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Alternaria species and related mycotoxin detection in Lebanese durum wheat grain

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Summary. *Alternaria* is a ubiquitous genus that may infect wheat in many countries, causing the disease black point. The present study aimed to assess contamination by fungi, of durum wheat kernels from Lebanon, and identify the main *Alternaria* species contaminants. *Alternaria* was detected in the majority (97%) of the inspected fields. Contamination by *Alternaria* differed among the samples according to their geographical origins. The greatest contamination was detected in the West Bekaa area (average 59%), followed by Akkar (55%), and lowest was observed in Baalbeck (2%). HPLC-DAD analyses performed on grain samples showed that altenuene, alternariol, alternariol monomethyl ether, and tenuazonic acid were not detected in any sample. Phylogenetic analyses, based on DNA sequences of *β-tubulin*, *glyceraldehyde-3-phosphate dehydrogenase* and *calmodulin* gene fragments, showed that *Alternaria* field strains belonged to two major sections: *Alternaria* (51%) and *Infectoriae* (40%). The remaining strains were in separate clades in sections *Ulocladioides* (3%), *Chalastospora* (3%) and *Pseudoalternaria* (3%). Although this study revealed no contamination of wheat kernels by *Alternaria* mycotoxins, the potential risk of mycotoxin accumulation remains high due to the widespread occurrence of toxigenic *Alternaria* species on kernels.

Keywords. Multi-locus gene sequencing, toxigenic fungi, *Ulocladioides, Chalastospora, Pseudoalternaria, Infectoriae*.

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INTRODUCTION

Alternaria are ubiquitous fungi, generally detected as saprophytes in soil or in plant debris, and are pathogens of several crops, including cereals, oil crops, ornamentals, vegetables, fruit trees (Logrieco *et al.*, 2009) and plants

used for their medicinal properties (Chalbi *et al*., 2020). *Alternaria* species are the main causal agent of black point in wheat, causing harvest and post-harvest damage of wheat grains. Economic damage caused by *Alternaria* infections is generally not associated with yield losses but is related to grain reduced quality of cereals (Kosiak *et al.,* 2004; Vučković *et al.,* 2012). Wheat kernels colonized by *Alternaria* species are characterized by black pigmentation in the underlying embryo regions, which compromises flour quality and grain nutritional values (Kashem *et al*., 1999).

Contamination of wheat kernels by *Alternaria* has been reported in several countries with different climates, including Italy, China, Russia, Argentina, Tunisia and Slovakia (Li *et al.,* 2000; Gannibal *et al.,* 2007; Patriarca *et al.,* 2007; Bensassi *et al.,* 2009; Mašková *et al.,* 2012; Vučković *et al.,* 2012; Ramires *et al.,* 2018; Somma *et al.,* 2019).

Several *Alternaria* species have been associated to black point symptoms, although *A. alternata* has been isolated with the greatest frequency (Somma *et al*., 2019). In addition, *A. triticina* was reported to be one of the most important causal agents of wheat leaf blight, and has been involved in black point in different geographic areas (Chaurasia *et al.,* 2000; Mercado Vergnes *et al.,* 2006; Somma *et al*., 2019). Also, *A. arborescens*, *A. infectoria* and *A. tenuissima* morpho-species have been identified in several studies (Gannibal *et al.,* 2007; Bensassi *et al.,* 2009; Scott *et al.,* 2012; Somma *et al.,* 2019; Masiello *et al*., 2020).

Some of *Alternaria* species produce secondary metabolites, including host specific toxins, which are required for fungal pathogenicity, and mycotoxins (Escrivà *et al*., 2017). The most important *Alternaria* mycotoxins are alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), and tenuazonic acid (TA), occurring with high frequency on wheat (Somma *et al.,* 2011). Based on *in vitro* and *in vivo* assays, toxicity, mutagenicity and genotoxicity of these metabolites have been proven (Lehmann *et al.,* 2006; Ostry, 2008; Zhou and Qiang, 2008), and the risks for human and animal health have been studied (Ostry, 2008; Alexander *et al.,* 2011). *Alternaria* conidia are also airborne allergens related to respiratory diseases and skin infections in humans (Cramer and Lawrence, 2003; Kilic *et al.,* 2010).

For the *Alternaria* species occurring on wheat kernels, species-specific mycotoxin profiles have not been clearly assessed. However, the morpho-species *A. alternata, A. tenuissima,* and *A. arborescens* are able to produce AOH, AME, TA, tentoxin and altertoxin (ATX)-I, II, III, whereas *A. infectoria*, a widespread species, is apparently not able to synthetize any mycotoxin (Logrieco *et al.,* 2009; Da Cruz Cabrala *et al.,* 2016).

