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Characterization of *Stemphylium* spp. associated with tomato foliar diseases in Algeria

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Summary. Leaf blight and spot caused by *Stemphylium* spp. and *Alternaria* spp. are the most common destructive tomato diseases in north-western Algeria. During 2018 growing seasons, more than 30% of samples collected from plants grown in greenhouses or open fields were infected with *Stemphylium*. Initial symptoms were small, multiple, irregular to oval, yellow leaf spots, which enlarged to brown lesions later. In these lesions, *Stemphylium* mostly co-occurred with *Alternaria* spp. Twenty nine *Stemphylium* isolates were characterized based on morphological features, and multi-locus phylogenies using *ITS*, *gpd*, and *cmdA* genomic loci. Five *Stemphylium* species (*S. lycopersici*, *S. gracilariae*, *S. eturmiunum*, *S. vesicarium*, *S. lycii*) were associated with tomato leaf spot, of which *S. lycii* is a new report for tomato. Pathogenicity tests on healthy 2-months-old tomato seedlings reproduced symptoms similar to those observed in tomato crops. The tested fungus isolates differed in pathogenicity. Two isolates of *S. lycopersici* were more aggressive than those of the other species, causing major lesions on tomato plants. The five identified *Stemphylium* species are reported for the first time as new pathogens for tomato in Algeria, and *S. lycopersici*, *S. gracilariae, S. eturmiunum,* and *S. lycii* as new species of Algerian mycoflora.

Keywords. Tomato leaf spot, disease complex, pathogenicity test.

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INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the most common temperate vegetable crop cultivated in Mediterranean countries. In Algeria, demand for tomato is increasing as a result of human population growth, where tomato is the fourth most commonly grown crop, after potato, watermelon and onion (Messak, 2014). Tomato crops covered 24,996 ha and yielded was 59,124 kg ha^{-1} in 2019 (FAO, 2019). However, heavy crop losses are caused by pests and fungus diseases every year. Leaf blight caused by *Stemphylium* spp. and *Alternaria* spp. has become of increasing importance in recent years. These fungi are serious pathogens on tomatoes and other *Solanaceae* in the north-western growing regions of Algeria, viz. Mostaganem, Oran, Mascara,

Tlemcen and Ain Témouchent (Bessadat *et al*., 2016; Bessadat *et al*., 2019).

Stemphylium (*Pleosporaceae*) was first described in 1833 by Wallroth and then by Wiltshire (1938). This genus includes saprophytic and pathogenic fungi (Inderbitzin *et al*., 2009). These fungi reproduce asexually through production of conidia, and in some species through *Pleospora* teleomorphic stages (Simmons, 2007; Inderbitzin *et al*., 2009). *Stemphylium* is morphologically similar to the related genus *Alternaria*. The conidia of both genera are multiseptated and pigmented, and form on conidiophores generating from hyphae in the mycelium. *Stemphylium* is distinguished from *Alternaria* based on apically percurrent conidiophores which produce successive conidia (Simmons, 2007). Because of its widespread distribution and pathogenicity, *Stemphylium* is an important and destructive pathogen, causing leaf spot diseases in several crop hosts (Ellis, 1971). Leaf spot has the potential of becoming a threat to tomato production, especially in regions where susceptible cultivars are grown. Incidence of *Stemphylium* has been reported to reach as high as 100% (Cedeño and Carrero, 1997).

Symptoms are often first seen on young tomato seedlings. Conidia of the pathogen invade leaves primarily through stomata, and a vesicles developed inside the substomatal cavities (Benets and Matsuoka, 2005). Symptoms appear as small brown or grayish spots with yellow halos that later expand into necrotic lesions, sometimes with gray centres and dark brown borders. As the disease progresses, affected leaves become chlorotic, and the lesions dry out and usually crack. Severely infected leaves die and fall off (Blancard *et al*., 2012). The pathogen can persist on dead or dying plant material on alternative hosts (pepper, eggplant and other solanaceous crops or weeds) where pesudothecia can form (Cerkauskas, 2005). Conidia and ascospores are the primary inocula in the following season (Basallote-Ureba *et al*., 1999; Brahamanage *et al*., 2018), and these are disseminated by wind or rain (Rossi *et al*., 2005; Cerkauskas, 2005). The disease is favoured by extended periods of leaf wetness from rains or dew, and by moderate to warm temperatures (20–30°C), all of which characterize the temperate climate of Mediterranean basin regions.

These environmental conditions also enhance the appearance and development of other diseases on tomato, including *Alternaria* (Chaerani and Voorrips, 2006) and bacterial leaf spots (Huang and Tsai, 2017). *Stemphylium* and *Alternaria* have been shown to cause similar symptoms in the field (Fernández and Rivera-Vargas, 2008). *Alternaria* spp. were associated with *Stemphylium* spp. in more than 25% of analyzed samples from tomato producing areas of Algeria between 2011 and 2013 (Bessadat *et al*., 2016). *Stemphylium* disease of tomato may be confused with symptoms of bacterial leaf spot or late blight (Huang and Tsai, 2017). Thus, it is often difficult to accurately diagnose the disease caused by *Stemphylium* through visual observations.

Stemphylium spp. have wide host ranges, with cases of multiple pathogens on individual hosts, as well as single species on diverse hosts (Ellis and Gibson, 1975; Köhl *et al*., 2009; Brahmanage *et al.,* 2018; Farr and Rossman, 2021). Many *Stemphylium* species have been reported on tomato, including *S. solani* (Weber, 1930; Cedeño and Carrero, 1997), *S. floridanum* syn. *lycopersici* (Rotem and Bashi, 1977; Blancard *et al*., 1986), *S. vesicarium* (Blancard *et al*., 1986), *S. lycopersici* (Enjoji, 1931; Al-Amri *et al.,* 2016; Huang and Tsai, 2017), *S. botryosum* (Dickens and Evans, 1973; Rotem and Bashi, 1977), *S. tomatonis* syn. *vesicarium* and *S. eturmiunum* (Simmons, 2001; Andersen and Frisvad 2004).

Characterization and taxonomic determination of *Stemphylium* spp. has relied mainly on morphological characteristics including conidium shape, size, septation, length/width ratio and ornamentation (Simmons, 1969). However, high variability of species in culture and plasticity in host preference for these fungi have made it difficult to identify isolates to species (Chowdhury *et al*., 2015; Poursafar *et al*., 2016; Subash and Saraswati, 2016). Molecular tools have been widely implemented for accurate differentiation between *Stemphylium* spp., with species identification relying on sequence analyses of various DNA regions (Câmara *et al*., 2002; Inderbitzin *et al*., 2009; Woudenberg *et al*., 2017) and metabolite profiling (Andersen *et al*., 1995; Olsen *et al*., 2018). Several molecular-based studies have confirmed that combining the internal transcribed spacers (*ITS*) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) regions could help resolve *Stemphylium* to species level (Câmara *et al*., 2002; Wang *et al*., 2010). More than 90 *Stemphylium* species have been isolated from different host plants (Poursafar *et al.* 2016). However, understanding of phylogenetic relationships within the genus has evolved. Woudenberg *et al*. (2017) revised *Stemphylium* taxonomy based on multi-gene phylogeny, and showed that only 28 species can be distinguished based on combined DNA sequences of the *ITS*, *gpd* and calmodulin (*cmdA*) gene regions. In that study, 22 species names were synonymized, and two new combinations were proposed. More recently, five new species were described based on morphological and on multi loci phylogeny (Brahmanage *et al.,* 2019; Marin-Felix *et al*., 2019; Crous *et al*., 2020).

