit	0	0	0	0	0	0	0
Eureka lemon							
Inoculated fruit	24.0 ± 0.3 B	24.3 ± 0.3 B	22.6 ± 0.3 B	20.4 ± 0.3 B	36.8 ± 0.3 A	19.0 ± 0.3 B	13.6 ± 0.3 B
Control fruit	0	0	0	0	0	0	0

[a] Each value represents the mean of five replicates.
[b] SE = standard errors of means.
[c] Different letters accompanying means in each row indicate statistically significant differences for ($P < 0.05$), Tukey's *post hoc* test.

ity were statistically different as demonstrated in ANOVA ($P < 0.05$). For mean lesions diameter, on Valencia orange, the most aggressive isolates were C35 and C31 (Table 3), whereas no lesions were obtained with the isolate C5. On Eureka lemon, the most aggressive isolate was C31, which produced lesions three times larger than those of isolate C35. The data also showed that Eureka lemon was more susceptible to all of the *Colletotrichum* isolates compared to Valencia orange. For example, isolate C35 caused smaller lesions than the other isolates (Table 3). Isolations from lesions of inoculated fruits of the two citrus types yielded colonies identical to the original inoculated isolates, confirming Koch's postulates for these pathogenic fungi.

DISCUSSION

Symptoms of anthracnose, such as wither-tip of twigs, have been observed on cultivated citrus crops in Tunisia since 2009. *Colletotrichum gloeosporioides* was confirmed as the principal pathogen responsible for wither-tip of twigs and lesions on fruits and leaves of Encore mandarin and Minneola tangelo in Northern Tunisia (Rhaiem and Taylor, 2016). The present study has confirmed that *C. gloeosporioides* is the main causal agent of anthracnose also in clementine and sweet orange, and records for the first time in Tunisia that *C. karstii* is an anthracnose pathogen on citrus. Although ITS regions are not variable enough to separate closely related species in *Colletotrichum* (Crouch *et al.*, 2009), phylogeny obtained in the present study clearly showed that the strains were monophyletic in the *C. gloeosporioides sensu stricto* and *C. karstii* clades. Isolate C35 could also belong to *C. phyllanti*, a sister species of *C. karstii*, with identical ITS sequence, but *C. phyllanti* has only been recorded found in India on *Phyllanthus acidus*, *Bahuinia variegate* and *Bougainvillea glabra* (Damm *et al.*, 2012b; Sharma and Shenoy, 2013). *Colletotrichum karstii* could represent a species complex, although more isolates need to be analysed to validate this hypothesis (Sharma and Shenoy, 2013).

Severe symptoms of anthracnose were observed on fruits, flowers, leaves and twigs in different citrus types and varieties in Northern Tunisia. These included Malti, Meski, Thomson oranges and clementine Cassar. *Colletotrichum gloeosporioides* was isolated from citrus fruits, flowers, leaves and twigs, while the unique isolate of *C. karstii* was found on fruits.

Colletotrichum karstii is a morphologically diverse species (Alizadeh *et al.*, 2015). Colour of the upper surfaces of the colonies varies from white to grey, and pink on the reverse sides (Velho *et al.*, 2014), or also white to grey, usually with pink conidium masses, and reverse sides yellow to dark brown (Yang *et al.*, 2011). According to Taheri *et al.* (2016), the surfaces of colonies vary from white to grey with orange conidium masses and entire margins, and with pale orange reverse sides. Orange conidiomata are seen on PDA with filter paper. These variations have also been observed in other *Colletotrichum* spp. (Taheri *et al.*, 2016). In *C. karstii*, conidia also have different size ranges, including: (11.5–14.5) × (5–6.5) µm (Damm *et al.*, 2012b), (13.5–15) × (4–5) µm (Aiello *et al.*, 2015) or (10–16.5) × (4–6.5) µm (Taheri *et al.*, 2016).

During the last 2 to 3 decades, *Colletotrichum* spp. associated with citrus anthracnose have attracted considerable attention from researchers (Ramos *et al.*, 2016). Post-bloom Fruit Drop (PFD) and Key Lime Anthracnose (KLA) caused by *C. acutatum sensu lato* were known to affect citrus production in many countries (Peres *et al.*, 2008). However, these diseases were not previously reported in Tunisia. *Colletotrichum* fungi belonging to the *C. gloeosporioides* species complex include a number of economically important posthar-

vest pathogens (Gan *et al*., 2017). This species is very common on *Citrus* spp. in many countries, where this fungus is responsible for KLA and postharvest diseases. It also occurs on other hosts (Weir *et al*., 2012). In Southern Italy, *C. gloeosporioides* is commonly isolated from citrus, especially from *Citrus sinensis* (Aiello *et al*., 2015; Cannon *et al*., 2008; Peng *et al*., 2012). *Colletotrichum gloeosporioides* was also reported to cause preharvest symptoms in Southern Italy, including severe lesions on fruits of sweet orange (Aiello *et al*., 2015), and wither-tip of twigs and tear stain and stem-end rot of fruits (Benyahia *et al*., 2003; Huang *et al*., 2013). *Colletotrichum karstii* has been isolated from citrus plants in South Africa, New Zealand (Damm *et al*., 2012b) and China (Peng *et al*., 2012). In Iran, five species of *Colletotrichum*, including *C. gloeosporioides sensu stricto* and *C. karstii*, were found from leaves, fruits and stems of citrus species (Taheri *et al*., 2016). Recently, *C. karstii* was described as a causal agent of citrus anthracnose in Italy (Aiello *et al*., 2015). In Portugal, *C. gloeosporioides* and *C. karstii* were identified from symptoms on leaves, branches, flowers and fruit, with *C. karstii* frequently occurring in branches and leaves of lemon in specific geographic locations (Ramos *et al*., 2016).

The pathogenicity tests carried out in the present study showed that *C. karstii* and *C. gloeosporioides* isolates were virulent for the two studied citrus fruits. In Portugal, Ramos *et al.* (2016) reported that the importance of *C. karstii* was greater in lemons compared to other citrus types, although *C. karstii* can be as virulent as *C. gloeosporioides* for some citrus organs and varieties. In Italy, pathogenicity tests with isolates of *C. gloeosporioides* and *C. karstii* clearly showed their ability to cause lesions on fruits (Aiello *et al*., 2015). However, these data demonstrated that *C. gloeosporioides* was more aggressive than *C. karstii*. Our study has shown that in Tunisia, *C. karstii* was one of the most virulent isolates on fruits of Valencia orange but it caused less severe symptoms in fruits of Eureka lemon.

Anthracnose caused by different *Colletotrichum* species could become severe threats for citrus production and for other crops when they establish in particular horticulturally important areas. Further studies on the development and spread of anthracnose caused by *C. karstii* in Tunisia are necessary, to provide knowledge of the implications of anthracnose for citrus production, and to define suitable disease management strategies.

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