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Research Paper

Characterization of mycotoxigenic *Alternaria* species isolated from the Tunisian halophyte *Cakile maritima*

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Summary. *Cakile maritima* is a typical halophyte of the Mediterranean coasts. In addition to its ecological and industrial properties, *C. maritima* has antiscorbutic, diuretic and purgative roles in folk remedies. This plant is infected by different fungal species, mainly belonging to *Alternaria* genus. Two-hundred *Alternaria* strains were collected from four different pedo-climatic areas in Tunisia, from *C. maritima* fresh plant tissues showing symptoms of *Alternaria* infection. Phylogenetic analyses of 79 representative *Alternaria* strains, were carried out using multi-locus gene sequencing. All the strains clustered in the *Alternaria* Section: 47 strains had high homology with *A. alternata* reference strain, 13 grouped with *A. arborescens* reference strain, 12 grouped with *A. mali* reference strain, and seven strains were not well defined with *A. mali* as their closest species. *In vitro* production of tenuazonic acid (TA), alternariol (AOH), alternariol-monomethyl ether (AME), and altenuene (ALT) was evaluated. Approx. 68% of strains simultaneously produced AOH, AME and TA. Only two *A. alternata* and one *A. mali* strains were ALT producing. Pathogenicity tests on leaves of *C. maritima* were carried out with 41 representative strains. *Alternaria arborescens* showed the greatest pathogenicity compared to *A. alternata* and *A. mali*, although no statistically significant differences in pathogenicity were observed. This is the first study on Tunisian populations of *Alternaria* species isolated from the extremophile *C. maritima*.

Keywords. *Alternaria* section, *Alternaria arborescens*, *Alternaria mali*, *Alternaria* mycotoxins.

Key contributions. Four clades of *Alternaria* species were identified from the halophyte plant *Cakile maritima*.

Alternaria alternata was the most abundant (60%) and especially in stems and leaves.

A new genetic entity among the *Alternaria* strains studied needs to be further characterized. The majority of *Alternaria* strains (74%) produced the AME mycotoxin.

AME and AOH were the most frequently produced mycotoxins by all the *Alternaria* strains.

Alternaria arborescens strains were the most pathogenic.

INTRODUCTION

Cakile maritima (sea rocket) is an extremophile C3 halophyte (Brassicaceae) which is widely distributed on sandy coasts (Clausing *et al.*, 2000; Kadereit *et al.*, 2005). This plant, together with *Xanthium italicum* and *Eryngium maritimum*, is typical coastal vegetation of several regions including the Black Sea coasts, the Mediterranean basin, the Atlantic coasts of North Africa and Europe, and the North Sea and Baltic Sea coasts (Clausing *et al.*, 2000). These plants grow a few meters from shorelines, and are useful barriers defending coastal ecosystems. Sea rocket is an annual succulent plant, growing in geographical areas characterized by high salinity and low soil fertility (Barbour *et al.*, 1970). It is tolerant to abiotic stress conditions such as high salinity, water stress and high temperature, which are all the characteristics of halophyte plants (Debez *et al.*, 2012). *Cakile maritima* is a good candidate model plant for understanding botanical biochemical and physiological mechanisms.

In Tunisia, *C. maritima*, in addition to its role in ecosystem coastal preservation, is also considered an edible plant, used traditionally as a green vegetable for human and animal consumption. Dried root powder is also mixed with cereal flour to make bread (Zarrouk *et al.*, 2003; Debez *et al.*, 2004). The whole plant, harvested at flowering for its high content of iron, ascorbic acid and iodine, is traditionally used in antiscorbutic, diuretic and purgative folk remedies (Kubiak-Martens *et al.*, 1999).

Spontaneous plants of *C. maritima* are colonized by fungi causing necrotic lesions on above-ground parts. Among fungal species, *Alternaria brassicicola* (Schwein.) Wiltshire (1947) has been often associated to *C. maritima* infections (Thrall *et al.*, 2000), so several studies have been carried out to evaluate this host-pathogen

interaction (Thrall *et al.*, 2000; 2002; 2005; Oliver *et al.*, 2001; Bock *et al.*, 2005; Linde *et al.*, 2010).

Alternaria is ubiquitous and abundant in the atmosphere and in soil, seeds, and agricultural commodities. This genus includes plant pathogenic and saprophytic species that may affect crops in the field or can cause harvest and postharvest decay of plant products (Logrieco *et al.*, 2009; Patriarca, 2016; Somma *et al.*, 2019). *Alternaria* spores have been related to human infections, such as rhinosinusitis, asthma, cutaneous and subcutaneous infections, and oculomycosis (Pastor and Gaurro, 2008; Canova *et al.*, 2013; Hattab *et al.*, 2019). Mycotoxins are major food contaminants affecting global food security, especially in low and middle-income countries (Moretti *et al.*, 2019). Several studies have confirmed the toxic effects of *Alternaria* metabolites for animals and humans, but these toxins are not regulated by legislation, and acceptable levels in food are not determined. *Alternaria* mycotoxins are arousing interest worldwide, and the European Food Safety Authority (EFSA) has provided scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in food and feed (Arcella *et al.*, 2016).