Due to the range of demonstrated toxic effects of *Alternaria* mycotoxins, the high field occurrence of toxigenic *Alternaria* species requires correct identification to evaluate the risks they pose. *Alternaria* taxonomy is also confused. *Alternaria* species have been classified based only on morphological and physiological traits. About 280 species of *Alternaria* were described by Simmons (2007). However, due to environmental influences on the morphological traits, the close similarity between some species, and the presence of several strains with intermediary traits, many errors in *Alternaria* taxonomy and identification have occurred (Andrew *et al.,* 2009).

In consequence of molecular studies carried out using multi-locus gene sequence approaches, important taxonomic revision of *Alternaria* genus has occurred. At first, *Alternaria* morpho-species were phylogenetically analyzed and defined as species-groups (Lawrence *et al*., 2013; 2014). Further studies then elevated the species-groups to section status, defining 27 sections within *Alternaria*, also including species belonging to previous closely related genera (Woudenberg *et al*., 2015). According to this taxonomic revision, different morpho-species were synonymized, as for *A. alternata* that now includes more than 35 species (Woudenberg *et al*., 2015; Somma *et al*., 2019). The *Infectoriae* section, which mainly includes species with low or no mycotoxin capability, always forms a well-defined clade, to indicate a genus different from *Alternaria* (Somma *et al*., 2019). On the other hand, the toxigenic species are mainly placed in Section *Alternaria*, which includes the most common species on wheat, and detected in different countries.

In Lebanon, wheat grown on a total area of 41,000 ha, and producing approx. 140,000 tons of grain (FAOSTAT, 2020), is an important cereal crop used primarily for human consumption. *Alternaria* was reported on wheat kernels in Lebanon by Joubrane *et al.* (2011), but incidence was low, and data on its geographical distribution and species identification were not reported. The aims of the present study were: a) to assess contamination of durum wheat kernels by *Alternaria* species; b) to characterize, using a multi-locus sequence approach, the main *Alternaria* species occurring on wheat in Lebanon; c) to determine phylogenetic relationships between the main *Alternaria* species associated with black point; and d) to evaluate occurrence of *Alternaria* mycotoxins in wheat kernels.

MATERIALS AND METHODS

Wheat sampling and isolation of fungi

During the 2018 crop season, at harvesting time, 36 durum wheat fields were randomly selected from

the five most important Lebanese wheat production areas: Central Bekaa (ten fields), West Bekaa (12 fields), Baalbeck (five fields), Marjeyoun (five fields) and Akkar (four fields). From each field, one sample of wheat kernels was collected. Each sample consisted of approx. 1 kg of kernels, randomly gathered from five different sampling locations. In this way, 36 wheat samples were analyzed as representative of Lebanese wheat production.

For each sample, fungus isolation was carried out from 100 randomly selected kernels. The kernels were surface decontaminated with 70% ethanol (for 1 min) and 2% sodium hypochlorite (for 1 min), and then washed twice in sterile distilled water. The grains were dried on sterile filter paper under the laminar flow hood, and were then aseptically plated on potato dextrose agar (PDA) amended with 0.10 g L^{-1} streptomycin sulphate salt and 0.05 g L^{-1} neomycin. The isolation plates were then incubated at 25±1°C under a 12 h light, 12 h darkness regime for 5 d, for development of fungus colonies. The percentage of kernels contaminated by *Alternaria* species and other endophytic fungi was then determined.

Alternaria colonies originating from colonized kernels were selected to obtain mono-conidium cultures. A set of 75 representative *Alternaria* strains were selected for phylogenetic analyses, based on their geographical origins and their macro- and micro-morphological traits, according to Simmons (2007).

DNA extractions and PCR amplifications

Genomic DNA was extracted and purified from 2-d-old colonies grown on cellophane disks overlaid on PDA at 25±1°C (Habib *et al.,* 2021).

Four informative gene fragments were amplified for each fungus strain using the primer pairs gpd1/gpd2 for *glyceraldehyde-3-phosphate dehydrogenase* (*gpd*) (Berbee *et al.,* 1999), caldf1/caldr1 for *calmodulin* (Lawrence *et al.,* 2013), T1/T2 for β-*tubulin* (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997), and alt-for/alt-rev for *allergen alt 1a* (*alt-a1*) (Hong *et al.,* 2005).