Stemphylium species associated with gray leaf spot symptoms of tomato have been reported in several countries of the Mediterranean basin (Blancard *et al*., 1986

2012), but distribution of many species has not been thoroughly investigated in Algeria. The main purpose of the present study was to evaluate the composition of *Stemphylium* spp. on leaves and fruits of tomato collected in the northwest regions of the country. Specific objectives were: (1) to identify *Stemphylium* species using sequence analyses of combined datasets of the *ITS*, *gpd*, and *cmdA* gene regions, because morphological criteria were not reliable enough for this purpose; and (2) to test and compare the pathogenicity of different isolates under experimental conditions. Knowledge obtained will provide a basis for establishing effective management strategies for leaf spot diseases of tomato.

MATERIALS AND METHODS

Sample collection and isolation

Samples with characteristic symptoms of leaf spot were collected from different tomato fields in Mostaganem and Oran regions of Algeria during the 2018 growing season. To isolate the causal agent, pieces of diseased leaf or fruit samples collected randomly from the prospected fields were disinfected with 2% NaOCl for 2 min, rinsed with sterile distilled water three times, plated on potato carrot agar (PCA), and then incubated at room temperature (18–25°C). After 1 to 3 weeks, mycelium of growing fungi with typical characteristics of *Stemphylium* was transferred with a fine sterile needle onto new PCA plates. The incidence of fungi associated with the diseased tomato material was summarized as isolation frequency (number of diseased leaves from which a species was isolated compared to the total number of leaves incubated × 100). Pure cultures of the resulting fungi were obtained transferring single conidia onto 2% water agar or hypha tips onto potato dextrose agar (PDA). Purified isolates were subcultured onto PDA slants and kept at 4°C for further examination. Pure cultures of all identified species were deposited in fungal culture collection COMIC (SFR QUASAV, Angers, France) (Table 1).

Morphological assessments

For macroscopic descriptions (colony colour, shape, texture and diameter), purified cultures were grown on PDA and incubated for 7 d at 25°C in continuous darkness. Microscopic characters were assessed based on the standardized conditions suggested by Simmons (2007). Isolates were incubated at room temperature on PCA under a light/dark photoperiod for 5 to 7 d. The standard technique of adhesive tape preparation (Scotch tape prep or cellophane tape prep) was used for slide preparation (Forbes *et al*., 2002), with lactic acid solution as mounting fluid. Morphological observations were made with a light microscope (Optika 190 B) and a stereomicroscope. Microscopic features recorded were compared with available literature.

DNA extractions and PCR amplifications

Genomic DNA of each *Stemphylium* isolate was extracted using a microwave mini-prep extraction method (Goodwin and Lee, 1993). The primers ITS1/ITS4 (White *et al*., 1990) were used for amplification of the internal transcribed spacer regions of ribosomal DNA (*ITS rDNA*), primers gpd1/gpd2 (Berbee *et al*., 1999) for the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene region, and CALDF1 /CALDR1 (Lawrence *et al*., 2013) for the calmodulin (*cmdA*) gene region. Polymerase chain reaction (PCR) amplification was carried out using the primer and PCR protocols described in Woudenberg *et al*. (2017). Each PCR amplification was carried out in a total volume of 25 µL, containing 75mMTris-HCl pH 9.0, 20 mM $(NH_4)_2SO4$, 0.01 % (w/v) Tween 20, 1.5 mM $MgCl_2$, 200 µM desoxyribonucleotide triphosphate, 1 unit of thermostable DNA polymerase (GoTaq, Promega), and 400 nM of each relevant oligonucleotide primer. PCR products were sent to GATC lab for sequencing. The newly generated sequences of *ITS, gpd* and *cmdA* were submitted to GenBank (Table 1).

Phylogenetic analyses

The resulting sequences from each locus were concatenated and aligned through ClustalW algorithm and refined manually using MEGA 7 (Kumar *et al*., 2016). Sequences of the type isolates were retrieved from GenBank and included in the analysis. *Alternaria alternata* isolate GV14-634a1 was used as an outgroup. Phylogenetic analyses were carried out using the maximum likelihood (ML) under IQTree v.1.6.11 (Nguyen *et al*., 2015), and Bayesian inference (BI) with MrBayes v.3.2.1 (Ronquist and Huelsenbeck, 2003). The best-fit evolutionary models for each dataset calculated by ModelFinder (Kalyaanamoorthy *et al*., 2017) under the Bayesian Information Criterion (BIC) selection procedure were K2P + G4 for *ITS* and *gpd*, and HKY + F + G4 for *cmdA*. The ML analysis was carried out with 1000 ultrafast bootstrap replicates, and only values above 70% were considered significant. BI analyses were carried out to estimate the posterior probabilities (PP) of tree topologies based on the Markov Chain Monte

Table 1. *Stemphylium* isolates from tomato characterized in this study, their geographical origins, and the GenBank accession numbers of their internal transcribed spacer (*ITS*), glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and calmodulin marker (*cmdA*) genomic sequences.

