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Occurrence and characterization of *Stemphylium* and *Alternaria* species associated with lettuce leaf spot in Algeria

Nabahat BESSADAT[1,2], Nelly BATAILLÉ-SIMONEAU[1], Justine COLOU[1], Bruno HAMON[1], Mabrouk KIHAL[2], Philippe SIMONEAU[1,*]

[1] *University of Angers, Institut Agro, INRAE, UMR IRHS, SFR QUASAV, 42 rue G. Morel, CS60057, 49071 Beaucouzé, France*
[2] *Laboratoire de Microbiologie Appliquée, University Oran1 Ahmed Ben Bella, BP1524 El M'naouer, Oran 31000, Algeria*
*Corresponding author. E-mail simoneau@univ-angers.fr

Summary. Lettuce leaves (*Lactuca sativa*) with leaf spot symptoms were collected from an organic farm and from farm markets in Oran Province, Oran, northwest Algeria, during the 2022 and 2023 growing seasons. A total of 119 isolates with morphological characteristics of *Stemphylium* and *Alternaria* were obtained from brown necrotic lesions on lettuce leaves. Based on morphological and multi-gene sequencing analyses, these isolates were identified as *Stemphylium lycopersici*, *S. vesicarium*, *S. eturmiunum*, *S. amaranthi*, and *S. gracilariae*, and *Alternaria* in sections *Alternaria* and *Eureka*. Pathogenicity tests with 24 representative isolates were conducted on young lettuce plants under greenhouse conditions, and lesions similar to those observed on the field and market collected plants developed on leaves of inoculated plants. *Stemphylium gracilariae* and *S. lycopersici* were the most aggressive fungi, causing extensive leaf necroses and defoliation, while other *Stemphylium* and *Alternaria* isolates develop small necrotic spots on mature leaves. A series of cross-inoculation experiments compared disease severity when plants were inoculated with one of four *Stemphylium* species alone or paired with one of five *Alternaria* isolates (20 combinations total). Differences in pathogenicity of mixed inoculations compared to single inoculation were detected, but synergistic increases in leaf spot severity were not detected for any mixed inoculations. These results demonstrate the potential of emerging fungal pathogens to cause lettuce leaf spot, and that *Stemphylium* and *Alternaria* species combinations can damage economically important lettuce crops.

Keywords. Foliar disease, *Lactuca sativa*, morphology, *Pleosporaceae*, phylogeny, pathogenicity.

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ORCID:
NB: 0000-0001-7795-2606
NB-S: 0000-0001-6200-4259
JC: 0000-0002-9623-4412
BH: 0000-0001-5868-0676
MK: 0000-0003-2901-373X
PS: 0000-0002-3890-9848

INTRODUCTION

Lettuce (*Lactuca sativa*) is an economically important and widely grown leafy vegetable crop (Shatilov *et al*., 2019), for fresh use in salads, sandwiches, and wraps, or cooked (Katz and Weaver, 2003; Fearnley-Whittingstall, 2013). Lettuce has antioxidant, anti-diabetic, anti-inflammatory, anti-cardiovascular

disease, anti-cancer, and health-promoting effects (Kim *et al*., 2016), and is a low-calorie, low-fat, and low-sodium salad vegetable, rich in fiber, folate, vitamin C, and essential minerals, (Kim *et al*., 2016). Lettuce tissues also contain essential nutritional bioactive compounds (Yang *et al*., 2022). Several lettuce types and cultivars are grown throughout each year in Algeria, in open fields or greenhouses (Institut Technique des Cultures Maraîchères et Industrielles, 2010; Lallouche *et al*., 2020).

Lettuce production can be exposed to several biotic stresses. Among these, leaf spot diseases caused by fungi can be damaging in certain cropping situations (Subbarao *et al*., 2017), but little research has been carried out to characterize these diseases in Algeria.

Lettuce leaf spot symptoms are brownish angular or circular lesions, which are slightly sunken and have brown necrotic centers that can become holes with time. Fungal pathogens of *Pleosporaceae* (*Pleosporales*, *Dothideomycetes*, *Ascomycetes*), including *Stemphylium* and *Alternaria*, are reported to cause the most common foliar diseases of lettuce in temperate zones (Nasehi *et al*., 2013; 2014; Koike *et al*., 2017; Liu *et al*., 2019). In fact, *Alternaria* spp. and *Stemphylium* spp. are prevalent in Northwest of Algeria and cause major crop major losses in this country (Bessadat *et al*., 2017; 2019; 2022). These pathogens infect lettuce foliage and other plant tissues. *Alternaria* and *Stemphylium* are also abundant in the atmosphere (Damialis *et al*., 2017) and are important human allergens and pathogens (Gutiérez-Rodriguez *et al*., 2011; Grewling *et al*., 2020; Sánchez *et al*., 2022).

Alternaria and *Stemphylium* are introduced into lettuce fields by windblown conidia from neighbouring plants (including weedy hosts) and crop debris, or as seed-borne inoculum (Blancard, 2021; Roberts and Punja, 2021). Development of both fungi is favoured by warm weather and high humidity (Singh *et al*., 2015; Blancard, 2021). Reduced photosynthetic area leads to varying crop losses and economic impacts (Das *et al.* 2019). In lettuce, fungal toxins can inhibit seedling growth (Das *et al*., 2019; Wang *et al*., 2022). Additionally, small plants and cosmetic damage, such as leaf spotting, can reduce yields and render affected lettuce unsalable.

Alternaria and *Stemphylium* induce small necrotic spots on lettuce leaves that can enlarge over time, often appearing dark brown due to pathogen conidium production. Optimum temperatures for infection are from 18 to 25°C which are typical of Mediterranean climates, and these temperatures extend during winter in Algeria (Algeria Climate Fact Sheet, 2023). In severe cases, lesions enlarge and coalesce to blight entire leaves. As leaves age, they increase in susceptibility. Both fungi can overwinter on infested plant residue and infected volunteer plants (Hay *et al*., 2021; Matić *et al*., 2020). In the next life cycle stage, secondary conidia and ascospores are released and dispersed from dead plant tissues of the previous season or from leaf lesions of nearby plants (Basallote-Ureba *et al*., 1999; Damialis *et al*., 2017). Leaf infections from conidia can occur by various mechanisms such as entry through stomata (Thomma, 2003; Tayviah, 2017), wounds caused by other diseases, insect pest feeding, movement of workers or farm equipment, or by the action of wind (Thomma, 2003; Nischwitz, 2016).

Identification of *Stemphylium* and *Alternaria* spp. has been based on morphological characteristics including conidium shape, size, septation, length to width ratio, and conidiophore dimensions (Simmons, 1969; 2001; 2007). However, many of these characteristics overlap among species due to varying environmental conditions, which complicates species identification, so different species can appear to be similar and may be misidentified when relying on morphological traits. Beyond morphological analyses, the relationships among these fungi have been unclear, prompting discussion and analysis using molecular markers (Pryor and Gilbertson, 2000; Camara *et al*., 2002; Inderbitzin *et al*., 2009). Molecular methods combined with phylogenetic analyses are considered more accurate for delineating species within several related fungi, re-defining and expanding the morphological group concept and other taxonomic hypotheses for both genera (Woudenberg *et al*., 2013, 2017; Li *et al*., 2023). This has separated *Alternaria* into 29 sections and seven monotypic lineages (Woudenberg *et al*., 2013; Al Ghafri *et al*., 2019; Marin-Felix *et al*., 2019; Gannibal *et al*., 2022), while *Stemphylium* is now classified into 33 species, distinct from *Alternaria* (Ariyawansa *et al*., 2015; Woudenberg *et al*., 2017; Brahmanage *et al*., 2019; Marin-Felix *et al*., 2019; Crous *et al*., 2020).

Alternaria alternata is considered the main causal agent of lettuce leaf spot, but other species such as *A. cichorii* (Pegg *et al*., 2014), *A. sonchi* (Subbarao *et al*., 2017), *A. solani, A. brassicae*, *A. brassicicola* (Moses *et al*., 2016; Guo *et al*., 2018), and *A. dauci* (Koike *et al*., 2017) have also been found on symptomatic leaves. Similarly, several species of *Stemphylium* are associated with the disease on cultivated lettuce, including *S. lycopersici*, *S. vesicarium* (Liu *et al*., 2019), *S. solani* (Nasehi *et al*., 2013), *S. gracilariae* (Woudenberg *et al*., 2017), and *S. lactucae* (Zhang and Zhang, 2002).