Amplification of the *gpd* partial gene was performed using GoTaq G2 Colorless Master Mix (Promega), where 25 ng of DNA template were added in mixture with a final concentration of 1× ready Master Mix, 2 mM $MgCl_2$ and 0.4 µM of each primer. *alt-a1* gene amplification was achieved using Taq DNA Polymerase (Takara) in mixture with 1× Takara PCR Buffer, 0.075 µL of Hot Master Taq DNA Polymerase (1 U μL^{-1}; 5Prime), 0.3 µL of dNTPs (10 mM), 0.45 µM of each primer and 15 ng of DNA template. *Calmodulin* and β-*tubulin* genes were amplified using Taq PCR Master Mix (Qiagen) in mixture with 1× ready Master Mix, 0.4 µM of each primer, and 50 ng of DNA template.

Amplifications were carried out in T^{100} MyCycler thermal cycler (BioRad). The PCR reactions of *gpd*, *tub* and *alt-a1* were carried out using the PCR parameters reported by Somma *et al.* (2019). PCR reactions for the *calmodulin* partial gene were: initial denaturation for 4 min at 95°C, followed by 35 cycles of 95 °C for 30 sec, 58.5°C annealing for 30 s, 72°C for 1 min, each and a final extension for 5 min at 72°C. After electrophoretic separation on agarose gel (1.5% Molecular Biology Certified Agarose, Bio-Rad Laboratories), PCR products were visualized by UV after GelRed (Biotium Inc.) staining to confirm the expected products.

Sequencing and phylogenetic analyses

Before sequencing, each PCR product was purified with the enzymatic mixture Exo/FastAP (Exonuclease I, FastAP thermosensitive alkaline phosphatase; Thermo Scientific). Sequencing of both strands of each gene was carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), according to the manufacture's protocol, and the fragments were purified through Sephadex G-50 (5%) (Sigma-Aldrich), and then sequenced in an "ABI PRISM 3730 Genetic Analyzer" (Applied Biosystems). Partial sequences were assembled using the BioNumerics v. 5.1 software (Applied Maths, Inc.). A phylogenetic tree of concatenated gene sequences was generated using the maximum likelihood statistical method and bootstrap analysis (1000 replicates, removing gaps) with MEGA7 (Kumar *et al.,* 2016).

Appropriate gene sequences were downloaded from the National Center for Biotechnology Information (NCBI), and from the "*Alternaria* Genomes Database" (AGD), and were included in a phylogenetic analysis. These sequences were for the following *Alternaria* species reference strains: *A. alternata* EGS 34-016, *A. tenuissima* EGS 34-015, *A. tangelonis* EV-MIL-2s; *A. arborescens* EGS 39-128, *A. cerealis* EGS 43-072, *A. angustiovoidea* EGS 36-172, *A. consortialis* JCM 1940, *A. dumosa* EGS 45-007, *A. malorum* CBS 135.31, *A. rosae* EGS 41-130, *A. abundans* CBS 534.83, *A. triticina* EGS 17-061, *A. photistica* EGS 35-172, *A. infectoria* EGS 27-193, *A. novae-zelandiae* EGS 48-092, *A. intercepta* EGS 49-137, *A. viburni* EGS 49-147, *A. graminicola* EGS 41-139, *A. hordeicola* EGS 50-184, *A. ethzedia* EGS 37-143, *A. metachromatica* EGS 38-132, *A. oregonensis* EGS 29-194, *A. californica* EGS 52-082 and *A. conjuncta* EGS 37-139.

Mycotoxin extractions and HPLC analyses

Mycotoxin analyses were carried out for the 36 samples of wheat kernels, based on the method of Rubert *et al.* (2012), with modifications. To evaluate recovery of mycotoxins from the extraction method used, wheat samples contaminated at different amounts (0.1, 0.2 and 0.4 ppm) (n = 3) of AOH, AME, ALT and TA (Sigma-Aldrich) were assessed. Four wheat samples, G27, G25, G26 and G28 from Masiello *et al.* (2020), were also included in the analysis as positive controls for, respectively, the mycotoxins AOH, AME, ALT and TA.

Each sample of kernels (200 g) was thinly ground with an Oster Classic grinder (Madrid, Spain). Five g of each ground sample were weighed into a 50 mL capacity plastic tube containing 25 mL of methanol. For each extraction, an Ultra Ika T18 basic Ultra-turrax, Ika, (Staufen), was used for 3 min. After centrifugation, 1 mL of the supernatant was filtered on a 13 mm/0.22 µm 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. Tenuazonic acid, AME and AOH standards were provided by SIGMA Chemical Company.