Among the secondary metabolites produced by *Alternaria* species, there are both phytotoxins, that can play an important role in the plant pathogenesis processes, and mycotoxins, that can be harmful to humans and animals (Logrieco *et al.*, 2009). Therefore, since *C. maritima* is used in traditional food, consumption of *Alternaria* toxin-contaminated plants can represent a serious toxicological risk for consumers (Wang *et al.*, 1996; Ostry, 2008; Logrieco *et al.*, 2009; Lou *et al.*, 2013).

The dibenzopyrone derivatives alternariol (AOH), alternariolmonomethyl ether (AME) and altenuene (ALT), and the tetramic acid derivative tenuazoic acid (TA), are among the most important mycotoxins produced by *Alternaria* species. Alternariol and AME are usually found in combination, and have teratogenic and fetotoxic effects (Fehr *et al.*, 2009). Genotoxic activity on human colon cancer cell lines has also been observed (Wang *et al.*, 1996; Ostry, 2008; Fehr *et al.*, 2009; Bensassi *et al.*, 2012). A recent study also reported that AOH was an androgen agonist in *in vitro* assay (Stypula-Trebas *et al.*, 2017).

Tenuazonic acid is a well-known mycotoxin and phytotoxin, produced primarily by *A. alternata* (Fr.) Keissl. (1912) and by other phytopathogenic *Alternaria* species, including *A. japonica* Yoshii (1941), detected on wheat in Tunisia (Bensassi *et al.*, 2009), *A. longipes* (Ellis & Everh.) E.W. Mason (1928), *A. radicina* Meier, Drechsler & E.D. Eddy (1922), and *A. tenuissima* (Kunze) Wilt-

shire (1933). In central and southern Africa, TA has been associated with human hematologic disorder known as “onalay”, a thrombocytopenia (Steyn and Rabie, 1976). The presence of these mycotoxins in wheat has also been related to elevated levels of human esophageal cancer in China (Liu *et al.*, 1992).

Morphological identification of *Alternaria* species is often difficult due to interspecific similarities and intraspecific polymorphisms (Simmons, 1990; Simmons and Roberts, 1993; Roberts *et al.*, 2000; Pryor and Michailides, 2002; Serdani *et al.*, 2002; Belisario *et al.*, 2004; Hong *et al.*, 2006). The high sensitivity of *Alternaria* to environmental factors may lead to ambiguities if taxonomy is based only on phenotype (Andrew *et al.*, 2009). “Polyphasic” approaches using morphological, molecular and chemical techniques could therefore be useful for characterization of *Alternaria* species (Andersen *et al.*, 2008; Brun *et al.*, 2013).

Although several studies have focused on the occurrence of *Alternaria* species and *Alternaria* metabolites in food commodities, little knowledge is available on the *Alternaria* species living on extremophile plants. Santiago *et al.* (2018) suggested that *Alternaria* species that colonize extremophile plant environments show great adaptation to survival under extreme conditions, such as high temperature, low water and nutrient availability, osmotic stress, desiccation, and exposure to high levels of UV radiation, so these fungi are interesting examples of fungal diversity.

Based on this lack of knowledge, the aims of the present study were: i) to isolate and estimate the distribution of *Alternaria* species on *C. maritima* growing in different bioclimatic conditions, in Tunisia; ii) to identify *Alternaria* at species level using a polyphasic approach; iii) to analyze the profile of the main mycotoxins produced by the species isolated from *C. maritima*; and iv) to test their pathogenicity on *C. maritima* leaves.

MATERIAL AND METHODS

Origin of the samples

Leaves, stems and seeds of spontaneous *C. maritima* plants, showing typical symptoms of *Alternaria* infections, were collected arbitrarily in Tunisia. Approximately 20 plants were collected per province, and five portions of each plant were used for fungal isolation. A total of 100 *C. maritima* samples were collected per province. Four provinces, from the north to the south of Tunisia and with two pedo-climatic conditions, were considered: Rades and Raouad, characterized by temperate winters and annual rainfall of 200–700 mm (semi-arid regions),

and Sfax and Djerba, characterized by temperate winters and annual rainfall of 100–200 mm (arid regions).

Fungus isolation and growth conditions

After surface-disinfection with 2% sodium hypochlorite solution for 2 min and two washings with sterilized distilled water for 1 min, symptomatic plant tissues were cut into small pieces (5 mm diam.) with a sterilized scalpel and dried on a sterile filter paper in a laminar flow cabinet. Single tissue pieces were then transferred into Petri dishes containing Potato Dextrose Agar (PDA) amended with 0.10 g L⁻¹ streptomycin sulphate salt and 0.05 g L⁻¹ neomycin. The dishes were incubated at 25 ± 1°C for 7 d under an alternating light/darkness cycle of 12 h photoperiod. From the 100 samples selected per province, 50 isolates were kept as monocolonidium and homogeneous cultures. Two hundred *Alternaria* strains were collected from the four sample sites.