During 2022 and 2023, severe necroses were observed on lettuce plants at an organic farm and in commercialized head lettuce. Precise identification of the responsible pathogens was necessary to implement effective disease management. The objectives of the present study were to characterize the causal agents of emerging

leaf spot diseases on lettuce, using morphological analysis, multi-gene sequencing, and pathogenicity tests of representative isolates to assess fulfilment of Koch's postulates. To achieve these objectives, random samples of lettuce were obtained from fields and markets in Oran province, Algeria, and related studies were initiated.

MATERIALS AND METHODS

Sample collection and fungal isolation

In the autumn of 2022 and spring of 2023, symptomatic romaine lettuce plants were sampled from fields or markets in Oran Province, northwest Algeria. Symptoms on leaves were brown to dark brown, irregular to circular, ranging from 2 to 18 mm in diameter, across much of the leaf surface. Fungi were isolated by cutting one or two fragments (5–10 mm^2) from the margins of lesions. The samples were surface disinfected by immersing in 2% sodium hypochlorite solution for 2 min, rinsing three times in sterile distilled water, drying with sterilized paper towels, and then placing them on potato carrot agar (PCA) in Petri plates (Simmons, 2007). The plates were then incubated at room temperature (18–25°C) under natural daylight for 7 to 14 d. Conidia that developed were observed under a light microscope (Optika B-190 series, OPTIKA Srl) to identify species based on conidium morphology, and to estimate the frequencies of *Stemphylium* and *Alternaria* in the different plant samples. The frequencies of isolated fungi per lesion were calculated using the formula of Saleemi *et al.* (2012):

$$\text{Isolation Frequency (IF)} = \frac{\text{Number of samples with a species or genus} \times 100}{\text{Total number of samples}}.$$

Hyphae of prevalent fungi were picked from the peripheries of colonies and inoculated onto new PCA plates. Pure cultures were grown on Potato Dextrose Agar (PDA) slants for 7 to 14 d and then stored at 4°C and -80°C in 30% glycerol at the fungal culture collection COMIC (Collection of Microorganisms) of the SFR 4207 QUASAV (Angers, France) for future experiments.

Micro- and macro-morphological examination of isolates

Twenty-three representative isolates (five of *Alternaria* and eighteen of *Stemphylium*) were each inoculated into plastic plates (90 mm diam.) (n = 3) each containing 15 mL of PCA, and were grown under standardized conditions (Simmons, 2007). Micro-morphological characteristics of the isolates were observed after 7–14 d. Conidiophore lengths (n = 30), and conidium (n = 30) dimensions , shapes (L/W ratio), colours, and ornamentation, were determined at ×400 magnification from microscope slide preparations in lactic acid, using clear Scotch tape (Samson *et al*., 2010). The PCA plates were further incubated and checked for ascomata after 1 month of incubation at room temperature. Colony characteristics (colour, texture and diameter) were recorded on PDA plates (n = 3) after 7 d incubation at 25°C. Colour determinations were made using the colour charts of Kornerup and Wanscher (1978). The morphological characteristics of each isolate were registered and compared to strains previously identified on *Solanaceae* hosts including *S. gracilariae* (NB717, NB690), *S. lycopersici* (NB751), *S. eturmiunum* (NB709, NB735), or *S. vesicarium* (NB713, NB737) (Bessadat *et al*., 2022).

Pathogenicity assessments

Pathogenicity testing was carried out using two techniques. A primary set of inoculations experiments was conducted using six *Alternaria* and 18 *Stemphylium* representative isolates for individual inoculations. The isolates were grown on PCA under natural day light for 7–14 d at ambient temperature to promote conidium production. Conidia were then gently dislodged from cultures using a rubber spatula and suspended in sterilized distilled water supplemented with 0.01% Tween20 (Bessadat *et al*., 2017; 2022). Inoculations were carried out by spraying 10 mL of conidium suspension of each isolate (1×10^4 mL^{-1}) onto three 50-d-old lettuce plants cv. Romaine grown in sterilized commercial potting mix. The second set of inoculations involved cross-inoculation experiments to assess how *Alternaria* spp. affected the virulence of *Stemphylium* isolates. These were conducted using a completely randomized experimental design. Representative isolates of *Alternaria* (n = 5) or *Stemphylium* species (n = 4) were used in 50:50 mixtures ratios. Inoculated plants were then covered with polyethylene bags for 48 h to maintain high relative humidity (90%), and were then grown at 12–20°C, 55% relative humidity (night), and 24–33°C, 72% relative humidity (day). Plants inoculated with sterile water served as controls. Five to six replicate plants were inoculated with each isolate, and the experiment was repeated three times. Leaf spot progression was recorded at 7, 14 and 21 d post inoculation (dpi). Percent leaf necrotic area (l. n. a.) was calculated by dividing the area of the lesions on each leaf by the total leaf area, and multiplying by 100. The areas under disease progress curves (AUDPC) were calculated using the open source 'agricolae' package in R software (Campbell and Madden, 1990). Data analyses were carried out using Kruskal-Wallis and Dunn's post-hoc tests with R (R4.3.1) (Faraway,

2002). Conidia formed on diseased plant tissues were observed after 21 dpi.

Extraction, PCR amplification and sequencing of isolate DNA

Total genomic DNA was extracted from 14–21 d -old PDA cultures of isolates, using the NucleoSpin® Genomic DNA kit (MACHEREY-NAGEL), according to the manufacturer's protocol. For *Stemphylium* spp. identification, amplification of the internal transcribed spacer regions of ribosomal DNA (*ITS rDNA*), glyceraldehyde-3-phosphate dehydrogenase (*gpd*), and calmodulin (*cmdA*) gene regions, was conducted using, respectively, the primer pairs ITS1/4, gpd1/gpd2, and CALDF1/CALDR1 (White *et al*., 1990; Berbee *et al*., 1999; Lawrence *et al*., 2013). *Alternaria* spp. characterization at section level was based on glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene sequences, after amplifications using gpd1/gpd2 primer pairs. Polymerase chain reaction (PCR) amplification procedures were set as those of Woudenberg *et al.* (2017). Each PCR amplification was carried out in a total volume of 25 µL, containing 75mM Tris-HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 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 amplification was carried out in a Bio-Rad T100™ Thermal Cycler (Bio-Rad Inc.). The products were analyzed by electrophoresis in 1.2% (w/v) agarose in 0.5× TAE buffer, were visualized by ethidium bromide staining and UV illumination, and were then sent to Eurofins Genomics (Germany) for sequencing. The generated DNA sequences were submitted to GenBank, and additional DNA sequences from type strains for species of *Alternaria* and *Stemphylium* genera were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Phylogenetic analyses

DNA sequences of isolates and reference species retrieved from GenBank were concatenated and aligned by the MUSCLE algorithm using MEGA 7 (Kumar *et al*., 2016). Phylogenetic analysis used the maximum likelihood (ML) approach within IQTree v.1.6. (Nguyen *et al*., 2015). The best-fit evolutionary models for each dataset were calculated by ModelFinder (Kalyaanamoorthy *et al*., 2017) within the Bayesian Information Criterion (BIC) selection procedure. The ML analysis was carried out with 1000 ultrafast bootstrap replicates and only values greater than 90% were considered significant. Phylogenetic tree construction was carried out using the online tool Interactive Tree of Life (ITOL) version 6 (Letunic and Bork, 2024).

RESULTS

Stemphylium lettuce leaf spot symptoms can be confused with early symptoms of *Alternaria* leaf spot. Both pathogens cause large, brown or dark brown (Figure 1, A and B), and round spots with a light brown to beige necrotic center (Figure 1 C). The necrotic centre of each lesion often abscises and falls out leaving a hole in the spot centre. Heavily infected leaves turn yellow to brown and dry, with tendency to crack and tear in the necrotic tissue (Figure 1, B and D). A total of 106 naturally infected lettuce leaf samples were collected from five markets and one organic farm in Oran, and were analyzed. Fungi isolated from these samples using PCA showed varying frequencies. *Stemphylium* spp. had the greatest frequency (48.2%) followed by small-spored *Alternaria* species (35.2%). Other genera, including *Cladosporium* sp., *Mucor* sp., *Fusarium* sp., and *Botrytis* sp. were isolated at the least frequency (16%). Most of the symptomatic lesions (80%) had mixed infections of *Alternaria* and *Stemphylium* spp. Twenty-seven isolates with morphological characteristics of *Stemphylium* and *Alternaria* were selected for further characterization (Table 1).