Isolate	Location	Species	*ITS*	*gpd*	*cmdA*
NB644	Mostaganem	*Stemphylium gracilariae.*	MZ093115	MZ152654	MZ152682
NB646	Mostaganem	*Stemphylium gracilariae*	MZ093112	MZ152649	MZ152677
NB649	Mostaganem	*Stemphylium gracilariae*	MZ093113	MZ152653	MZ152681
NB650	Mostaganem	*Stemphylium gracilariae*	MZ093115	MZ152652	MZ152680
NB651	Mostaganem	*Stemphylium vesicarium.*	MZ099818	MZ152669	MZ152697
NB654	Mostaganem	*Stemphylium vesicarium.*	MZ099817	MZ152668	MZ152696
NB682	Mostaganem	*Stemphylium vesicarium*	MZ099816	MZ152667	MZ152695
NB683	Mostaganem	*Stemphylium lycii*	MZ090945	MZ130945	MZ152704
NB690	Oran	*Stemphylium gracilariae*	MZ093117	MZ152651	MZ152679
NB696	Oran	*Stemphylium gracilariae*	MZ090945	MZ152648	MZ152676
NB709[1]	Mostaganem	*Stemphylium eturmiunum*	MZ093122	MZ152670	MZ152698
NB710[1]	Mostaganem	*Stemphylium lycopersici*	MZ093124	MZ152657	MZ152685
NB711[2]	Mostaganem	*Stemphylium lycopersici*	MZ093125	MZ152658	MZ152686
NB712[2]	Mostaganem	*Stemphylium eturmiunum*	MZ093119	MZ152671	MZ152699
NB713[3]	Mostaganem	*Stemphylium vesicarium*	MZ099814	MZ152663	MZ152691
NB714[3]	Mostaganem	*Stemphylium eturmiunum*	MZ093118	MZ152672	MZ152700
NB715	Mostaganem	*Stemphylium vesicarium*	MZ099812	MZ152666	MZ152694
NB717	Mostaganem	*Stemphylium gracilariae.*	MZ093114	MZ152650	MZ152678
NB719	Mostaganem	*Stemphylium lycopersici*	MZ093126	MZ152659	MZ152687
NB720	Mostaganem	*Stemphylium vesicarium*	MZ099811	MZ152665	MZ152693
NB731	Mostaganem	*Stemphylium eturmiunum*	MZ093121	MZ152675	MZ152703
NB735	Mostaganem	*Stemphylium eturmiunum*	MZ093123	MZ152674	MZ152702
NB736	Mostaganem	*Stemphylium eturmiunum*	MZ093120	MZ152673	MZ152701
NB737	Mostaganem	*Stemphylium vesicarium*	MZ099815	MZ152662	MZ152690
NB744	Mostaganem	*Stemphylium lycopersici*	MZ093129	MZ152661	MZ152689
NB747	Mostaganem	*Stemphylium lycopersici*	MZ093127	MZ152655	MZ152683
NB748	Mostaganem	*Stemphylium lycopersici*	MZ093130	MZ152660	MZ152688
NB751	Mostaganem	*Stemphylium lycopersici*	MZ093128	MZ152656	MZ152684
NB754	Mostaganem	*Stemphylium vesicarium*	MZ099813	MZ152664	MZ152692

[1], [2] and [3]: Isolates recovered from a same leaf necrosis.

Carlo (MCMC) analysis with four chains, 1M generations, sampled every 1000 generations. Burn-in was set to 25% and only PP values greater than 0.95 were considered significant.

Pathogenicity tests

To confirm the disease and its causal agents, a series of pathogenicity tests was conducted under greenhouse conditions. These used 2-month-old seedlings of tomato var. 'Saint Pierre' planted in pots (two seedlings per pot) containing 3:1 sterilized potting media:sand. Inoculum was prepared of each of the 28 representative isolates as described (Bessadat *et al*., 2016; 2019). For each trial, three plants were inoculated with 10 mL of a 1×10^4 conidia mL^{-1} suspension by spraying foliage with a hand atomizer. Three plants were also sprayed with sterile distilled water as experimental controls. Plants were then covered with polyethylene bags for 2 d to maintain a high relative humidity. Leaf spot progression was evaluated at 7, 14 and 21 d after inoculation (dai). The area under disease progress curves (AUDPC) was calculated using the open source image analysis software ImageJ (https://imagej.nih.gov/ij/plugins/). Symptoms expressed were studied and compared with those that occurred on the infected leaves collected from field-grown plants. Isolations were made from diseased inoculated plants for assessing fulfilment of Koch's postulates. Mean percentage of necrotic leaf area (n.l.a) and AUDPCs were determined for three replicates. Statistical analyses of obtained data were carried out using R software for ANOVA and Tukey post-hoc tests.

RESULTS

Pathogen isolation and disease incidence

During the 2018 growing season, severe leaf chlorosis and necrosis, characterized by brown to dark brown colour (Figure 1, a to f), followed by defoliation, were observed on tomato plants in the Mostaganem and Oran regions of northwest Algeria. In many cases, the symptoms were also associated with *Alternaria* diseases (leaf spot and blight) and other pests including *Tuta absoluta*. Field observers had difficulty distinguishing *Alternaria* leaf blight and spot symptoms from those caused by *Stemphylium* spp., because symptoms caused by fungi of both genera strongly resemble each other. However, the typical symptoms of *Stemphylium* on ripe and unripe fruit (brown specks, Figure 1, g and h) were easily distinguishable from the black mould symptoms caused by *Alternaria* spp. Fruit with physiological conditions allowing secondary infection by fungi, such as blossom end rot pathogens (Figure 1, i), were mainly colonized by *Stemphylium* and *Alternaria* species with small conidia. The brown lesions observed on tomato stems and petioles were induced by *Alternaria* sp. only.

Figure 1. Symptoms caused by *Stemphylium* spp. on tomato leaves and field-grown fruit. Developing lesions on tomato leaves (**a** and **b**). Darkening lesions on a tomato leaflet (**c**). Advanced necroses on tomato leaves (**d**, **e**, and **f**). Brown speck on ripe (**g**) and unripe tomato fruit (**h**). Dead tissues of a tomato fruit, colonized by *Stemphylium* with black felting corresponding to conidium production (**i**).

After 1 to 3 weeks, a total of 949 colonies were obtained from 370 repetition pieces of symptomatic tomato tissues. We frequently observed morphologically distinct fungal colonies on PCA plates originating from a single lesion; these primarily consisted of *Stemphylium* and *Alternaria* species. *Stemphylium* sp. were associated with *Alternaria* spp. in more than 29% of the samples. Simultaneous isolation of the two fungi from symptomatic tomato leaves in Algeria has been previously reported (Bessadat *et al*., 2019), but co-infection of individual lesions has not. *Stemphylium* sp. were recovered from 20 to 47% of lesions on tomato (average = 34%), whereas *Alternaria* spp. were isolated at a frequency of approx. 41%. Secondary leaf invaders were isolated at an average frequency of 25%, and these included *Penicillium* sp., *Cladosporium* sp. and *Chaetomium* sp.

A total of 93 isolates with *Stemphylium* characteristics were obtained by single conidium or hyphal tip methods. Cultural and macro-morphological features of strains were characterised by mycelium colour (white, beige, orange, olivaceous, grey, pink or greyish green) and texture. Diffusible pigmentation into culture medium produced by some isolates was also observed (Figure 2). Twenty-nine isolates were selected for further characterization (Table 1). Preliminary identification made by ITS sequencing confirmed that all these isolates were *Stemphylium*.