Morphological characterization

Following procedures of Simmons (2007), *Alternaria* strains were grown on PDA and on Potato Carrot Agar (PCA) to characterize their morphological traits. After 7 d of incubation at 25 ± 1°C, under an alternating light/darkness cycle of 12 h photoperiod, conidium dimensions, colour, septation, conidiophore branching, and dimensions of catenulate conidia were determined using a light microscope (×40 magnification).

Molecular characterization and phylogenetic analysis of Alternaria strains

Based on morphological traits, 79 representative strains were selected for phylogenetic and chemical analyses. Thirty-eight *Alternaria* strains were from the semi-arid region (Rades: 21 and Raouad: 17) and 41 were from the arid region (Djerba: 19 and Sfax: 22).

Mycelium of 3-d-old colonies, grown on sterilized cellophane disks overlaid on PDA plates, was collected by scraping and then frozen. Genomic DNA was extracted and purified from powdered lyophilized mycelia (each sample, 10–15 mg) using the Wizard Magnetic DNA Purification System for Food kit (Promega Corporation), according to the manufacturer’s protocol. Quantity and integrity of DNA were checked at Thermo-Scientific Nanodrop (LabX, Midland, Ontario, Canada), and by comparison with a standard 1 kb DNA Ladder (Fermentas GmbH) on 0.8% agarose gel, after electrophoretic separation.

For each *Alternaria* strain, the informative target genes, allergen *alt-a1* (*alt-a1*), glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and translation elongation factor 1- α (*tef*), were amplified, respectively, with the following primer pairs: *gpd1/gpd2* (Berbee *et al.*, 1999), *alt-for/alt-rev* (Hong *et al.*, 2005), and *Alt-tef1/Alt-tef2* (Somma *et al.*, 2019), and were sequenced for phylogeny analyses.

The Polymerase Chain Reaction mixture (15 μ L), contained 15 ng of DNA template, 0.45 μ L of each primer (10 mM), 0.3 μ L of dNTPs (10mM) and 0.075 μ L of Hot Master Taq DNA Polymerase (1 U μ L⁻¹; 5 Prime). The three fragment genes were amplified using the PCR conditions of Ramires *et al.* (2018). All PCR products were visualized under UV light after electrophoretic separation in 1 \times TAE buffer on 1.5% agarose gel.

Each PCR product was purified with the enzymatic mixture Exo/FastAP (Exonuclease I, FastAP thermostable alkaline phosphatase, Thermo Scientific) and then sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), according to the manufacturer's recommendations. Both strands were purified by filtration through Sephadex G-50 (5%) (Sigma-Aldrich) and sequenced in an "ABI PRISM 3730 Genetic Analyzer" (Applied Biosystems). The FASTA sequences were obtained with BioNumerics software (Applied Maths). Phylogenetic trees of single and combined genes, following a multi-locus sequence approach, were generated using the Maximum Likelihood statistical method and bootstrap analyses (1000 replicates, removing gaps) with MEGA5 (Tamura *et al.*, 2011).

Phylogeny analysis was carried out considering the gene sequences of the following reference strains: *A. alternata* E.G.S.34.016, *A. tenuissima* E.G.S.34.015, *A. arborescens* E.G.S.39.128, *A. infectoria* ex type E.G.S.27.193, *A. brassicicola* ATCC96836, *A. capsici* BMP0180, *A. carthami* BMP1963, *A. citriarbusti* BMP2343, *A. crassa* BMP0172, *A. fragariae* BMP3062, *A. gaisen* BMP2338, *A. limoniasperae* BMP2335, *A. longipes* BMP0313, *A. macrospora* BMP1949, *A. mali* BMP3064, *A. solani* BMP0185, *A. tagetica* BMP0179, *A. tangelonis* BMP2327, *A. tomatophila* BMP2032, and *A. turkisafrina* BMP3436. The gene sequences of these reference strains were downloaded from the National Center for Biotechnology Information (NCBI) and "Alternaria Genomes Database" (AGD). *Alternaria infectoria* ex type E.G.S.27.193 was used as the outgroup in phylogenetic analyses.

Mycotoxin extraction and chemical profile analyses

Chemical analyses of the 79 *Alternaria* strains were carried out to evaluate their capabilities to produce

AOH, AME, ALT and TA. Following the methods of Li, *et al.* (2001), all the strains were inoculated onto sterilized rice grain and grown for 21 d at 25 \pm 1°C in darkness. The cultures were then dried and powdered before mycotoxin extraction.

Mycotoxin analyses were based on the methods of Rubert *et al.* (2012), with modifications. The samples were each finely ground with an Oster Classic grinder (220–240V, 50/60 Hz, 600W). Five grams of each homogenized sample were weighed in a 50 mL capacity plastic tube, and 25 mL of methanol was added. The extraction was carried out using an Ultra Ika T18 basic Ultra-turrax for 3 min. The extract was centrifuged at 4000 rpm for 5 min at 5°C. The supernatant (1 mL) was filtered through a 13 mm \times 0.22 μ m nylon filter, and diluted before injection into high performance liquid chromatography associated with a diode array detector (LC-DAD). All the extractions were carried out in triplicate.