AOH, AME, ALT, and TA were determined using Merk HPLC through 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 analyses. As the stationary phase, a Gemini C18 column (Phenomenex) 4.6 × 150 mm, 3 µm particle size was used. Themobile phase consisted of two eluents: eluent A (water with 50 µL L^{-1} trifluoroacetic acid) and eluent B (acetonitrile with 50 µL 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. After that, 100% B was maintained for 1 min. The gradient was then returned to 10% B in 1 min and permitted to equilibrate for 3 min before the next analysis (Myresiotis *et al.*, 2015). The limits of detection (LOD) and quantification (LOQ) of the method used were, respectively, 0.01 and 0.1 ppm.

RESULTS

Fungus contamination of wheat kernels

The microbiological analyses carried out on the 36 wheat kernel samples showed that *Alternaria* species were the most frequent contaminants of the durum wheat kernels sampled in Lebanon. This genus was present in all the inspected fields except one, which was located in Baalbeck district. The contamination by *Alternaria* was generally high and differed among the samples according to geographic origin (Table 1). The greatest contamination was detected from West Bekaa area (average 59%; range: 36–84%), whereas the least contamination was from Baalbeck (average 2.2%; range: 0–4%).

Table 1. Fungus contamination detected in durum wheat kernels collected from different sampling areas of Lebanon.

Sampling area	Number of Fields	Fungus contamination (%)			
		Alternaria spp.		Other fungi	
		Range	Average	Range	Average
Akkar	4	46-64	55.3±5.1	0-3	1.3±0.8
Baalbeck	5	0-4	2.2±0.7	0-3	1.2±0.7
Central Bekaa	10	1-64	25.6±7.1	0-18	6.5±1.7
Marjeyoun	5	9-48	39.8±2.5	1-8	3.4±0.4
West Bekaa	12	36-84	59.0±4.1	1-25	10.2±2.4

Other fungi were also recovered from the kernels, with proportions of contamination less than that of *Alternaria*. From almost all fields, the contamination by these fungi did not exceed 10%, except in few fields located in Central and West Bekaa areas. The average values and ranges of contamination are shown in Table 1. Among these fungi, *Cladosporium* was the most frequent genus, whereas the potentially toxigenic genera *Penicillium* and *Aspergillus* were detected at low levels (1%), in, respectively, one and two fields in Central Bekaa.

Further details on the fungal contamination of kernels are provided in the Supplementary Table 1.

Phylogenetic analyses

In total, 1395 colonies of *Alternaria* were recovered from the wheat kernels assessed. From each site, pure colonies of *Alternaria* were obtained, and these were grouped based on their macro- and micro-morphological traits. Single-conidium isolates were then obtained from each group, and one to two strains per morphotype were selected from each site. In this way, 75 strains representing the population of *Alternaria* from durum wheat kernels in Lebanon (Central Bekaa, 19 strains; West Bekaa, 25 strains; Baalbeck, three strains; Marjeyoun, 12 strains; Akkar, 16 strains) underwent the phylogenetic analyses (Supplementary Table 2).

All *Alternaria* strains gave the specific PCR amplicons of the expected size for *gpd*, *calmodulin*, and *beta-tubulin* genes. Unexpectedly, 26 out of 75 field strains did not give PCR products when amplified with Alt-for/Alt-rev primer pair, so phylogenetic analyses were car-

ried out excluding *alt-a1* locus. The sequences of the three selected genes were aligned and cut at the ends to consider a common fragment for all strains.

The phylogenetic analysis of the concatenated sequences of 1451 positions resulted in a phylogenetic combining dataset comprising 90 taxa, including: 74 *Alternaria* field strains, 24 *Alternaria* reference sequences and one strain of *Stemphylium* also isolated from wheat (Altern1392) considered together with *Stemphylium vesicarium* 173-1a-13FI1M3 strain as the outgroup taxon. The phylogenetic tree, rooted to *Stemphylium* reference strain, was resolved in five well-separated clades (A-E), corresponding to *Infectoriae*, *Pseudoalternaria*, *Chalastospora*, *Ulocladioides* and *Alternaria* Sections, supported by high bootstrap values (Figure 1).