Alternaria *spp. isolates*

Most of the *Alternaria* isolates from lettuce were small-spored *Alternaria* species which were morphologically identified as members of section *Alternaria* (NB1149, NB1150, NB1151) or section *Eureka* (NB1088, NB1089, NB1090).

Phylogenetic analyses of *gpd* sequences from these isolates and corresponding sequences from strains representative of the 29 sections in *Alternaria* confirmed that they belonged to these two sections (Figure 2). Isolates from section *Alternaria* developed olivaceous and dull, velvety to cottony mycelium, with colonies having white regular margins (Figure 3, A and B). Conidiophores were short to long, simple or branched, septate, and with 1–2 (–3) apical conidiogenous loci. Conidia were obclavate, ellipsoid, with 1 (–3) transverse and 0–3 longitudinal septa, and mean dimensions of 18.8–35.2 × 7.5–12.6 µm. They were medium brown, punctate, small or moderate in size, septate, slightly constricted near some septa, in moderately long to long, and simple or branched chains. Conidia each had a tapered beak or secondary conidiophore, each with one or a few conidiogenous loci from

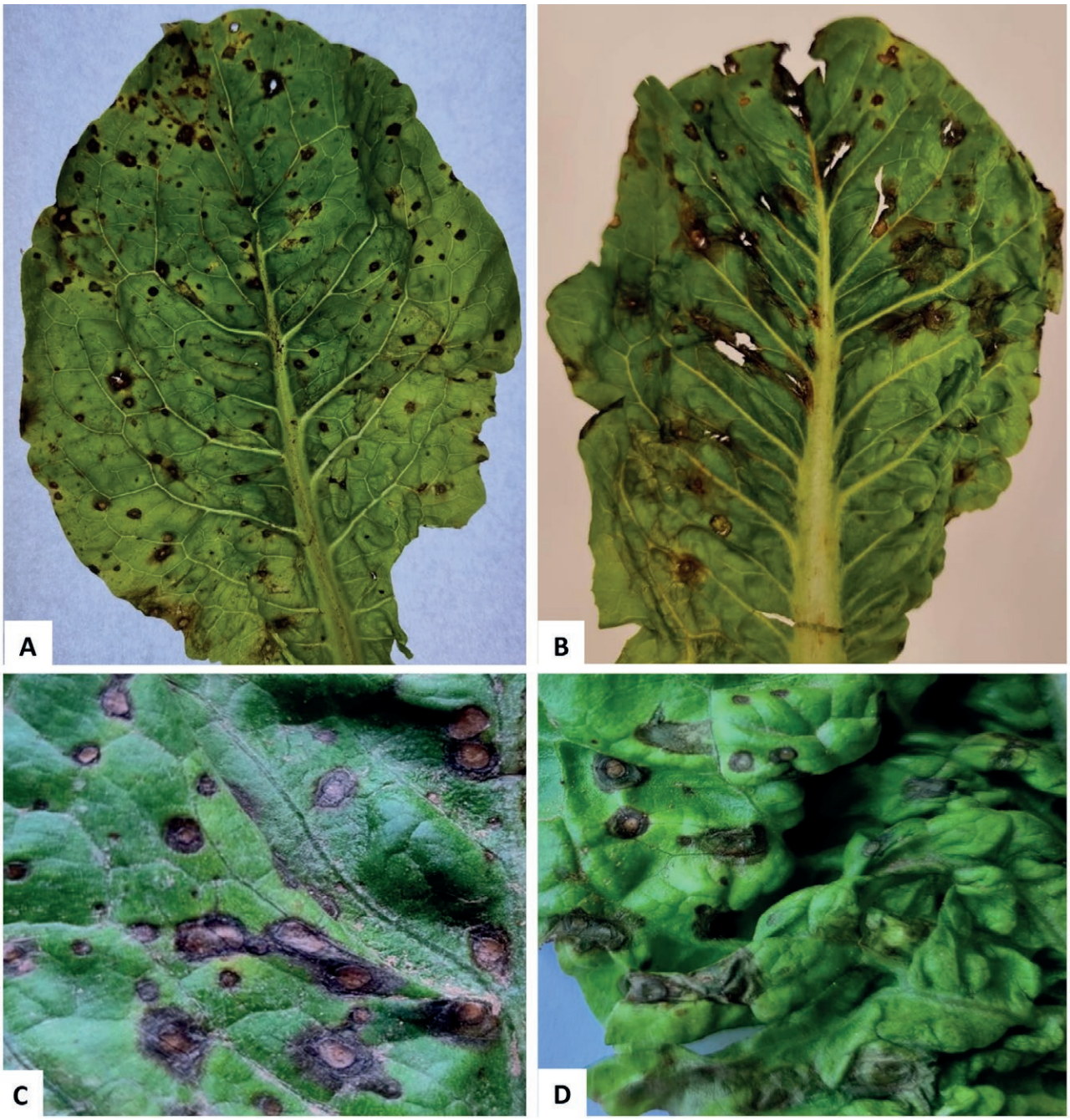

Figure 1. Foliar symptoms on leaves of field-grown lettuce: **(A)** circular to oval dark brown spots with chlorosis; **(B)** extended brown necrosis with numerous cracks and perforations; **(C)** brown necrosis with beige lesion centres caused by *Stemphylium* spp.; **(D)** brown and dark brown necroses caused by *Stemphylium* and *Alternaria* spp.

the apex or lateral cells, bearing one to three conidiogenous loci (Figure 4A).

The three isolates from section *Eureka* developed grayish brown to olive brown, cottony mycelium (Figure 3C). The conidiophores were simple or branched, straight or geniculate, medium brown, and were 17.6–113 × 4.5–6.3 µm, with 1–2 (–3) apical proliferations (Figure 4B). Conidia were ovoid, narrowly ellipsoid, with 1 (–3) transverse and 0–3 longitudinal septa per transverse segment, were 27.6– 42.7 × 13.8–25 µm, brown, constricted at the major transverse septa, and were solitary or in short chains of 2–3 conidia.

Table 1. Isolates characterized in this study and their GenBank accession numbers.

Isolate	Section/Species	Year of isolation	GenBank accession numbers		
			ITS	*gpd*	*cmd*
NB1088	*A.* section *Eureka*	2022	NA	PV007868	NA
NB1089	*A.* section *Eureka*	2022	NA	PV007869	NA
NB1090	*A.* section *Eureka*	2022	NA	PV007870	NA
NB1102	*S. lycopersici*	2022	PQ963026	PV007826	PV007847
NB1103	*S. lycopersici*	2022	PQ963027	PV007827	PV007848
NB1104	*S. lycopersici*	2022	PQ963028	PV007828	PV007849
NB1105	*S. lycopersici*	2022	PQ963029	PV007829	PV007850
NB1107	*S. gracilariae*	2022	PQ963030	PV007830	PV007851
NB1108	*S. gracilariae*	2022	PQ963031	PV007831	PV007852
NB1109	*S. lycopersici*	2022	PQ963032	PV007832	PV007853
NB1110	*S. gracilariae*	2022	PQ963033	PV007833	PV007854
NB1111	*S. eturmiunum*	2022	PQ963034	PV007834	PV007855
NB1116	*S. lycopersici*	2023	PQ963035	PV007835	PV007856
NB1117	*S. gracilariae*	2023	PQ963036	PV007836	PV007857
NB1118	*S. gracilariae*	2023	PQ963037	PV007837	PV007858
NB1120	*S. gracilariae*	2023	PQ963038	PV007838	PV007859
NB1122	*S. gracillariae*	2023	PQ963039	PV007839	PV007860
NB1123	*S. gracilariae*	2023	PQ963040	PV007840	PV007861
NB1124	*S. gracilariae*	2023	PQ963041	PV007841	PV007862
NB1149	*A.* section *Alternaria*	2023	NA	PV007871	NA
NB1150	*A.* section *Alternaria*	2023	NA	PV007872	NA
NB1151	*A.* section *Alternaria*	2023	NA	PV007873	NA
NB1153	*S. amaranthi*	2023	PQ963042	PV007842	PV007863
NB1154	*S. vesicarium*	2023	PQ963043	PV007843	PV007864
NB1155	*S. vesicarium*	2023	PQ963044	PV007844	PV007865
NB1156	*S. vesicarium*	2023	PQ963045	PV007845	PV007866
NB1157	*S. eturmiunum*	2023	PQ963046	PV007846	PV007867