Alternariol, AME, ALT, and TA were determined using a Merk HPLC with a diode array detector (LC-DAD) L-7455 (Merk), at 256 nm, and Hitachi Software Model D-7000 version 4.0 was used for data analysis. A Gemini C18 column (Phenomenex) 4.6 \times 150 mm and 3 μ m particle size was used as the stationary phase. The mobile phase consisted of two eluents, eluent A (water with 50 μ L L⁻¹ trifluoroacetic acid), and eluent B (acetonitrile with 50 μ L L⁻¹ trifluoroacetic acid). A gradient programme with a constant flow rate of 1 mL min⁻¹ was used, starting with 90% A and 10% B, reaching 50% B after 15 min and 100% B after 20 min which was maintained for 1 min. The gradient was then returned to 10% B for 1 min and allowed to equilibrate for 3 min before the next analysis (Myresiotis *et al.*, 2015). The limit of detection (LOD) for this method was 0.01 mg L⁻¹ and the limit of quantification (LOQ) was 0.1 mg L⁻¹. The data of mycotoxin production were statistically processed using the Prism 5 software (La Jolla; www.graphpad.com).

Pathogenicity tests

A set of 41 representative strains, belonging to all phylogenetic groups previously characterized (Figure 2), were tested for pathogenicity on *C. maritima* leaves. Twenty-four strains of the *A. alternata* group, eight of the *A. arborescens* group, five of the *A. mali* group, and four strains of unknown identity were tested. Seeds were sown into plastic pots (diam, 29 cm; height, 23 cm), filled with inert sand, which were then watered each day with distilled water until germination. The pots were maintained in a greenhouse under controlled conditions

of 16 h light/8 h dark cycles, $25 \pm 1^\circ\text{C}$ and 60% relative humidity. Resulting seedlings were irrigated each day for 2 weeks with the nutrient solution (Hewitt, 1996). At 6 weeks after sowing, leaves of the plants were cut and placed on glass slides in Petri dishes (one slide per dish) containing a moist filter paper. The leaves were then inoculated with *Alternaria* conidium suspension (10^8 conidia mL^{-1}). Control plants were grown in similar condition without fungal inoculation. Separate leaves (six replicates per inoculation treatment) were inoculated. A 10 μL droplet of conidium suspension (10^8 conidia mL^{-1} ; 0.05% Tween 20) was placed in the centre of each leaf. Controls were inoculated by a droplet of sterile distilled water 0.05% Tween 20 solution. The Petri dishes containing leaves were then incubated under laboratory conditions ($25 \pm 3^\circ\text{C}$; 12h light/12 h dark photoperiod). Disease progress was evaluated at 7 d post-inoculation (dpi) (Taheri, 2019).

After the incubation period, lesion appearance and external and internal lesion diameters were assessed. The degree of pathogenicity of each inoculated strain was assessed by calculating the diameter of the lesion. Statistical analyses of data were performed using the statistical package Statistix8. A one-way ANOVA was used to test the hypothesis of *Alternaria* species differentiation with respect to the lesion diameter, and means were compared at $P < 0.05$.

RESULTS

Host symptoms and morphological characterization of Alternaria strains infecting Cakile maritima

Symptomatic plants of *C. maritima* were sampled from two different pedo-climatic conditions (arid and semi-arid). All collected samples (stems, leaves and seeds) showed blight symptoms typical of *Alternaria* infections (Figure 1). Infected plant tissues had necrotic flecks surrounded by chlorotic halos which coalesced into nearly circular or irregularly shaped lesions.

Conidium lengths of *Alternaria* strains ranged from 6 to 29 μm (greatest average = 17.45 μm). Conidium widths ranged from 3 to 10 μm (greatest average = 5.52 μm) (Supplementary Table S1). The numbers of transverse septa varied from 4 to 11 and longitudinal septa varied from 1 to 3. Different margin types, (smooth and rough surfaces) were observed and the colour of margins varied from brown to dark brown or blackish brown. Different forms of conidia were also observed, including ellipsoidal to long ellipsoidal, subspherical, base conical, rounded or obtuse, and apex rounded.

Based on morphological traits, all the strains were identified as *A. alternata*, *A. arborescens* E.G. Simmons or *A. tenuissima*. However, variations in culture characteristics such as margin types and colony colours were observed.



Figure 1. Symptoms of *Alternaria* infections on *Cakile maritima*. Whole plant (a), stem (b), seed (c) and leaves (d).

Molecular and phylogenetic analyses of *Alternaria* strains

Seventy-nine *Alternaria* strains were identified by sequencing informative fragments of the *alt-a1*, *gpd* and *tef* genes. All PCR reactions gave amplicons of the expected sizes of approx. 620 nucleotides for *alt-a1*, 510 for *gpd* and 590 for *tef*.

For sequence alignment of the *gpd* gene, a fragment of about 470 nucleotides was analyzed; however, in the *A. brassicicola* (ATCC96836) sequence, beginning at position 104 of the fragment considered, a short deletion of 27 nucleotides was observed. For *tef* and *alt-a1* sequence alignments, common fragments of about 490 nt (*tef*) and 460 nt (*alt-a1*) were considered. The *tef* gene showed low variability with few polymorphic sites, whereas the *alt-a1* gene showed greater variability among *Alternaria* species.