The clade A (*Infectoriae* Section) grouped 30 out of 74 field strains (41%) in three not well-supported sub-clades, except for Altern1395 which did not cluster with any strain included in this Section. The sub-clade A1 grouped 12 field strains together with the reference strains of *A. conjuncta* (EGS 37-139), *A. californica* (EGS 52-082), *A. oregonensis* (EGS 29-194), *A. hordeicola* (EGS 50-184), *A. metachromatica* (EGS 38-132), *A. graminicola* (EGS 41-139), and *A. ethzedia* (EGS 37-143). The sub-clade A2 included 13 field strains that did not cluster with any of the reference strains included in the analysis. The sub-clade A3, supported by a bootstrap value of 87, comprised four field strains showing high homology with *A. triticina* EGS 17-061 reference strain.

The two field strains Altern 1481 and Altern 1484 clustered together with *A. rosae* EGS 41-130 reference strain (clade B) belonging to *Pseudoalternaria* Section.

A well-defined clade (clade C) clustered two field strains, Altern 1381 and Altern 1382, with *A. malorum* CBS 135.31 reference strain (*Chalastospora* Section). A well-supported clade (clade D), defined as *Ulocladioides* Section, grouped *A. consortialis* JCM 1940 reference strain and two field strains, Altern 1397 and Altern1408 (Figure 1).

Of 74 *Alternaria* field strains, 38 (51%) were assigned to the *Alternaria* Section (clade E). In this section, a sub clade (Sub-Clade E1) clustered six field strains together with *A. tangelonis* (synonym of *A. gossypina*) EV-MIL-2s reference strain and *A. dumosa* EGS45-007 reference strain. A different sub-clade (Sub-Clade E2) included the reference sequences of *A. arborescens* EGS 39.128, *A. cerealis* EGS 43-072, *A. angustiovoidea* EGS 36.172 and four field strains. The strain Altern1474 showed 100% of homology with *A. arborescens* reference strain. The majority of the strains belonging to *Alternaria* section (28 of 38 strains) shared high similarity with *Alternaria* reference strains *A. alternata* EGS 34.016, and *A. tenuissima* EGS 34.015 (Sub-Clade E3). The high level of homology of these strains did not allow distinction between *A. alternata* and *A. tenuissima* morpho-species.

Mycotoxin contamination of wheat kernels

All the durum wheat samples were analyzed for the mycotoxins AOH, AME, ALT, and TA, but none of the samples were contaminated with these mycotoxins. This result was supported by inclusion of mycotoxin-positive grain samples in the same detection experiment. The grain samples G25, G26, G27, and G28 (Masiello *et al*., 2020) showed contamination with some *Alternaria* toxins. In particular, the sample G27 showed 152 ± 6 mg kg^{-1} of AOH, 231 ± 4 mg kg^{-1} of AME, 23 ± 3 mg kg^{-1} of ALT, and 189 ± 6 mg kg^{-1} of TA.

Mean recovery percentage of AOH, AME, ALT, and TA mycotoxins in the grain contaminated with 0.1 ppm was 82 ± 4%, with 0.2 ppm was 85 ± 6%, and with 0.4 ppm was 93 ± 5%.

DISCUSSION

Wheat and wheat products are among the most important staple foods consumed in Lebanon. The present study focused on characterization of *Alternaria* species isolated from durum wheat kernels during the 2018 crop season, from the five main Lebanese areas of wheat production. Almost all previous studies of mycotoxins on Lebanese wheat were focused on *Fusarium*, *Aspergillus, Penicillium*, and related mycotoxins (Joubrane *et al*., 2011; Elaridi *et al*., 2019), while occurrence of *Alternaria* has been little investigated, and has long been considered as only a quality problem for commerce.

International reports of natural occurrence of *Alternaria* species and related mycotoxins on wheat have increased. Serious toxicological risks of *Alternaria* mycotoxins for human and animals have been demonstrated (Osrty, 2008; Arcella *et al.,* 2016; Solhaug *et al.,* 2016). These risks originating from toxin levels produced by *Alternaria* species in wheat produced in Lebanon should therefore be monitored to prevent their potential harmful effects on public health.