Species identification

A multi-gene phylogeny approach was used to identify *Stemphylium* isolates at the species level, using currently accepted methods to differentiate *Stemphylium* species (Woudenberg *et al*., 2017). The combined dataset of *ITS*, *gpd,* and *cmdA* sequences from 29 tomato isolates and from 68 representative strains of 31 recognized species of *Stemphylium* were compared. The dataset had a total length of 1462 bp (*ITS*: 451 bp, *gpd*: 394 bp, *cmdA*: 617 bp) of which 265 bp were phylogenetically informative (*ITS*: 20 bp, *gpd*: 89 bp, *cmdA*: 156 bp). The topologies of the trees inferred by the two phylogenetic methods (ML and BI) were similar. The ML phylogenetic tree with high bootstrap support (99%) and posterior probability values (1.0) indicated that the 29 tomato isolates grouped into five well-supported phylogenetic species (Figure 3). Additional analysis of morphological characteristics was carried out to describe the isolates from tomato.

Stemphylium eturmiunum

Six isolates (NB709, NB712, NB714, NB731, NB735, NB736), all from Mostaganem, were attributed to *S. eturmiunum*. Colonies were cottony to sub-aerial, dark green with greyish surfaces and white regular margins, reaching 56 to 76 mm diam., and occasionally producing yellow pigment on PDA after 7 d at 25°C in the dark (Figure 2, a to e). Conidium production was moderate on PCA after 7 d. Conidiophores were swollen at their tips, brown in colour, cylindrical, occasionally branched, and of short to moderate length, measuring 25–120 × 5–8 µm, and with two to eight transverse septa. Conidiophore each generally produced a single apical conidium. Conidia were ovoid to oblong, ellipsoid, occasionally rectangular, muriform upon aging, light to dark brown in colour (Figure 4 a), and measuring 22–38 × 10–22 µm. Mean conidium length/width ratio was 1.7 ± 0.3, and conidia had one to four transverse septa and zero to five longitudinal or oblique septae per transverse segment. The conidia were mostly constricted at the median transverse septae. Short conidium chains of two to four conidia, and lateral or apical secondary conidiophores were frequently observed at the centres of colonies. Ascomata observed on agar were spherical, subspherical, and dark brown, with dark hyphal outgrowths (Figure 5 a), and were single or aggregated, ranging from 200 to 980 µm in length. Asci and ascospores were observed on PCA after 4 to 5 weeks incubation. Asci were cylindrical with bitunicate and broadly rounded apices, gradually narrowed near the bases (Figure 5 e), and measured 145–221 × 21–37 µm. Ascospores were oblong, ellipsoid, with obtusely rounded ends, yellowish brown with darkened septae, constricted mostly at the 2nd transverse septae, and had zero to three longitudinal septa per transverse segment. Ascospores had dimensions of 27–46 × 10–20 µm.

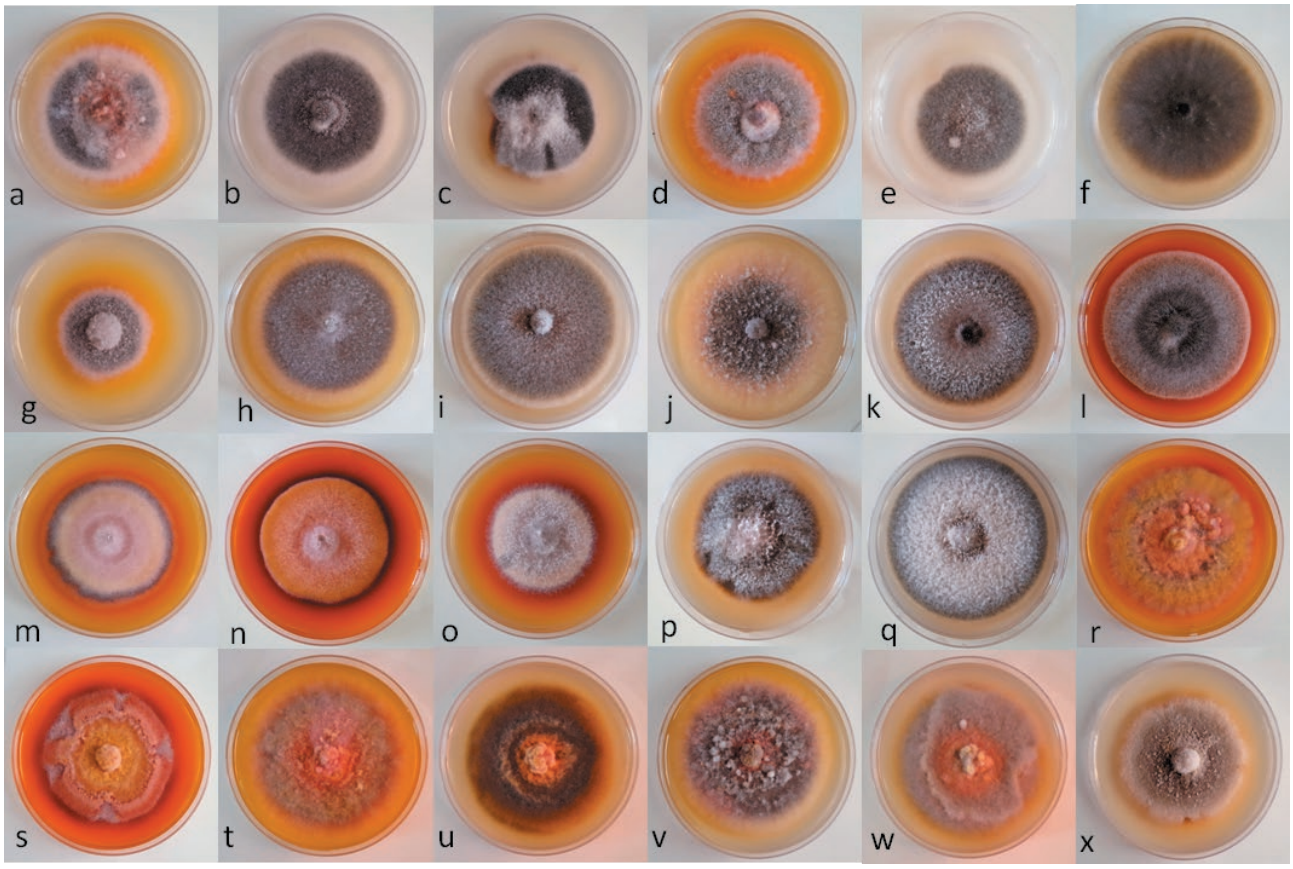

Figure 2. Colonies of 21 field isolates of *Stemphylium* spp. on potato dextrose agar after 7 d incubation at 25°C in the dark. *Stemphylium eturmiunum*, NB709 (a); NB712 (b), NB714 (c), NB731 (d), NB736 (e): *S. vesicarium,* NB654 (f), NB713 (g), NB715 (h), NB720 (i), NB737 (j): *S. gracilariae*, NB644 (k), NB646 (l), NB649 (m), NB650 (n), NB690 (o), NB696 (p), NB717 (q): *S. lycopersici*: NB710 (r), NB711 (s), NB719 (t), NB744 (u), NB747 (v), NB748 (w): *S. lycii*: NB683 (x).