To further determine the identity of the strains, phylogenetic analysis of the concatenated sequences of the three fragments was carried out. The phylogenetic tree, obtained with Mega5 software using the Maximum Likelihood method, allowed definition of four well-separated clades, corresponding to Section *Alternaria*, Section *Porri*, Section *Brassicicola*, and Section *Infectoriae*, as determined using reference strains (Figure 2). The resolution of all the clades was supported by high bootstrap values. *Alternaria brassicicola* ATCC 96836 and *A. infectoria* E.G.S.27.193 did not cluster with any *Alternaria* strains isolated from *C. maritima*. In the Section *Porri*, only reference strains were clustered together (Figure 2). All *Alternaria* strains clustered in Section *Alternaria*. Forty-seven strains showed homology close to 100% with *A. alternata* E.G.S. 34.016, *A. tenuissima* E.G.S.34.015, *A. limoniasperae* BMP2335, *A. turkisasfria* BMP3436 (Figure 2).

A well-supported group clustered 13 field strains with *A. arborescens* E.G.S.39.128.. However, *A. arborescens* was not detected in Raouad region. There was high variability in this group (Figure 2). In *Alternaria* Section, a sub-clade grouped together 12 *Alternaria* strains showing 100% homology with *A. mali* BMP3064 and *A. citriarbusti* BMP2343A, and seven *Alternaria* strains (ITEM17797, ITEM17798, ITEM17799, ITEM17800, ITEM17801, ITEM17803, ITEM17809), showing 100% of homology among them, all of which were collected in Sfax region.

Based on these data, four phylogenetic *Alternaria* groups can be discriminated. The *A. alternata* group was the most frequent (60% of strains), followed by *A. arborescens* (16%), and *A. mali* (15%). Seven strains (9%), phylogenetically closely-related to *A. mali*, were not identified at species level (Figure 2).

Mycotoxin production profiles of *Alternaria* strains

Data of mycotoxin production are summarized in Table 1 and in Supplementary Table S2. Alternuene was produced by two *A. alternata* strains (39.2 mg kg⁻¹ by ITEM17789 and 43.1 mg kg⁻¹ by ITEM17855) and one *A. mali* strain (77.3 mg kg⁻¹ by ITEM17788). The amounts of AME, AOH and TA were very variable. These were: for AOH, from 4.4 mg kg⁻¹ (*A. alternata* ITEM17782) to 1,856.8 mg kg⁻¹ (*A. alternata* ITEM17791); for AME, from 53.4 mg kg⁻¹ (*A. alternata* ITEM17848 to 9,149.4 mg kg⁻¹ (*A. mali* ITEM17835); and for TA, from 344.4 mg kg⁻¹ (*A. mali* ITEM17849) to 19,837.3 mg kg⁻¹ (*A. alternata* ITEM17808). Between the four clades of *Alternaria* species, qualitative and quantitative differences in mycotoxin production were examined. However, close correlation between phylogenetic clade and mycotoxin production was not detected.

Alternariol mono-methyl ether was produced by 63 of the 79 strains, (production ranges in parentheses): 38 of 47 *A. alternata* strains (53.4 to 8,766.6 mg kg⁻¹); seven of 13 *A. arborescens* strains (61.8 to 1,848.7 mg kg⁻¹); ten of 12 *A. mali* strains (54.0 to 9,149.4 mg kg⁻¹); and all seven *Alternaria* spp. strains (213 to 1,661.7 mg kg⁻¹). Alternariol was produced by 70 of the 79 strains: 42 of 47 *A. alternata* strains (4.4 to 1,856.8 mg kg⁻¹); nine 13 *A. arborescens* strains (9.8 to 514.4 mg kg⁻¹); all 12 *A. mali* strains (10.8 to 1,671.7 mg kg⁻¹); and all seven *Alternaria* spp. strains (40.9 to 179.6 mg kg⁻¹). Tenuazonic acid was produced by 64 of the 79 strains: 36 of 47 *A. alternata* strains (787.4 to 19,837.6 mg kg⁻¹); eight of 13 *A. arborescens* strains (953 to 6,202 mg kg⁻¹); 11 of 12 *A. mali* strains (344.4 to 6,692.3 mg kg⁻¹); and all 7 *Alternaria* spp. strains (1.941.6 to 8904.1 mg kg⁻¹).

Pathogenicity tests

Symptoms of tissue browning followed by necrosis were observed at 3 and 7 d after inoculation. Necrosis with yellowing occurred at 3 dpi which became more intense at 7 dpi. The control plants did not show disease symptoms (Figure 3).