The wheat samples analyzed in the present study were highly contaminated mainly by fungi belonging to *Alternaria*. This genus was detected from almost all sampled wheat fields, with proportions of contamination between 1 and 84%. Other reports of high levels of *Alternaria* contamination of grain (more than 90% of grains affected) have been from Serbia, Argentina, and Italy (Patriarca *et al.,* 2007; Levic *et al.,* 2012; Ramires *et*

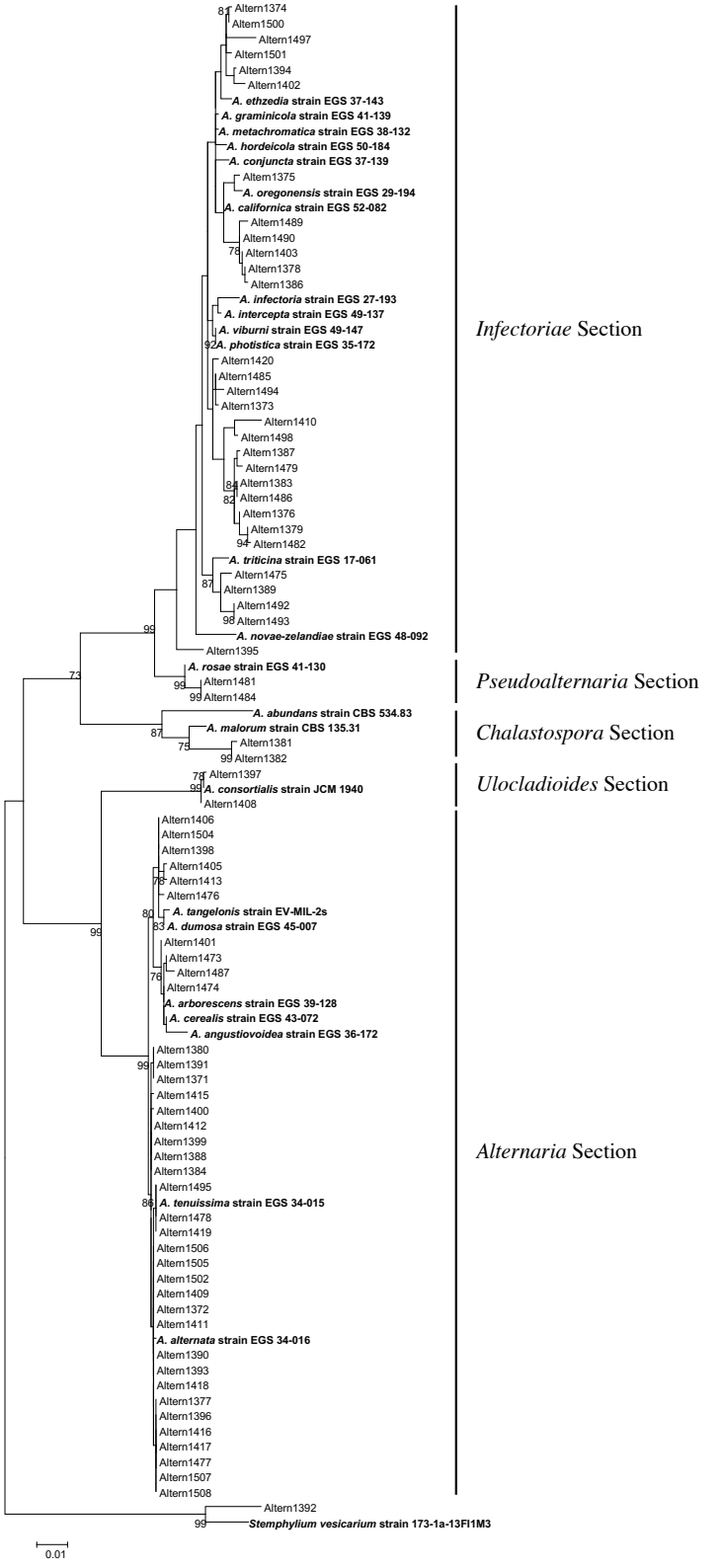

Figure 1. Phylogenetic tree generated by maximum likelihood analysis (bootstrap 1000 replicates) of combined *gpd*, *calmodulin* and β-tubulin gene sequences of 74 *Alternaria* field strains, 24 *Alternaria* reference strains, and rooted to *Stemphylium*. Bootstrap values greater than 70 are shown next to relevant branches.

al., 2018; Somma *et al.,* 2019). The results from the present study contradict those of Joubrane *et al.* (2011) for Lebanon, in which *Alternaria* species were rarely isolated (frequency of approx. 4%).