Stemphylium *spp. isolates*

Morphological analysis showed that most of the isolates collected from symptomatic lettuce belonged to *Stemphylium*. To further characterize these isolates, sequences of the rDNA ITS region and portions of the *gpd* and *cmdA* genes from the *Stemphylium* isolates were obtained, and a multi-gene phylogeny approach was used to identify the isolates at species level. The combined dataset of the three gene sequences from 21 lettuce isolates, and from 68 representative strains of 31 recognized species of *Stemphylium* retrieved from GenBank was used to compare the lettuce isolates and GenBank strains. Sequences from *Alternaria alternata* strain GV14-634a1 were used as the outgroup. The dataset had a total length of 1102 bp, of which 154 bp were parsimony informative. The resulting phylogenetic tree (Figure 5) indicated that the 21 lettuce isolates grouped into five well-supported phylogenetic species: *S. amaranthi* (NB1153), *S. eturmiunum* (NB1111, NB1157), *S gracilariae* (NB1107, NB1108, NB1110, NB1117, NB1118, NB1120, NB1122, NB1124), *S. lycopersici* (NB1102, NB1103, NB1104, NB1105, NB1109, NB1116), and *S. vesicarium* (NB1154, NB1155, NB1156). Colony measurements of selected isolates were conducted on the isolates grown on PDA (Table 2). This macro-morphological analysis was not efficient for isolate grouping due to high morphological variability within species. Additional analysis of morphological characteristics was carried out to describe the isolates from lettuce, and is provided below.

Stemphylium amaranthi Y.F. Pei & X.G. Zhang

One isolate (NB1153) had brown to grayish beige velvety mycelium, with regular white to transparent colony margins (Figure 3 D). Colonies were 71.0 ± 0.8 mm diam. after 7 d incubation on PDA at 25 °C in the dark. Conidiophores were solitary, simple or branched, pale brown, smooth, cylindrical, 3–10-septate, and measured 27.8–108.0 × 5.0–6.5 µm. Conidiogenous cells were each swol-

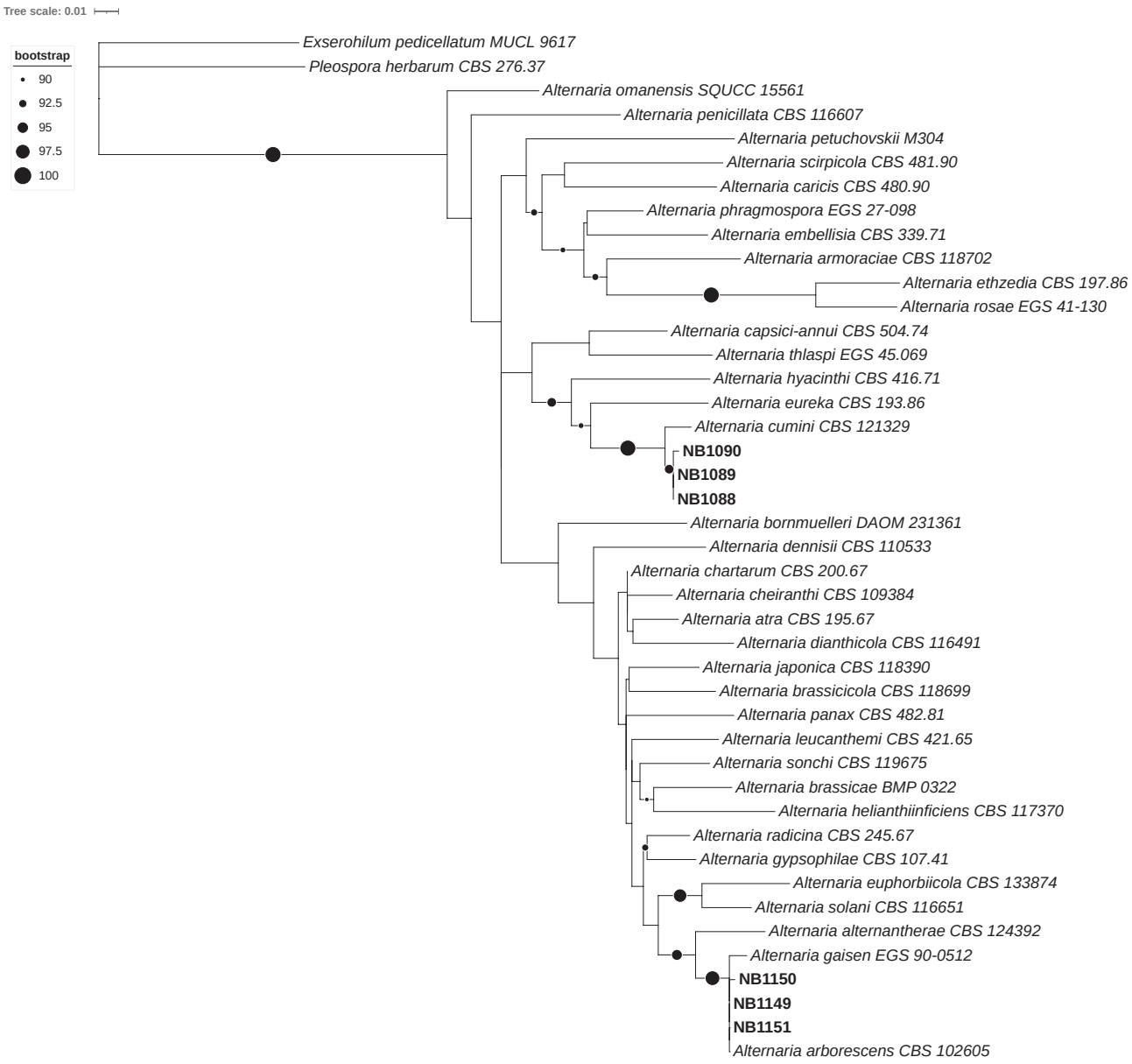

Figure 2. Phylogenetic tree reconstructed using the maximum likelihood method from the alignment of sequences of portions of the *gpd* gene from six small-spored *Alternaria* isolates and 35 *Alternaria* strains representative of 29 sections. Sequences of one isolate each of *Exserohilum pedicellatum* and *Pleospora herbarum* were included to root the tree. Sequences were retrieved from GenBank accessions reported in Woudenberg *et al.* (2013), Al Ghafri *et al.* (2019), Grum-Grzhimaylo *et al.* (2016). Bootstrap support values are indicated near nodes by black circles, whose sizes are proportional to the indicated support values. The tree scale bar indicates the expected number of substitutions per position.

len at the apex, medium brown, 5.0–7.5 µm wide, smooth, and occasionally had 1–5 apical proliferations. Conidia were, subspherical, oblong to oval, were each rounded at the base and spherical to conical at the apex. They had 1 (–4) transverse and 1–3 longitudinal/oblique septa, measured 21.4–38.4 × 9.0–18.8 µm, their L/W ratio was 1.4–4, they had one indistinctly constricted median septum, and were medium to dark brown (Figure 4 C). Conidia often generated short secondary conidiophores, which were 1–2-septate, 12.0–18.0 µm long, and were smooth. Sexual morphs were observed after 4 weeks in culture.