According to the phylogenetic strain identification, the 24 selected *A. alternata* strains produced a mean lesion diameter of 0.79 cm; the *A. arborescens* strains produced lesions of 0.86 cm mean diameter; the *A. mali* strains gave mean lesion diameter of 0.62 cm; and the *Alternaria* spp. strains produced lesions of 0.88 cm mean diameter. The statistical analyses did not show significant differences in pathogenicity among the different strains used in the tests. Only *A. alternata* and *A. mali* strains gave slight significant differences in mean lesion

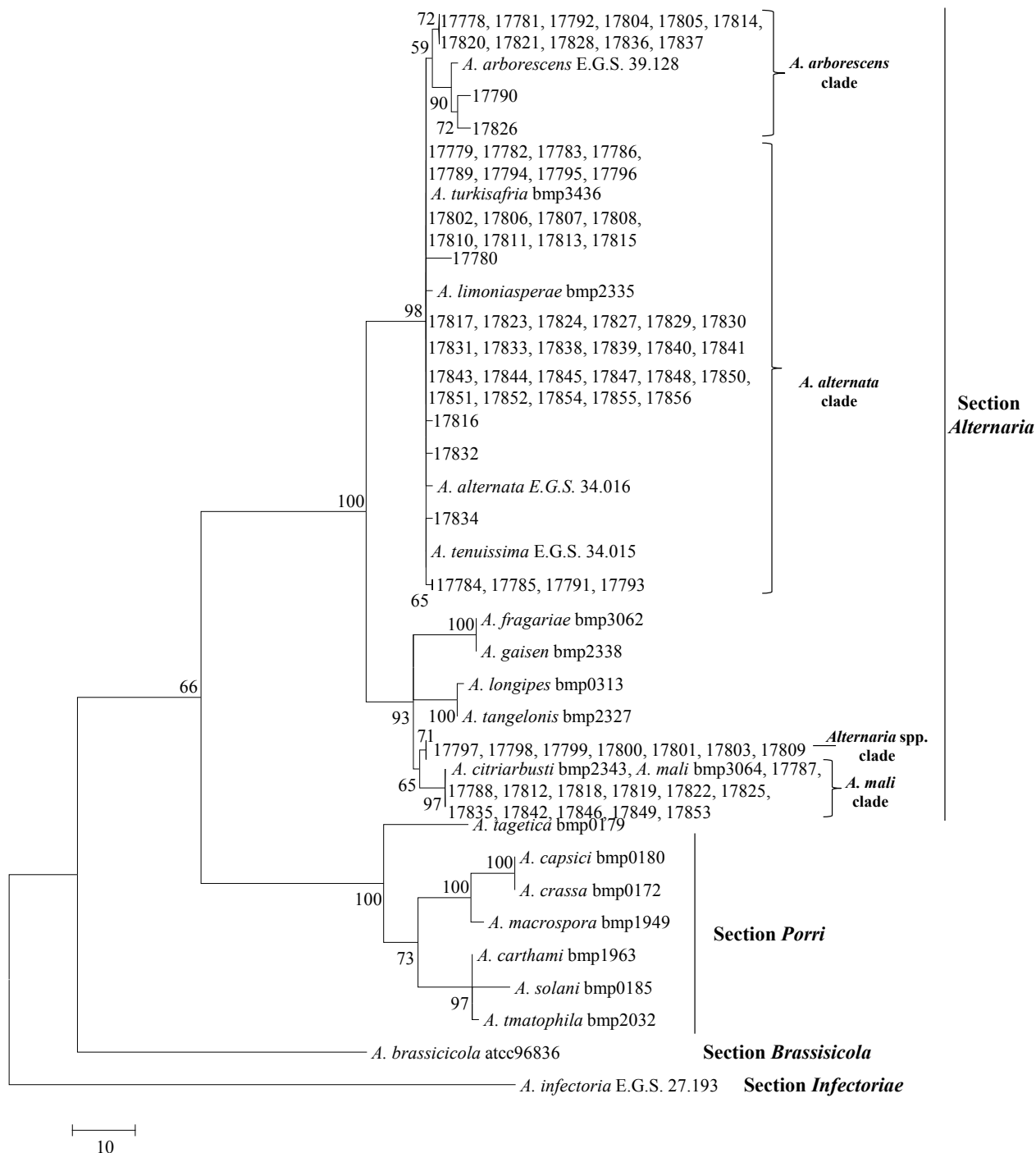


Figure 2. Phylogenetic tree generated by the Maximum Likelihood method (bootstrap 1,000 replicates) of combined *alt-a1*, *gpd*, and *tef* gene sequences from 79 *Alternaria* strains isolated from *Cakile maritima* in Tunisia. *Alternaria infectoria* E.G.S.27.193 was used as outgroup in tree.

sizes (Supplementary Table S3). A one-way ANOVA was performed using the lesion diameters as a factor to test the pathogenicity of the different species. The sig-

nificance level was set at $\alpha = 0.05$, and the strains were considered as replicates of the species. The probability ($P = 0.0846$) indicated that the species did not exhibit

Table 1. Mycotoxin production and mycotoxin incidence for different phylogenetic *Alternaria* clades. Proportions of strains, and mean amounts of four mycotoxins, are indicated of each clade