The high variability of proportions kernel fungus contamination of grains observed among the different Lebanese areas could be due to the different prevailing climatic conditions, as temperature and relative humidity are the most important physiochemical factors for *Alternaria* development (Gawai and Mangnalikar, 2018). The optimum temperature range for *Alternaria* growth is reported to be 22–28°C, and relative humidity between 60 and 100% (Gawai and Mangnalikar, 2018). This explains the lowest incidence detected in Baalbeck district, where a semi-arid climate prevails, and the average temperature and humidity were not favourable for disease development. In contrast, most of the fields with medium to high contamination percentages were mainly located in West Bekaa area, which is characterized by greater relative humidity. Nevertheless, *Alternaria* species have been isolated from other countries in a wide range of environmental conditions, because these fungi can develop in different climates at both high and low temperatures, with a wide range of relative humidity, and under multiple combinations of environmental factors (Rotem, 1994).

Although several studies have focused on *Alternaria* and closely related genera, *Alternaria* taxonomy remains controversial. Since morphological characterization can bring misidentification in taxonomy, genetic characterization is recommended for the identification at species level, or rather at Section level (Andersen *et al.,* 2009; Lawrence *et al.,* 2013; Woudenberg *et al*., 2015; Somma *et al.,* 2019). The identification of *Alternaria* species in the present study was based on sequence analyses of the fragments of *gpd* and *β-tubulin*, previously used for phylogenetic studies (Woudenberg *et al*., 2015; Somma *et al*., 2019). Based on Lawrence *et al.* (2013), the *calmodulin* gene was also included since it strongly supported the differentiation of *Alternaria* species, mostly those belonging to the *Infectoriae* Section (Lawrence *et al*., 2014). The *alt-a1* fragment, previously and widely used to characterize *Alternaria* species from wheat (Somma *et al*., 2019; Masiello *et al*., 2020), was also included in the present study. Several field strains did not amplify with *alt-a1* primers. The phylogenetic tree obtained with the remaining three gene fragments (*gpd, tub, calmodulin*) showed that all the strains that failed with *alt-a1* gene amplification belonged to *Infectoriae* Section, and were the majority of this section, i.e. 26 out of 30 strains (87%). Yet, the *alt-a1* gene was excluded from our phylogenetic analysis. A possible explanation to its failure in this study could be that a large proportion of strains of the *Infectoriae* Section from Lebanon had nucleotide polymorphisms in the sequence fragment within the annealing site of the primers. This hypothesis should be verified with further investigation.

The strains phylogenetically identified in the present study belonged mostly to *Alternaria* (51%) and *Infectoriae* (40%) sections. The *Alternaria* section clade showed that the sequences of *A. alternata* and *A. tenuissima* species are indistinguishable, and thus the two species can be merged in the same species *A. alternata*, as was proposed by Woudenberg *et al.* (2015) after whole genome and trancriptome analyses, and then confirmed by Somma *et al.* (2019). Furthermore, *A. tangelonis* synonymized under *A. gossypina* formed a supported clade from *A. alternata* in the present study. This species can be easily distinguished from *A. alternata*, in agreement with Woudenberg *et al.* (2015), who defined it as a new species. It is likely that *A. arborescens* and *A. cerealis*, defined as a species complex (Woudenberg *et al*., 2015), formed a well-separated clade from *A. alternata* in the present analysis.

On the other hand, *A. consortialis*, belonging to section *Ulocladioides*, also formed a separate clade, as in Woudenberg *et al.* (2013). Simmons *et al.* (1967) firstly established *Ulocladium* section, based upon morphological characteristics, and concluded that several atypical *Alternaria* and *Stemphylium* species should be classified as *Ulocladium*.

In addition, two field strains from wheat were assigned to a separate clade for *Chalastospora gossypii*, formerly *A. malorum* or *Cladosporium malorum* (Dugan *et al.* 1995), belonging to *Chalastospora* Section (Andersen *et al.* 2009, Lawrence *et al.* 2013). Only a few studies have reported the presence of *A. malorum* on wheat (Goetz and Dugan, 2006). This species was isolated from soil in Lebanon (Braun *et al.,* 2003), and has been reported on several host plants, including grape, chickpea, apple, and *Prunus*, and proven to be pathogenic (Zhang *et al.,* 2000; Dugan *et al.* 2002; 2005; Goetz and Dugan, 2006; Andersen *et al.,* 2009).