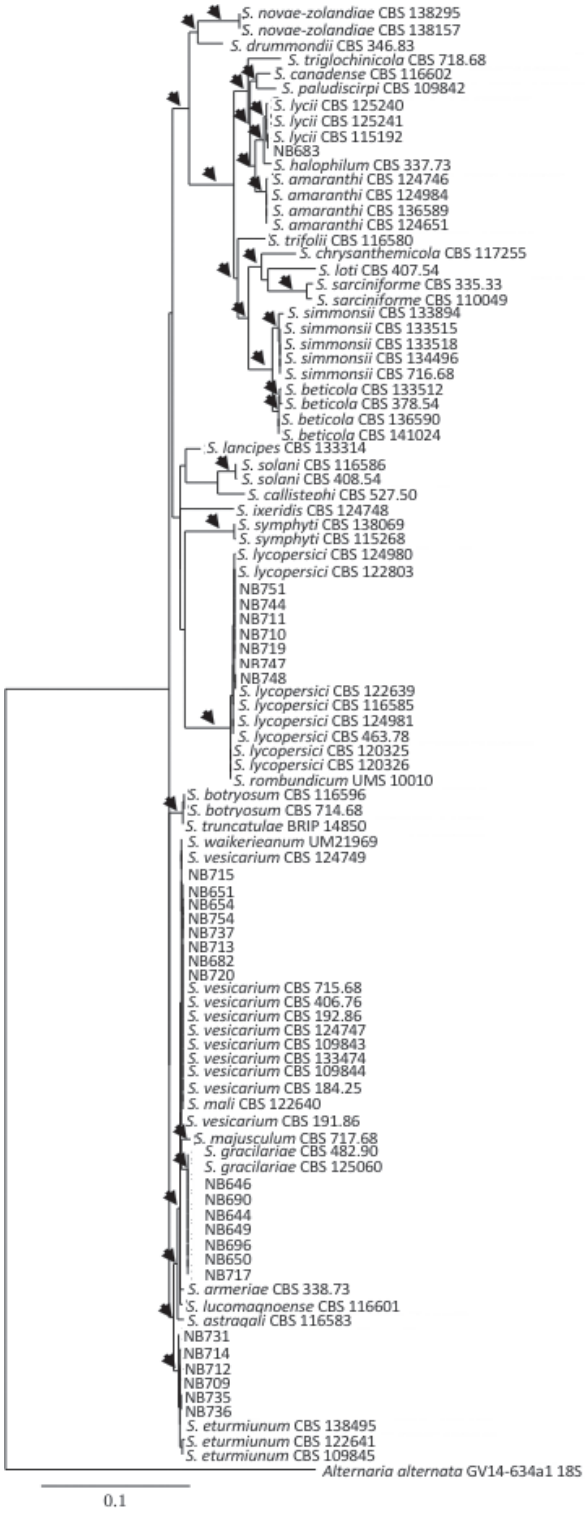

Figure 3. Phylogenetic tree reconstructed using the maximum likelihood method, from the alignment of *ITS*, *gpd* and *cmdA* sequences of *Stempylium* isolates. The tree was rooted with *Alternaria alternata* isolate GV14-634a1. Bootstrap support values greater than 0.7 are indicated by arrows near nodes. The scale bar indicates the expected number of substitutions per position.

Stemphylium vesicarium

Eight isolates (NB651, NB654, NB682, NB713, NB715, NB720, NB737, NB754), all from Mostaganem, were attributed to *S. vesicarium*. These isolates developed cottony colonies 49 to 80 mm diam. white regular margins on PDA after 7 d at 25°C in the dark (Figure 2, f to j). Colonies were olivaceous to greyish green with yellow pigmentation of the medium. Conidium production was moderate on PCA after 7 d. Conidiophores were straight or occasionally branched, each with one or two swollen apices and two to 11 septae, and were of short to moderate lengths (27–163 µm) and widths of 5–10 µm. Conidia were medium to dark drown, oblong, ellipsoid to muriform, with One to three (four) transverse and one to four longitudinal septae per transverse segment. The conidia were each constricted at one to three of the major transverse septae (Figure 4, b). Conidium dimensions were from 21–45 × 12–25 µm, with mean length/width ratio of 1.7 ± 0.3. Ascomata were formed after 7 d, and matured after 5 to 6 weeks. They were dark brown, thick-walled, spherical to sub-spherical, ranging from 356 to 700 µm in length (Figure 5, b). Asci were cylindrical to clavate, 138–243 × 22–32 µm with each containing eight ascospores. Mature ascospores were yellowish brown, oblong-ovoid, rounded at the bases and with conical apices (Figure 5 f), and measured 30–55 × 11–21 µm, with up to three transverse eusepta and zero to three longitudinal septae per transverse segment, and more or less constricted at the transverse septae. However, no fully mature ascospores could be found with NB713 and NB737 isolates in culture.

Stemphylium gracilariae

Seven isolates (NB644, NB646, NB649, NB650, NB690, NB696, NB717), from Mostaganem and Oran, were assigned to *S. gracilariae*. Colonies on PDA were cottony, sub-aerial, and olivaceous, with greyish or whitish surfaces and white to beige regular margins, reaching 59 to 78 mm diam. after 7 d at 25°C in the dark. Pigmentation of the growth media varied from pale yellow to dark orange (Figure 2, k to q). Abundant conidium production was observed on PCA. Conidiophores were brown, swollen, cylindrical, short (25–75 × 4–9 µm), with one to seven transverse septae, each bearing one or two conidia. Conidia were brown to dark drown, muriform, ovoid to oblong, ellipsoid, with one to three transverse and one to three (four) longitudinal septae per transverse segment. The conidia were constricted at the median transverse septae (Figure 4 c). Conidium dimensions were 20–37 ×

10–22 µm, with mean length/width ratio of 1.8 ± 0.3. Ascomata were abundantly formed, and were dark to black, covered with hyphal outgrows, and were spherical or irregular (Figure 5 c), broadly to narrowly oblong, single or aggregated, and ranged from 200 to 900 µm diam. Asci were cylindrical, bitunicate, hyaline (Figure 5, g), and measured 90–251 × 25–37 µm, and each ascus contained eight ascospores. Ascospores were 30–55 × 12–20 µm, yellowish brown in colour, each three main transverse septae which were thickened, darkened and constricted.