Clade		Mean amount of mycotoxin produced (mg kg ⁻¹) ^a			
		AOH	AME	TA	ALT
<i>A. alternata</i>	No. of positive/total strains	41/47	38/47	36/47	2/47
	Frequency (%)	87.2	80.8	76.6	4.2
	Mean Value	198.6	1632.0	3767.5	41.1
	Min Value	4.4	53.4	787.4	39.2
	Max Value	1856.8	8766.6	19837.6	43.1
<i>A. arborescens</i>	No. of positive/total strains	9/13	7/13	8/13	0/47
	Frequency (%)	69.2	53.8	61.5	0
	Mean Value	130.6	814.2	2993.5	-
	Min Value	9.8	61.8	953.0	-
	Max Value	514.4	1848.7	6202.0	-
<i>A. mali</i>	No. of positive/total strains	12/12	10/12	11/12	1/12
	Frequency (%)	100	83.3	91.7	8.3
	Mean Value	331.8	2108.7	4223.2	-
	Min Value	10.8	54.0	344.4	-
	Max Value	1671.7	9149.4	6692.3	-
<i>Alternaria</i> spp.	No. of positive/total strains	7/7	7/7	7/7	0/7
	Frequency (%)	100	100	100	-
	Mean Value	119.6	975.4	4631.5	-
	Min Value	40.9	213.0	1941.6	-
	Max Value	179.6	1661.7	8904.1	-

^a AOH, Alternariol; AME, Alternariol methyl ether; TA, Tenuazonic Acid; ALT, Altenuene.

a statistically significant difference in mean lesion sizes. However, the distributions of lesion diameters for *A. mali* was separated from those for the other species, as indicated by the box plots in Figure 4. To assess this difference a multiple comparison test was carried out. This was a pairwise comparison of the groups (species) using with different comparison procedures (Least Significant Difference; Bonferroni, Scheffe, Dunn and Sidak; or Fisher's least significant differences method). Only the Least Significant Difference method detected a statistically significant difference between *A. mali* and the other species. For the other comparison methods, the difference between the species were not statistically significant.

DISCUSSION

This study has characterized *Alternaria* species occurring on the halophyte *C. maritima* from different bioclimatic areas in Tunisia. *Alternaria* species are major contaminants and pathogens of a wide range of crops,

causing economic losses for producers, and health risks for consumers due to the accumulation of toxic metabolites (Da Cruz Cabrala *et al.*, 2016).

Morphological characterization of the representative strains *Alternaria* examined in this study has shown high variability in conidium size, shape, dimensions and septation, confirming that the species identification based on morphological characters is unreliable. Furthermore, there was no correlation between specific morphological traits of the different strains and their bioclimatic region origins. There was also no correlation with the different plant tissues from which the strains were isolated. Similar results were observed by Chethana *et al.* (2018), who found different morphological traits of *Alternaria* strains isolated from onion did not depend from their geographical or plant tissue origins. To perform a more discriminating identification at species level, a representative set of the *Alternaria* strains was investigated by using a polyphasic approach, based also on molecular and chemical characterization.

All the representative strains were phylogenetically identified in Section *Alternaria* (Lawrence *et al.*, 2013),

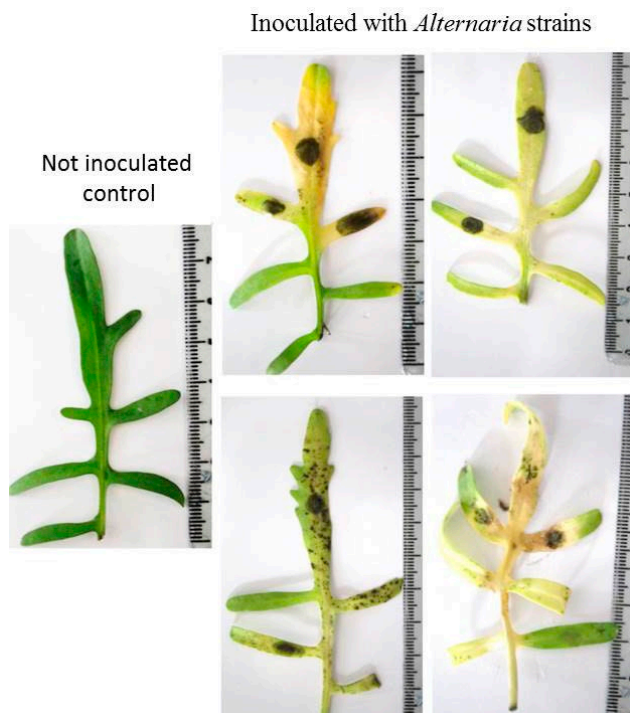


Figure 3. Symptoms resulting on *Cakile maritima* leaves 7 d after inoculation with *Alternaria* strains *A. alternata* (ITEM17830; two upper photographs) or *A. mali* (ITEM17842; lower photographs).