Stemphylium eturmiunum E.G. Simmons

Two isolates (NB1111, NB1157) had brownish orange to grayish brown cottony mycelium with regular

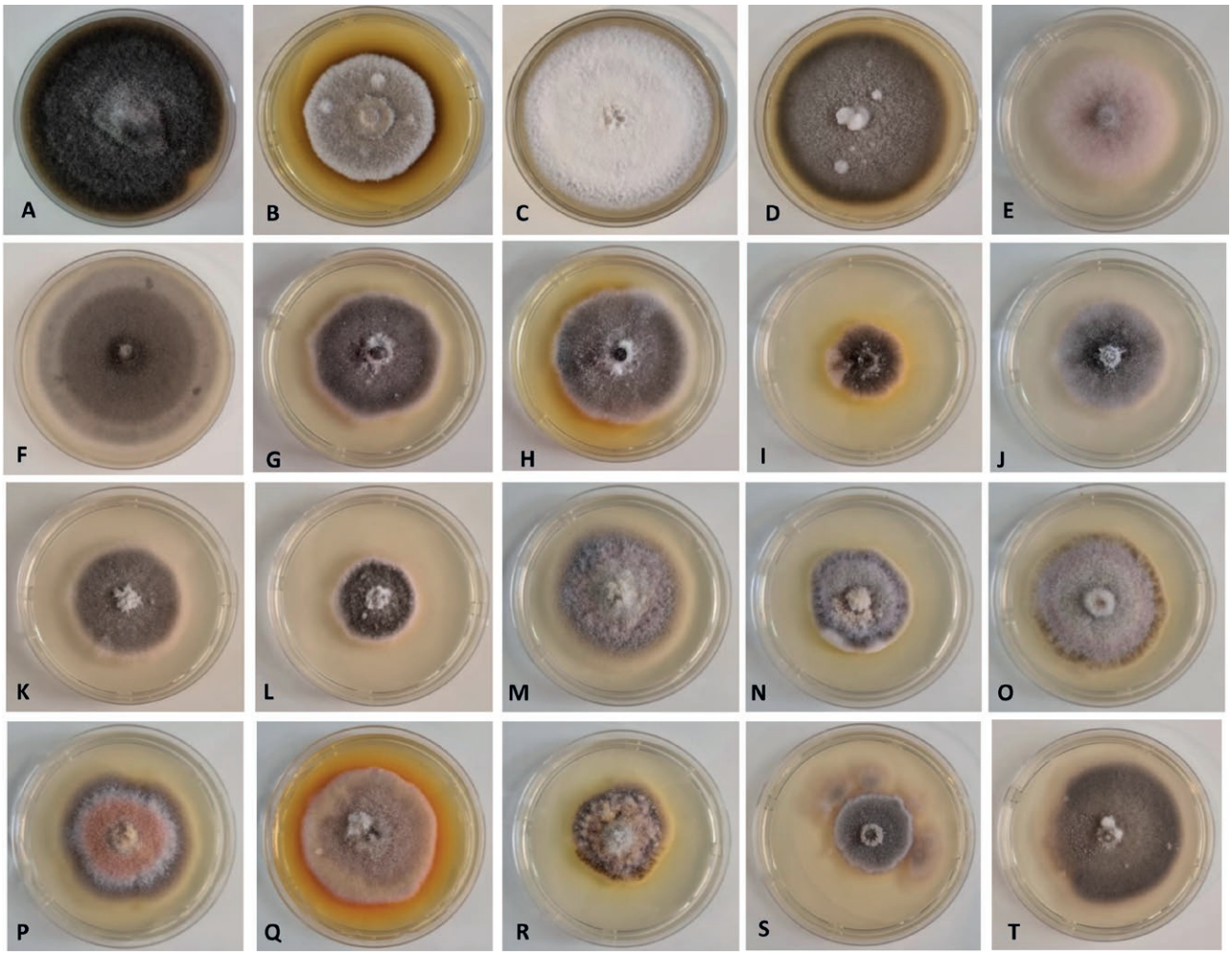

Figure 3. Morphology of selected *Alternaria* and *Stemphylium* isolates after 7 d growth on potato dextrose agar at 25°C. **(A)** *Alternaria* section *Alternaria*: NB1149; *A.* section *Eureka*: **(B)** NB1088, **(C)** NB1090; *S. amaranthi*: **(D)** NB1153, *S. eturmiunum*: **(E)** NB1111, **(F)** NB1157; *S. gracilariae*: **(G)** NB1107, **(H)** NB1110, **(I)** NB1117, **(J)** NB1118, **(K)** NB1120, **(L)** NB1122; *S. lycopersici*: **(M)** NB1102, **(N)** NB1103, **(O)** NB1104, **(P)** NB1105, **(Q)** NB1109, **(R)** NB1116; *S. vesicarium*: **(S)** NB1155, **(T)** NB1156.

colony margins, and sometimes produced yellow white pigmentation (Figure 3, E and F). Colonies were 62 to 77 mm in diameter after 7 d incubation, and occasionally produced yellowish white pigment on PDA at 25°C after 7d. Conidiophores were solitary, simple, brown, smooth, cylindrical, 3–10-septate, of short to moderate length of 21.4–70.0 × 5.0–12.6 µm. They were each swollen at the apex, medium brown, 5.0–7.5 µm wide, smooth, occasionally with 1–2 apical proliferations. Conidia were oblong to ovate, muriform, rectangular at the base and spherical at the apex, with 1–3 transverse and 1–3 longitudinal/oblique septa, measured 21.6– 34.0 (–41.0) × 10–20.1 µm, with L/W ratio of 1.3–2.5, one indistinctly constricted median septum, and were light to dark brown (Figure 4 D). Secondary conidiophores were rarely formed. Sexual morphs were observed after 4 weeks.

Stemphylium gracilariae E.G. Simmons

Eight isolates (NB1107, NB1108, NB1110, NB1117, NB1118, NB1120, NB1122, NB1124) developed olive brown, velvety to cottony mycelium, with white regular colony margins (Figure 3, G to L). The colonies were 37 to 60 mm diam. after 7 d incubation on PDA at 25°C in the dark. Conidiophores were solitary, simple or branched, pale brown, smooth, cylindrical, 1–5-septate, measuring 20–57 (–90) × 4.5–7.5 µm. Conidiogenous cells were swollen at the apex, medium brown, smooth, 5–7 µm wide, occasionally with 1–2 apical proliferations. Conidia were oblong to ovate, with 1(–3) transverse and 2–4 longitudinal septa, 17.6– 37.7 × 11.3–21.9 µm, with L/W

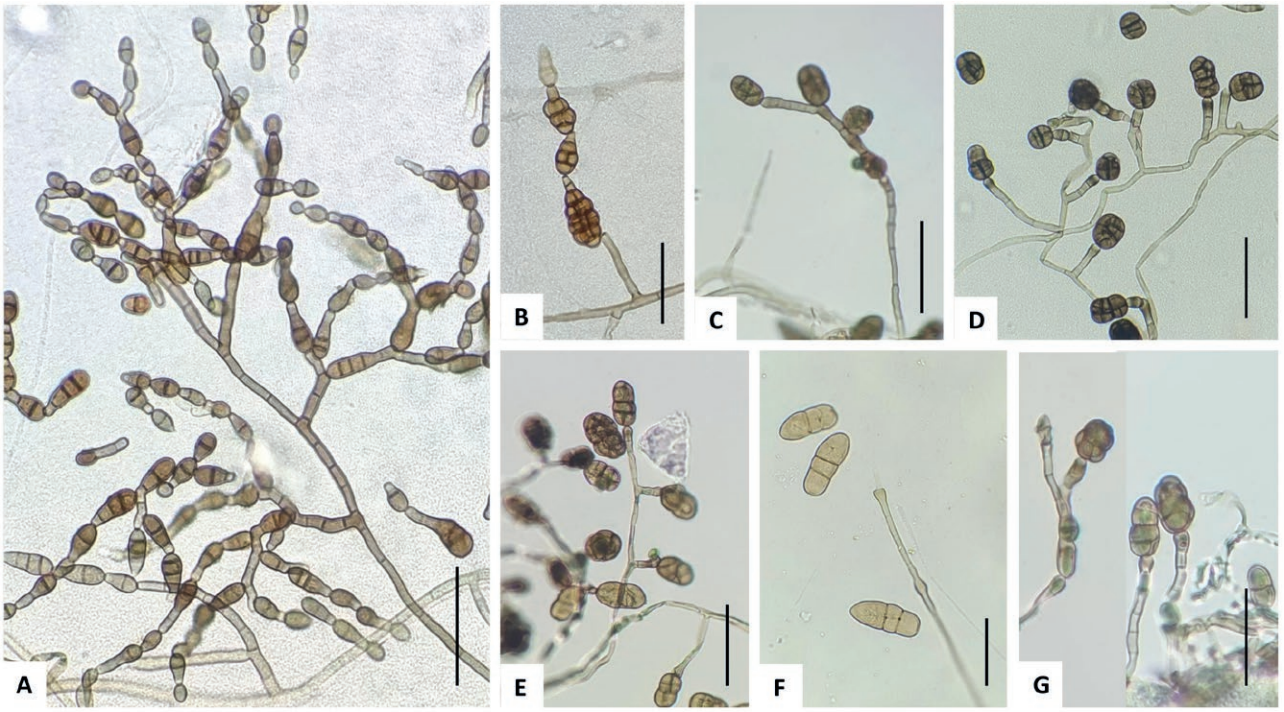

Figure 4. Conidiophores and conidia of *Alternaria* and *Stemphylium* spp. developed on potato carrot agar after 7 d incubation at room temperature. **(A)** *Alternaria* section *Alternaria* (NB1149), **(B)** *A.* section *Eureka* (NB1088), **(C)** *S. amaranthi* (NB1153), **(D)** *S. eturmiunum* (NB1111), **(E)** *S. gracilariae* (NB1122), **(F)** *S. lycopersici* (NB1104), **(G)** *S. vesicarium* (NB1156). Bars = 50 µm.

ratio of 1.2–2.5, one indistinctly constricted central transverse septum, medium brown, sometimes with punctuate ornamentation. Conidia often generated short secondary conidiophores, that were 1–2-septate, and 12–18 mm long. Secondary conidia formed in aged parts of colonies were ovoid or subcylindrical and smooth, with one or few transverse septa, and were 12.6–16.3 × 5–8 µm (Figure 4 E). Sexual morphs were observed after 3 to 4 weeks.