Stemphylium lycopersici

Seven isolates (NB710, NB711, NB719, NB744, NB747, NB748, NB751), all from Mostaganem, were identified as *S. lycopersici*. Colonies on PDA after 7 d at 25°C in the dark were 66 to 81 mm diam., cottony, compact, with sparse floccose aerial mycelium, and had regular to irregular velvety margins. Isolates were characterised by heterogeneous mycelium pigmentation (beige, orange and greyish pink to red), and each produced a yellow or a strong orange pigment that diffused into the culture medium (Figure 2, r to w). Conidium production was moderate to abundant on PCA after 7 d. Conidiophores were mostly solitary, straight, three to 17 septate, measuring 75–381 × 5–11 µm, with swollen apical conidiogenous cells and nodded appearance, sometimes due to successive periods of growth. Isolates did not form ascomata. Conidia were solitary, yellowish to brown, smooth or with a punctate ornamentation, mostly 42–87 × 13–22 µm, and with mean length/width ratio of 3.6 ± 0.5. On PCA, apical secondary conidiophores were occasionally observed. Conidia were oblong, cylindrical with conical apices and rounded bases, divided by three to eight transverse spetae and two to three longitudinal or oblique septae per transverse segment (Figure 4, d). Each conidium was constricted at the transversal septum. Isolates of this species did not form ascomata. This species can be distinguished its large conidia, long conidiophores and strong pigmentation of culture media.

Stemphylium lycii

Isolate NB683 was attributed to *S. lycii*. Colony diameter in 7-d-old PDA cultures was 62 to 68 mm. Colonies were cottony and compact with white irregular margins, and were grey with whitish centres and orange mycelial tufts (Figure 2x), and developed pale-yellow pigmentation. Conidium production was abundant on PCA after 7 d. Conidiophores had apically swollen conidiogenous cells, and were straight, cylindrical, unbranched, measuring 45–208 × 5–8 µm with three to 14 transverse septae, each bearing one to four conidiogenous sites. Conidia were solitary, brown, oblong to ellipsoid become muriform with age (Figure 4, e), measuring 26–45 × 13–20 µm, and with mean length/width ratio of 2.1 ± 0.4. Conidia had one to four transverse septae and zero to six longitudinal or oblique septae per transverse segment, and were usually constricted at the first or second major transverse septae. The sexual morph formed after 2 months. Ascomata were spherical to ovoid (Figure 5, d), single, ranging in size from 336–772 µm. Cylindrical to clavate asci measured 170–246 × 20–30 µm, and each contained eight ellipsoidal, muri-

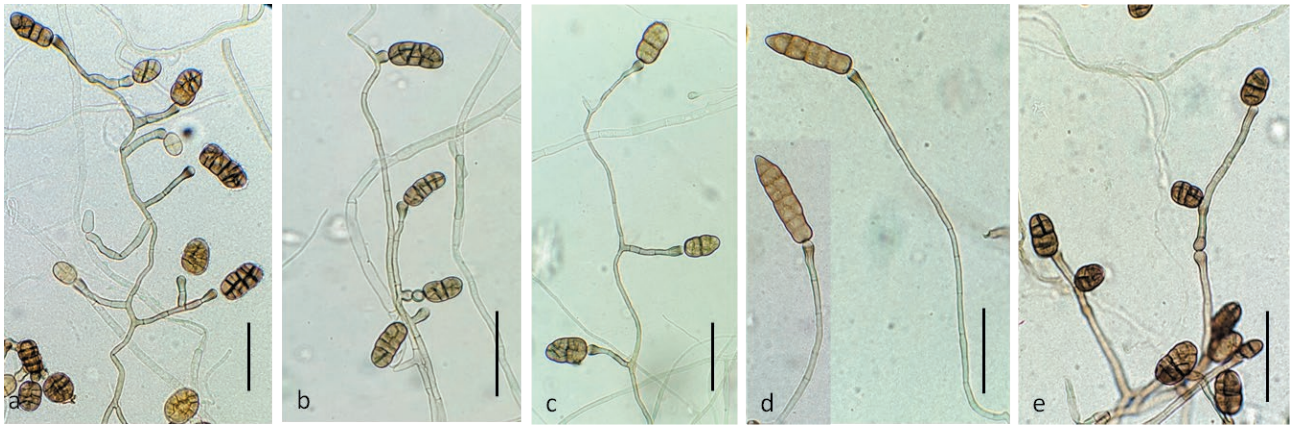

Figure 4. *Stemphylium* spp. on potato carrot agar, after 7 d incubation at room temperature; conidia and conidiophores with swollen apical cells. *Stemphylium eturmiunum* NB731 (a), and NB712 (b); *S. vesicarium* NB737 (c), NB713 (d), and NB654 (e)€; *S. gracilariae*, NB690 (f), NB696 (g), and NB644 (h); *S. lycopersici* NB711 (i); and *S. lycii* NB683 (j). Bars = 50 µm.

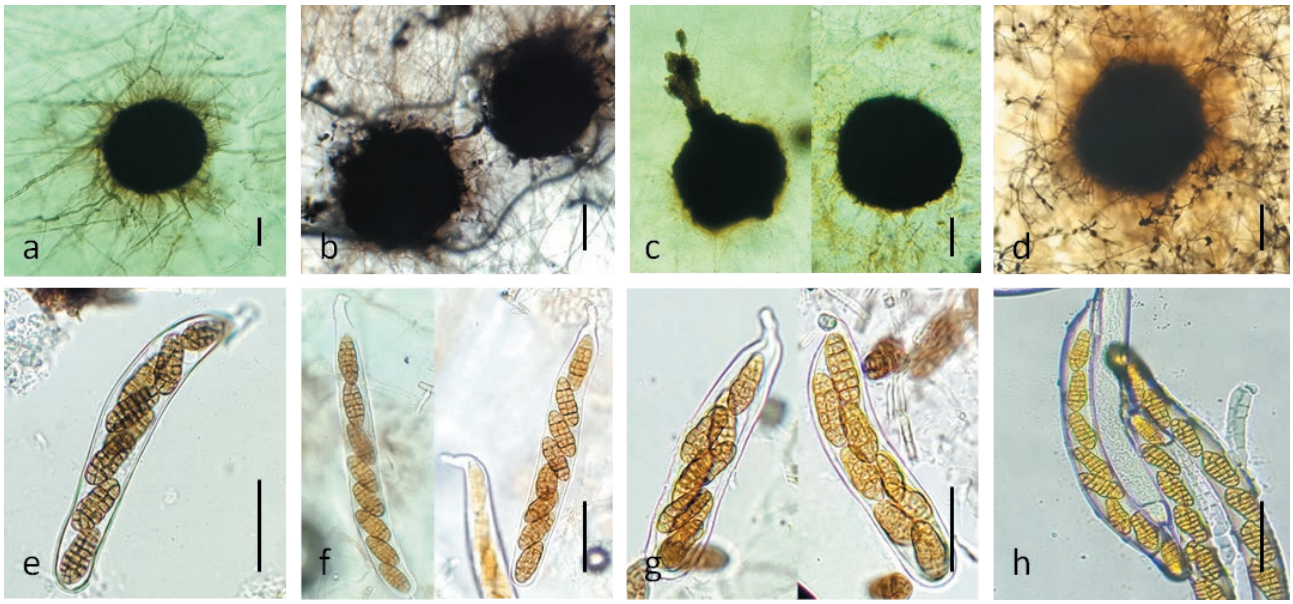

Figure 5. *Stemphylium* spp. ascoma, asci and ascospores of teleomorph stages developed on potato carrot agar after 1 to 5 weeks incubation at room temperature. *Stemphylium eturmiunum (Pleospora eturmiuna)* NB714 (a and e); *S. vesicarium (Pleospora herbarium)* NB654 (b and f); *S. gracilariae* NB696 (c and g); and *S. lycii* NB683 (d and h). Bars = 100 µm (a–d) and 50 µm (e–h).

form ascospores. Ascospores were yellowish brown and measured 32–42 × 12–17 µm, with three (four) transverse septae and one to three septae per transverse segment (Figure 5, h). This species is distinguished from *S. vesicarium* and *S. eturmiunum* by its long conidia and conidiophores formed on PCA, and colony characteristics on PDA.