Based on the data obtained with the multi-locus phylogenetic tree, differentiation between *Alternaria* and *Infectoriae* sections was clearly defined, in accordance with Lawrence *et al.* (2014) and Somma *et al.* (2019). These authors deeply investigated and clustered the species of *Infectoriae* section in a phylogenetically well-defined clade very distant from the *Alternaria* clade. Field strains belonging to *Infectoriae* section represented 40% of the total number of the *Alternaria* strains, although *Infectoriae* section was shown to be the most prevalent in cereals in other studies (Dugan and Peever, 2002; Perelló and

Sisterna, 2006; Perelló *et al*., 2008). Detection of *A. triticina* in wheat kernels from Lebanon, generally considered a weak pathogen of wheat (CPC, 2018) and reported to cause leaf spot on wheat plants (Perello and Larran, 2013), agrees with recent studies carried out in Italy (Ramires *et al.,* 2018; Somma *et al*., 2019).

A clade strongly supported as the sister group of section *Infectoriae* was defined in the present study as *Pseudoalternaria*. This group was introduced as a new asexual section by Lawrence *et al.* (2016), and was typified by *A. arrhenatheri, A. parvicaespitosa* and *A. rosae* (Gannibal and Lawrence, 2016). The presence of strains belonging to *Pseudoalternaria* section was therefore demonstrated in Lebanon. These strains were previously detected in wheat in Argentina (Perello *et al*., 2008), Iran (Poursafar *et al*., 2018) and Italy (Ramires *et al*., 2018; Masiello *et al*., 2020).

Therefore, data from the present study, on the clades identified by phylogenetic analyses, agree with previous taxonomic and phylogenetic studies, showing no correlation between geographic origins and species identifications.

Species belonging to *Alternaria* section have been widely shown to produce the mycotoxins TA, AME, AOH, ALT and ATX (Logrieco *et al.,* 2009). On the other hand, studies on species of section *Infectoriae* have never shown their capability to produce mycotoxins (Andersen *et al*., 2009), although some metabolite production (e.g. of infectopyrones and novae-zelandins, which are not produced by other *Alternaria* species) has been demonstrated (Andersen *et al*., 2009, Christensen *et al*., 2005). The present results disagree with many other reports on *Alternaria* from wheat (Patriarca *et al.,* 2007; Bensassi *et al.,* 2009; Kahl *et al.,* 2015; Ramires *et al*., 2018; Somma *et al*., 2019, Masiello *et al*., 2020), because all the samples of wheat kernels from Lebanon were free of *Alternaria* toxins. One explanation could be the *Alternaria* species composition of the whole Lebanese population. Species belonging to *Alternaria* section, known to be mycotoxin producers, were detected only at 51%. On the other hand, despite the high contamination of kernels by *Alternaria*, symptoms of black point were not as frequent. This could indicate that the infections by the pathogen at harvest were at early stages, so the contamination by mycotoxins could be still undetectable.

The absence of mycotoxin contamination in Lebanese wheat could also be related to environmental factors affecting *Alternaria* mycotoxin production, as reported in Amatulli *et al.* (2013). Mycotoxins can also be produced during either pre- or post-harvest stages of transportation and storage, or during processing procedures. In general, the main factors affecting mycotoxin production are nitrogen and carbon sources, temperature, water activity (a_w) and pH (Yu *et al*., 2005; Ogelgsang *et al*., 2008). As previously reported (Oviedo *et al*., 2009, 2010; Pose *et al*., 2010), the greatest toxin production occurs with 0.98 a_w and at 25°C. Lower a_w and temperature conditions probably characterize Lebanon regions, and this could have inhibited mycotoxin production. Also, Brzonkalik *et al.* (2011, 2012) showed that the production of AOH and AME was increased by phenylalanine, whereas some nitrogen sources can inhibit production. Carbon and nitrogen sources influenced the polyketide mycotoxin, and TA biosynthesis was mainly influenced by carbon source. Thus, further investigations are strongly encouraged to determine the environmental factors involved in the inhibition of mycotoxin production by these *Alternaria* strains in Lebanese wheat.

CONCLUSIONS

This study has highlighted the widespread occurrence of contamination of wheat grain by *Alternaria* species, with contamination rates ranging from 2 to 59% depending on location. Most of the isolated strains belonged to *Alternaria* and *Infectoriae* sections. Some strains (9%) belonging to sections *Ulocladioides*, *Chalatospora* and *Pseudoalternaria* were identified for the first time in Lebanon. All samples were assayed for the presence of the most important *Alternaria* mycotoxins (AOH, AME, ALT, and TA), but none of these mycotoxins were detected. Contamination by *Alternaria* spp. can reduce the quality of wheat grain. Further research is required to verify the mycotoxin production capability of *Alternaria* strains from Lebanese wheat.

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