Stemphylium lycopersici (Enjoji) W. Yamam

Six isolates (NB1102, NB1103, NB1104, NB1105, NB1109, NB1116) developed cottony and raised colonies with irregular margins, with olive brown to grayish yellow mycelial pigmentation and a diffused yellow pigment into the culture medium (Figure 3, M to R). Colonies were 51 to 73 mm diam. after 7 d incubation on PDA at 25°C in the dark (Table 2). Conidiophores were simple, straight, 4–14-septate, measuring 98–376 × 6–10 µm, with swollen apical conidiogenous cells each bearing a single conidium. One to 5-septate secondary conidiophores emerged from conidia in aged parts of colonies, and these measured 12–90 × 4–6 µm. Conidia were dark brown, solitary, oblong with conical ends at the apices and bluntly rounded or rectangular at the bases, and measured 36–70 × 13–18.8 µm, with L/W ratios of 2.1–5.1, and 2–3 darkened constrictions, 3–7 transverse septa and 1–3 longitudinal septa per transverse segment (Figure 4 F). A sexual morph was not observed.

Stemphylium vesicarium (Wallr.) E.G. Simmons

Three isolates (NB1154, NB1155, NB1156) had cottony mycelium, with colonies 28–62 mm diam. and grayish brown to grayish orange after 7 d incubation on PDA at 25 °C in the dark (Figure 3, S and T). Conidiophores were straight or occasionally branched, pale to brown with dark edges, each with a swollen apex and 3–9 septa, and dimensions of 30–70.3 × 5–6.8 µm. Conidia were medium to olive-brown, ovoid to muriform, with 1–3 transverse segments and 1–3 longitudinal septa per transverse segment, and measured 22.6– 30.1 × 8.8–16.8 µm, with mean length/width ratio of 1.3 –2.3. The conidia were each constricted at 1–2 of the major transverse septa (Figure 4G). Sexual morphs were observed after 4 weeks.

Pathogenicity tests

Individual inoculations Pathogenicity tests showed that all the tested *Alternaria* and *Stemphylium* isolates caused symptoms on romaine lettuce (Figure 6). Numerous small spots appeared on plant basal leaves 3–4 d of

Table 2. Morphological variability among *Alternaria* and *Stemphylium* isolates grown on potato dextrose agar.

Section/Species	Isolate number	Colony aspect				Pigmentation	Colony diameter after 7 days (mm)
		Surface			Reverse		
		Type	Colour [a]	Margin			
A. sect. *Eureka*	NB1088	Cottony compact	Greyish beige (4C2) with white surface	Regular	Brown (6E7/6E6)	Olive brown (4E8)	52.9 ± 2.4
	NB1089	Velvety to cottony	Olive brown (4E3) with brownish grey (4D2) margins	Irregular	Yellowish brown (5F4) with a black center	Olive brown (4D8)	35.3 ± 3.1
	NB1090	Cottony	Greyish brown (4C2)	Regular	Yellowish brown (5F4)	None	79.0 ± 0.8
A. sect. *Alternaria*	NB1149	Velvety to cottony	Olive brown (4F7) with yellowish grey surface (3C2/3D2) and olive margins (3E3)	Regular	Olive brown (4F3)	None	79.5 ± 0.6
	NB1150	Cottony centre and velvety margins	Olive brown (4E4/ 4E5)	Irregular	Olive brown (4F7)	None	78.6 ± 1.8
S. amaranthi	NB1153	Velvety	Brown (5E4) with greyish beige (4C2) spots	Regular	Olive brown (4F4) with black center	Yellowish white (3A2)	71.0 ± 0.8
S. eturmiunum	NB1111	Cottony compact	Brownish orange (5C3) centre with a reddish white (7A2) surface and orange grey (5B2) margins	Regular	Light orange (5A4) with brownish orange (5C3) center	Yellowish white (3A2)	62.0 ± 2.4
	NB1157	Cottony	Greyish brown (5D3/5E3) to brownish grey (5D2)	Irregular	Greyish brown (5B4) to light brown (5D4)	None	76.8 ± 1.3
S. gracilariae	NB1107	Cottony centre and velvety margins	Olive brown (4D4) with brownish grey (4D2) margins	Irregular	Light brown (5D7) with pale orange (5A3) margins	Pale yellow (3A3)	59.9 ± 2.6
	NB1110	Velvety	Olive brown (4D3/ 4E3) with white borders	Irregular	Grayish brown (5D3) with light brown (5C6) margins	None	59.8 ± 1.9
	NB1117	Velvety	Olive brown (4E4) center and greyish yellow (4B3) to white margins	Irregular	Yellowish brown (5D5/ 5F5)	Yellow (3A7)	37.1 ± 1.4
	NB1118	Velvety	Olive brown (4D3) center and brownish grey (5C2) margins	Regular	Yellowish brown (5F4) with orange grey (5B2) margins	None	54.0 ± 1.8
	NB1120	Velvety to cottony	Olive brown (4D4/ 4D3) with white borders	Regular	Yellowish brown (5E4) to grayish orange (5B3)	None	55.4 ± 1.7
	NB1122	Cottony compact	Olive brown (4D4/ 4D3) with white borders	Irregular	Yellowish brown (5E4) to pale orange (5A3)	Light yellow (3A5)	47.1 ± 0.8

(Continued)

Table 2. (Continued).

Section/Species	Isolate number	Colony aspect				Pigmentation	Colony diameter after 7 days (mm)
		Surface			Reverse		
		Type	Colour [a]	Margin			
S. lycopersici	NB1102	Cottony centre and velvety margins	Dull red (9B3) with grayish brown (5E3) surface and orange grey (5B2) margins	Regular	Olive brown (4D6/ 4E5) with grayish yellow (4B3) margins	Pale yellow (3A3)	70.3 ± 2.2
	NB1103	Cottony centre and velvety margins	Grayish yellow (4C3) with a brownish grey (9D2) surface	Irregular	Olive brown (4E5) with grayish yellow (4C4) margins	Light yellow (3A4)	51.0 ± 4.2
	NB1104	Cottony centre and velvety margins	Dull red (9B3) with a grayish yellow (4C3) surface and olive brown (4D5) margins	Irregular	Grayish yellow (4C6) with light brown (5D7) center	Light yellow (3A4)	52.0 ± 10.3
	NB1105	Cottony centre and velvety margins	Brownish orange (5C3) center with pastel red (8A4) and brownish grey (8D2) margins	Irregular	Grayish yellow (4B3) with olive brown (4D7) center	Yellow (3A6)	59.5 ± 7.2
	NB1109	Cottony compact	Brownish orange (7C3) center with grayish yellow (4B3) surface and pale orange (5A3) margins	Irregular	Reddish brown (9D4) center with pastel red (9A4) margins	Reddish orange (7A8)	58.0 ± 3.2
	NB1116	Cottony centre and velvety margins	Grayish orange (5B5) center with grayish yellow (4B3) surface and olive brown (4D4) margins	Irregular	Olive brown (4E7) with grayish yellow (4B5) margins	Yellow (3A6)	56.8 ± 10.2
S. vesicarium	NB1154	Cottony	Greyish orange to brownish grey (5B3/5D2)	Irregular	Brown to pale orange (5A4/5A3)	None	28.0 ± 1.6
	NB1155	Cottony to floccose	Greyish brown (5D3) to greyish orange (5B3) with grey spots	Irregular	Brown (5E4) to brownish orange (5C4)	None	56.3 ± 3.5
	NB1156	Cottony to floccose	Greyish brown (5D3) to orange grey (5C2) spots	Irregular	Yellowish to light brown (5F4/5D4)	None	62.0 ± 1.2

[a] Colour designations were made using the colour charts of Kornerup and Wanscher (1978).