Pathogenicity tests

All isolates produced symptoms on inoculated tomato plants. After 5 to 7 d, leaf spot symptoms appeared on plants, and the lesions increased progressively with time. Isolates differed in the severity of disease caused, as shown by proportions of leaf necrotic area (% l.n.a) and AUDPC values (Table 2; Figure 6). ANOVA of AUDPC data confirmed a strong "strain" effect ($P = 0.00448$) and no "repeat" effect ($P = 0.471$). Two isolates of *S. lycopersici* (NB744, NB747) were classified as highly virulent, with average AUDPCs >30 000. Aggressive isolates produced necrotic leaf lesions on the inoculated leaves which coalesced to encompass the entire leaves. Proportions of leaf area affected were >80% at 21 dai. No other statistically supported ($P > 0.05$) class of aggressiveness could be defined for all the other isolates, based on AUDPC data, and they were moderately virulent. However, three isolates, *S. gracilariae* NB649, *S. lycopersici* NB711 and *S. eturmiunum* NB714, produced small necrotic leaf lesions on leaves, which did not expand. These isolates were classified as minor pathogens for tomato, and the affected leaf areas for these isolates were less than 11% at 21 dai.

In general, *S. lycopersici* produced characteristic symptoms of dark brown spots on host leaves, which were most severe at 21 dai (Figure 7, a, b and c). Small and longitudinal, brown lesions were also observed on host stems. When plants were exposed to high relative humidity, *Stemphylium*-type conidia were produced on the lesions (Figure 7, l).

All isolates were re-isolated from 90 ± 15% of the inoculated infected leaves. The re-isolated fungi formed typical conidia on PCA and on diseased host leaflets from the artificial inoculations. No symptom development was observed on inoculation control leaves treated with sterilized water.

DISCUSSION

This study is the first report of presence and pathogenicity of five *Stemphylium* species on tomato in Algeria, highlighting the risk of these pathogens causing disease in the future. These pathogens were often recovered from tomato plant lesions along with the closely related *Alternaria* on *Solanaceae* (Bessadat *et al.*, 2016; 2019). Diseased materials collected in northwestern regions, during 2018 growing seasons, were infected by *Stemphy-*

lium spp. and *Alternaria* spp. The high isolation frequency of both genera from foliar lesions was irrespective of symptom morphology, but was most commonly associated with asymmetric, necrotic lesions.

Focusing on the *Stemphylium* spp. component, the polyphasic approach based on morphological and molecular analyses permitted the identification of five species among 29 tested isolates. Molecular data showed that species considered closely related based on morphological traits were phylogenetically distinct. Therefore, initial identifications on the basis of conidium dimensions alone were unreliable, as molecular data indicated that strains were *S. eturmiunum* while morphologically resembling *S. gracilariae* or *S. vesicarium*, and *vice versa*. Therefore, relying on morphological characters to identify species is not recommended (Köhl *et al*., 2009; Han *et al*., 2019; Jayawardena *et al*., 2020). Nevertheless, observations based on conidium size, shape and presence/absence of sexual states on PCA were reliable to distinguish *S. lycopersici* isolates from other species.

None of the isolates studied here were *S. botryosum*, although previous reports have indicated that this species was pathogenic for tomato (Dickens and Evans, 1973; Rotem and Bashi, 1977; Blancard *et al*., 2012; Blancard, 2017). This could be due to misidentifications with the closely related species *S. vesicarium* (Köhl *et al*., 2009; Woudenberg *et al*., 2017). Similarly, neither *S. solani* nor the newly described *S. simmonsii* that have been previously isolated from tomato (Woudenberg *et al*., 2017) were detected in the present study.

Several *Stemphylium* species have been identified as causal agents of tomato diseases in regions with differing climates, such as: South America (Venezuela, Argentina) (Cedeño and Carrero, 1997; Franco *et al*., 2017a, b), Europe (England, Russia) (Dickens and Evans,

Table 2. Mean percentages of leaf necrotic area (l.n.a) recorded on tomato plants inoculated with different *Stemphylium* isolates at 21 d after inoculations.

Isolate	Species	Mean*	Std dev
NB644	*S. gracilariae*	38.5	3.8
NB646	*S. gracilariae*	46.2	4.6
NB649	*S. gracilariae*	9.1	10.5
NB650	*S. gracilariae*	25.9	4.6
NB651	*S. vesicarium*	43.8	14.2
NB654	*S. vesicarium*	35.5	1.2
NB682	*S. vesicarium*	55.8	8.0
NB683	*S. lycii*	53.4	8.3
NB690	*S. gracilariae*	38.8	13.0
NB696	*S. gracilariae*	44.6	6.3
NB709	*S. eturmiunum*	29.0	3.5
NB710	*S. lycopersici*	30.7	7.2
NB711	*S. lycopersici*	10.4	7.9
NB712	*S. eturmiunum*	36.4	10.0
NB713	*S. vesicarium*	26.9	1.0
NB714	*S. eturmiunum*	6.4	2.3
NB715	*S. vesicarium*	50.8	6.1
NB717	*S. gracilariae*	44.3	0.5
NB719	*S. lycopersici*	40.8	2.3
NB720	*S. vesicarium*	32.6	6.1
NB731	*S. eturmiunum*	44.9	7.3
NB735	*S. eturmiunum*	45.9	4.9
NB737	*S. vesicarium*	58.7	5.4
NB744	*S. lycopersici*	89.9	8.8
NB747	*S. lycopersici*	86.5	6.4
NB748	*S. lycopersici*	63.1	5.7
NB751	*S. lycopersici*	38.9	5.0
NB754	*S. vesicarium*	49.4	11.6

* Each value is the mean of three repeats.