which includes the most important mycotoxigenic *Alternaria* species, and has *A. alternata* as the type species (Woudenberg *et al.*, 2015). The three genes (*gpd*, *tef*, *alt-1*) used for multi-locus sequence analyses were selected for their informative responses in *Alternaria*, as suggested by Somma *et al.* (2019). All the strains grouped into four phylogenetic clades: 13 strains were identified as *A. arborescens*, although they showed a great variability; the largest clade included 47 strains, with a high level of homology, and was identified as *A. Alternaria*; 12 strains were identified as *A. mali*; and seven strains were grouped in a clade closely related to, but different from, *A. mali*. These seven strains were not assigned to any species. The different identified *Alternaria* species could not be related to particular plant tissues and/or bioclimatic conditions. *Alternaria alternata* and *A. mali* strains colonize all types of *C. maritima* tissues, and were isolated from the two climatic regions. The exception was all the strains of the unidentified clade, (*Alternaria* spp.; Figure 2), which were isolated only from the seeds of *C. maritima* plants collected in the arid region of Sfax. Also, the *A. arborescens* strains were not detected in the semi-arid region of Raouad. Similar results were reported by Bensassi *et al.* (2009; 2011), who identified *A. alternata* as the most frequent *Alternaria* species

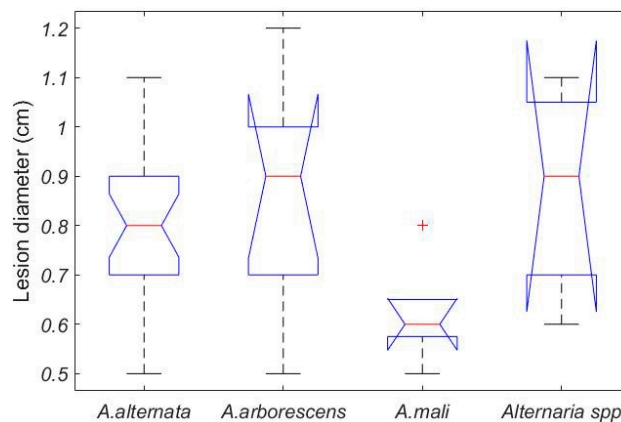


Figure 4. Boxplots (median (red), upper and lower 25th and 75th percentiles, 95% confidence intervals) of lesion diameters produced on inoculated *Cakile maritima* plants by different *Alternaria* species. The large overlap for *A. alternata*, *A. arborescens*, *Alternaria* spp. is obvious.

occurring on wheat growing in all regions of Tunisia sampled, which were characterized by different environmental conditions. Schiro *et al.* (2018) also showed that conidium production of several *Alternaria* spp. on wheat plants was not correlated with specific microclimatic conditions and was uniform in the field in all regions sampled.

Several authors (Andersen *et al.*, 2008; Brun *et al.*, 2013; Somma *et al.*, 2019), have shown that accurate identification of *Alternaria* spp. requires the polyphasic approach, using morphology, phylogeny, and mycotoxin profiling. *Cakile maritima* is also often used for human and animal consumption. The parts of the plants used for nutrition could become potential natural sources of *Alternaria* mycotoxins, if contaminated by *Alternaria* species. However, since there is still little knowledge of the natural *Alternaria* mycotoxin contamination in these plants, more investigations are required before conclusions can be drawn on the health risks associated to *C. maritima* consumption. Capability to produce mycotoxins by representative strains of the *Alternaria* population isolated in this study has showed that most (90%) of the strains, produced at least one of four assessed mycotoxins, sometimes at very high levels (Supplementary Table S1). This indicated the potential mycotoxicological risk of *Alternaria* strains isolated from these plants. Only three strains produced ALT, while production AME and AOH by the strains analyzed was proven strictly correlated (Supplementary Table S2). This confirms previous reports (Bensassi *et al.*, 2011; Ramires *et al.*, 2018). *Alternaria* mycotoxins have been frequently reported as natural contaminants

of agri-food crops (Puntscher *et al.*, 2018), and their production under natural conditions has been associated to several factors such as plant species and host cultivar, environmental conditions, cultivation techniques, plant growth stage, time of harvesting, and crop rotation (Lauren and Fadwa, 2008). The dominance of mycotoxigenic *Alternaria* species on *C. maritima*, reported here for the first time, suggests that this plant can potentially be a risk for human and animal health, if contaminated parts of plants are consumed. In addition, *C. maritima* could be a source of inoculum of *Alternaria* species, if the plants grow in the proximity of other important food plants such as wheat.

The pathogenicity tests showed that the levels of aggressiveness of different *Alternaria* strains from *C. maritima* leaves were different among species, with the pathogenicity of *A. mali* being less than for the other species.

The phylogenetic study indicated the occurrence of a set of strains closely-related to *A. mali*, that could represent a new phylogenetic species. This shows that *C. maritima* could be a source of *Alternaria* genetic diversity to be further investigated. This study also confirms that it is still important to provide genetically well-defined reference isolates for taxonomic investigations of *Alternaria*. These should be accurately validated by a standardized common approach in research dealing with *Alternaria* taxonomy (Somma *et al.*, 2019).

CONCLUSION

These results reported here show that the Tunisian halophyte *C. maritima* can be commonly infected by mycotoxigenic *Alternaria* species. Morphological, molecular and mycotoxin analyses confirmed variability within *Alternaria* species of Tunisian origin from different climates. However, no clear links between species with host or geographic origins could be established. This research has shown that *C. maritima* can be a source of *Alternaria* genetic diversity to be further investigated, to provide new information on the taxonomy of the genus.

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