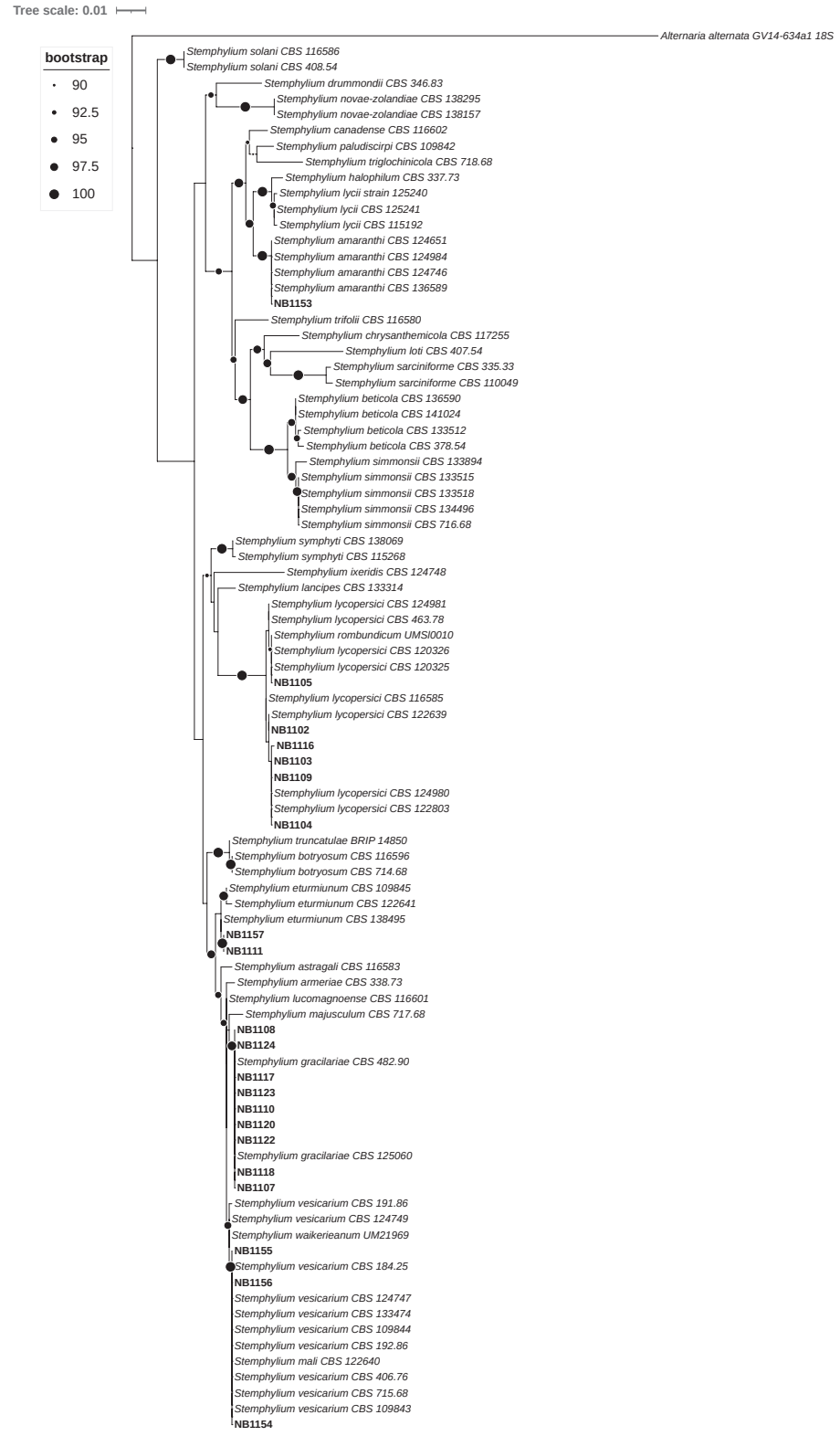

Figure 5. Phylogenetic tree reconstructed using the maximum likelihood method from the alignment of *ITS*, *gpd* and *cmdA* sequences of *Stemphylium* isolates. The tree was rooted with *Alternaria alternata* strain GV14-634a1. Sequences were retrieved from GenBank accessions reported in Woudenberg *et al.* (2017). Bootstrap support values are indicated near nodes by black circles whose size is proportional to the support values. The scale bar indicates the expected number of substitutions per position.

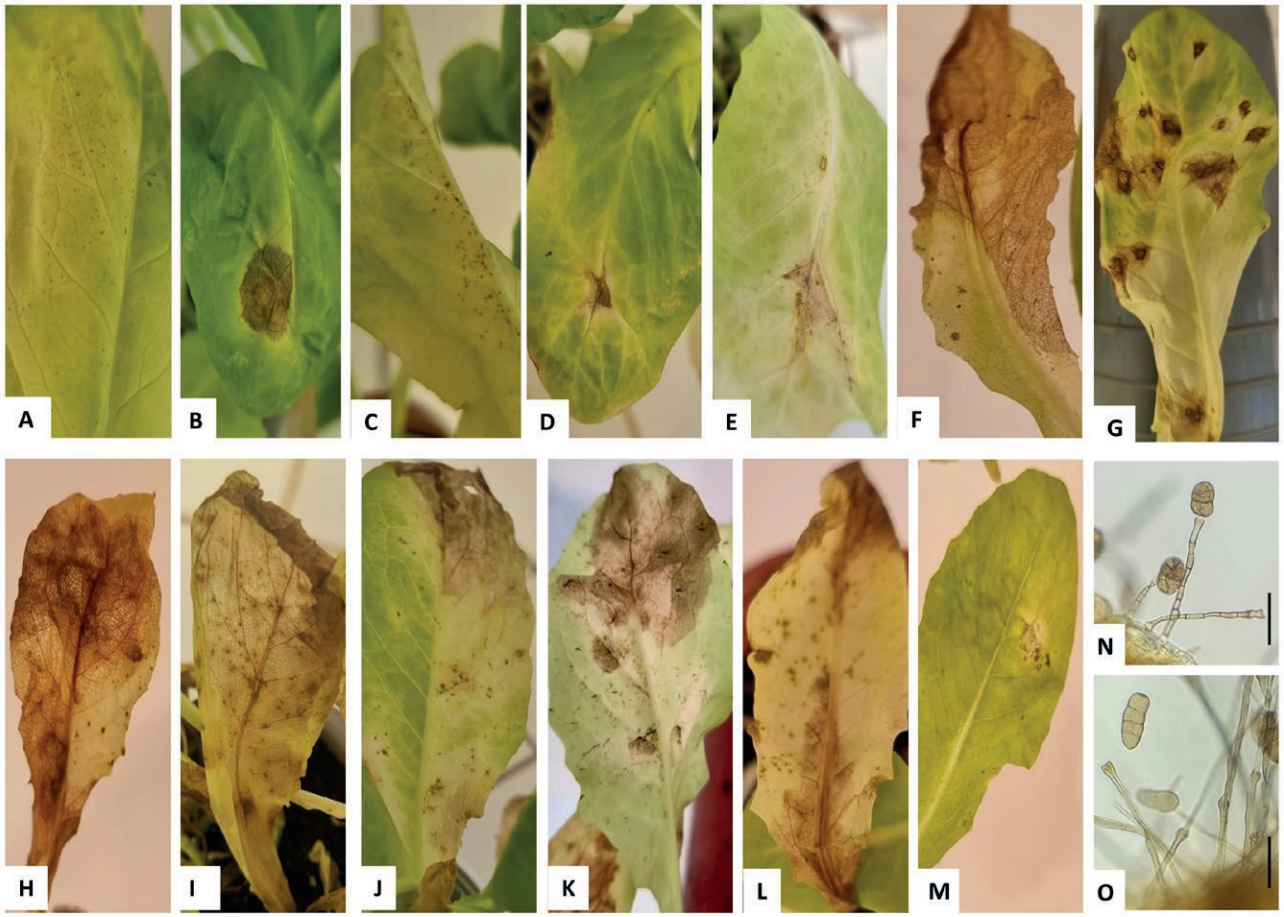

Figure 6. Foliar symptoms on inoculated romaine lettuce leaves after 21 dpi with different fungal isolates. **(A)** *Alternaria* section *Alternaria*: NB1149, **(B)** NB1150, **(C)** NB1151; *A.* section *Eureka*: **(D)** NB1089; *Stemphylium amaranthi*: **(E)** NB1153; *S. eturmiunum*: **(F)** NB1111; *S. gracillariae*: **(G)** NB1117, **(H)** NB1122, **(I)** NB1124; *S. lycopersici:* **(J)** NB1102, **(K)** NB1105, **(L)** NB1109; *S. vesicarium*: **(M)** NB1154. (**N**) Conidiophores and conidia of *S. gracilariae* (NB1120), and of **(O)** of *S. lycopersici* (NB1102); bars = 50μm.

inoculation. These spots expanded into long ellipsoid dark brown necrotic lesions. As these increase in size, entire leaves turned yellow, and the necrotic areas were easily ruptured. For isolates causing these symptoms, the lesions coalesced quickly into leaf blight. The symptoms were the same as observed on lettuce plants in the field and markets (Figure 6, A to M). Sporulation occurred in the necrotic lesions on basal leaves after inoculations with pathogenic isolates (Figure 6, N and O).