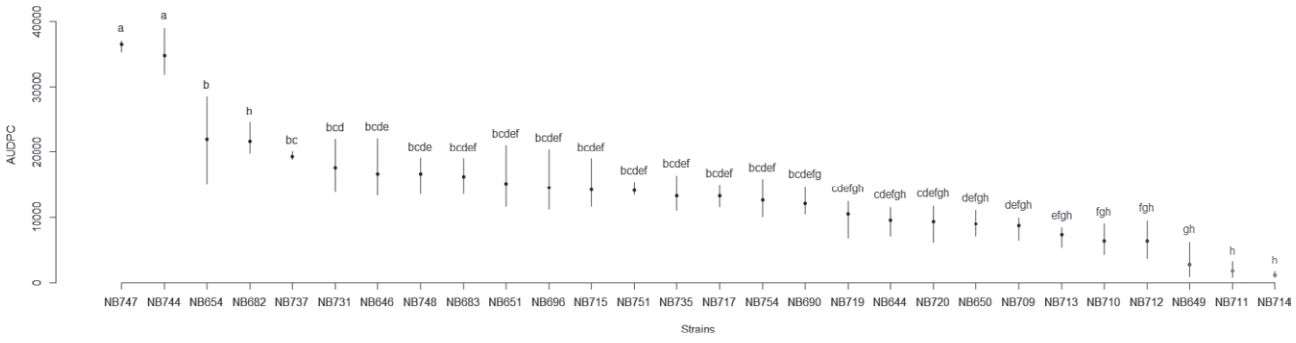

Figure 6. Mean areas under disease progress curves (AUDPCs) for disease progression on tomato plants inoculated with the different *Stemphylium* spp. isolates. The data were determined at 7, 14 and 21 d after inoculation for three replicates for each isolate. Means accompanied by the same letter are not different ($P > 0.05$).

Figure 7. Lesions caused by *Stemphylium* spp. on tomato leaves 21 d after inoculation (dai). *S. lycopersici*: NB744 (a), NB748 (b), NB747 (c); *S. vesicarium*; NB731 (d), NB720 (e), NB737 (f); *S. gracilariae*: NB644 (g), NB646 (h); *S. lycii*: NB683 (i); *S. eturmiunum*: NB709 (j), NB712 (k). Stereomicroscope view (×40 magnification) of an inoculated tomato leaflet at 21 dai (l).

1973; Gannibal, 2012), Asia (Oman, Taiwan, Malaysia, China) (Gannibal, 2012; Nasehi *et al*., 2012; Al-Amri *et al*., 2016; Sun *et al*., 2016; Huang and Tsai, 2017), Oceania (New Zealand) (Simmons, 2001) and Africa (Ivory Coast, Gambia, Tanzania, Tunisia, Morocco) (Blancard *et al*., 1986; 2012). All the isolates tested in the present study produced brown to dark necrotic lesions similar to those observed in fields, on tomato leaves under greenhouse conditions. This indicates the potential of these pathogens to cause disease in Algerian production areas. Huang and Tsai (2017) also reported that *S. lycopersici* was more likely to cause black/dark brown rather than gray lesions, suggesting that the disease name "gray leaf spot of tomato" should not be used for *Stemphylium* leaf spots. Rotem and Bashi (1977) suggested combining the diseases caused by three different *Stemphylium* species, including *S. lycopersici*, under the disease name, "*Stemphylium complex*" on tomatoes.

Stemphylium lycopersici is known to be pathogenic to tomato, and in the present study the most aggressive isolates all belonged to this species. The species was also isolated from all surveyed sites in Algeria. The sexual state of this species was not observed, which agrees with what other researchers have reported (Inderbitzin *et al*., 2009; Al Amri *et al*., 2016). This species is known to cause plant cell death through synthesis and release of phytotoxic secondary metabolites (Medina *et al*., 2019). Also, Zeng *et al*. (2018) predicted 511 secreted proteins putatively related to pathogenesis from *S. lycopersici*. This fungus can also infect a variety of host families, including ornamental and cultivated plants in the *Scrophulariaceae, Asteraceae, Caryophyllaceae, Juncaceae,*

Lamiaceae, Liliaceae, Araceae, Fabaceae, Malvaceae, Plantaginaceae, Rosaceae and *Solanaceae* (Nishijima, 1993; Nishi *et al*., 2009; Gannibal, 2012; Nasehi *et al*., 2014; Woudenberg *et al*., 2017; Kee *et al*., 2018).

Stemphylium eturmiunum was described on tomato fruit by Simmons (2001), with *Pleospora eturmiuna* as its teleomorph. This species has also been reported as a causal agent of postharvest mold in tomato (Andersen and Frisvad, 2004). *Stemphylium eturmiumum* was isolated from hosts in other families, including *Asphodelaceae* and *Amaryllidaceae* (Woudenberg *et al*., 2017). Similarly, the diverse host range of *S. vesicarium* and *S. gracilariae*, which includes solanaceous and non-solanaceous crops in different parts of the world, indicates the adaptability of these fungi to different environments.

Isolate NB683 from the present collection was identified as *S. lycii* and was found to be pathogenic on tomato. This species was described for the first time on leaves of *Lycinum chinense* leaves (Pei *et al*., 2011), and then identified on *Cucurbita moschata, Apium graveolens, Pistacia vera, Protea cynaroides*, *Triticum* sp. and *Hordeum vulgare* (Poursafar *et al*., 2016; Woudenberg *et al*., 2017), but not previously on tomato. The present report is therefore the first for *S. lycii* infecting tomato.

Symptoms observed in the field, such as brown/dark brown leaf necroses and defoliation, could be the result of interactions between different *Pleosporaceae* species with different virulence levels. *Stemphylium* and *Alternaria* spp. were always present and dominant in surveyed tomato fields from 2011 to 2017 (Bessadat *et al*., 2016; 2019). This indicates that these fungi may infect and develop on plant tissue causing a mixture of symptoms difficult to distinguish. *Stemphylium* and *Alternaria* spp. have been previously reported to simultaneously infect cultivated crops (Falloon *et al*., 1987; Hahuly *et al*., 2018; Das *et al*., 2019).

Abiotic factors are also likely to affect disease development. Temperature requirements for conidium germination of *Stemphylium* spp. vary within a range from 20°C to above 30°C (Bakr, 1991; Sinha and Singh, 1993; Mwakutuya and Banniza, 2010). These temperatures commonly occur in Algeria for extended periods from spring to autumn, due to global warming. *Alternaria* spp. have also been reported to be affected by temperature and incubation time in a similar manner to *Stemphylium* spp. (Montesinos and Vilardell, 1992; Mwakutuya and Banniza, 2010). Other factors could also be related to disease development, such as lack of crop rotation, type of fungicides used, dew periods, and host susceptibility. *Alternaria* and *Stemphylium* have capacity to produce very large amounts of secondary inoculum in short periods under favourable environmental conditions, which make tomato leaf blight and spot difficult to manage. Factors affecting disease development, and interactions with different hosts and pathogens have been little studied, so these factors require further study over long time periods.

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