Seven of the nine assessed *S. gracilariae* isolates (NB1107, NB1108, NB1110, NB1117, NB1118, NB1122, NB1124) and one of the six assessed *S. lycopersici* isolates (NB1105) were more aggressive than the other tested isolates (Figure 7). The *S. eturmiunum* isolate was moderately aggressive (mean AUDPC = 425). Isolates of *S. vesicarium* and *S. amaranthi* and the small-spored *Alternaria* spp. were less aggressive (i.e. weakly pathogenic) with a mean AUDPCs between 110 and 240. These isolates produced small, brown necrotic lesions on leaves, which did not expand. Inoculation control plants remained healthy. To determine fulfilment of Koch's postulates, isolations of fungi from lesioned areas were carried out, and the recovered pathogens were found to be morphologically consistent with the fungi used in the inoculations.

Cross inoculations Four *Stemphylium* isolates were inoculated alone or in combinations with one of five *Alternaria* isolates in cross-inoculation tests. Symptom morphology from the double inoculations did not differ from that of *Stemphylium* isolate alone. The small-spored *Alternaria* isolates did not enhance disease development on lettuce plants when mixed with pathogenic *Stemphylium* strains (Figure 8). In most cases infection rates from mixed *Stemphylium* plus *Alternaria* inoculations were similar or less than for individual inoculations with *Stemphylium* alone. Cross inoculations with

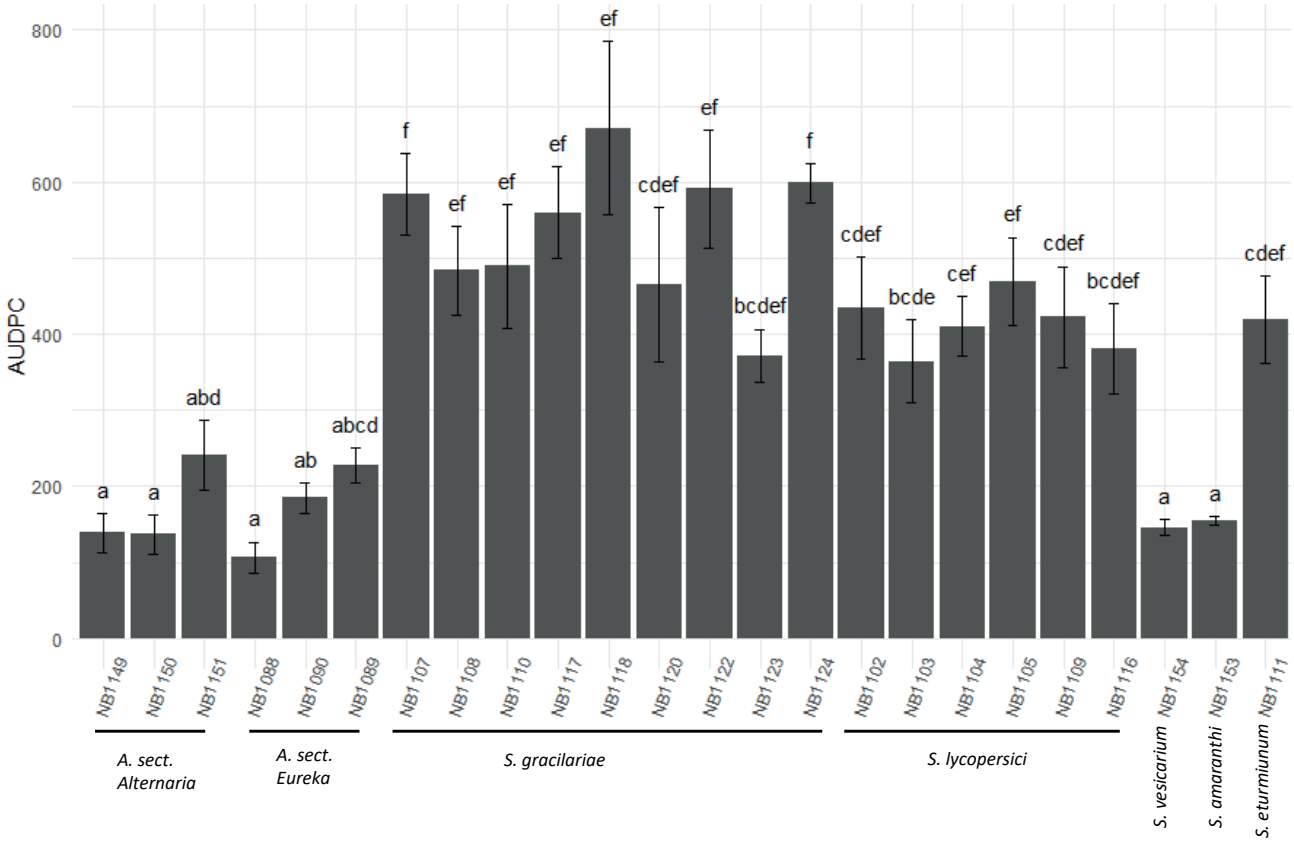

Figure 7. Mean areas under disease progress curves (AUDPCs) for romaine lettuce plants inoculated with the different *Alternaria* or *Stemphylium* spp. isolates after 7, 14 and 21 days. The letters above the standard deviation bars indicate significant differences ($P > 0.05$) between the means, as indicated by a Kruskal Wallis test and the Dunn *posthoc* test with Benjamini-Hochberg corrections.

S. lycopersici (NB1103, NB1104) and small-spored *Alternaria* isolates (respectively, NB1089, NB1090, NB1051, NB1089, NB1049) resulted in reduced AUDPCs. A similar trend was observed when *S. eturmiunum* (NB1111) was co-inoculated with isolates of section *Alternaria*. No differences were observed between the AUDPCs for plants inoculated by *S. gracilariae* alone or mixed with small-spored *Alternaria*, irrespective of the isolate.

DISCUSSION

In this study, the most commonly isolated fungi from lettuce plants with leaf spot symptoms were *Alternaria* spp. and *Stemphylium* spp. Different species were frequently isolated from the same leaf, or even from the same lesion. Morphologically, *Stemphylium* is distinguished from *Alternaria* by its percurrently or annellidically proliferating conidiophores that often each have a distinct swollen terminal apical cell or region (Simmons, 1967). However, based solely on morphological characteristics, reliable identification at species level is difficult, and sequence comparison at taxonomically informative loci is recommended (Woudenberg *et al*., 2017; Li *et al*., 2023).

Among the selected isolates, members of five *Stemphylium* spp. (*S. lycopersici*, *S. vesicarium*, *S. eturmiunum*, *S. amaranthi,* and *S. gracilariae*) and two *Alternaria* sections (sect. *Alternaria* and sect. *Eureka*), were identified using, respectively, multilocus *ITS-gpd-cmdA* and monolocus *gpd* analyses. Small-spored *Alternaria* spp. such as *A. alternata* have broad host ranges, and have been identified as causal agents of lettuce leaf spot (Guo *et al*., 2018; Naglaa and Safaa, 2020). Experimental inoculations of lettuce with Algerian isolates of sections *Alternaria* and *Eureka* showed that these fungi were weak pathogens. The weak pathogenicity of small-spored *Alternaria* spp. may be due to prolonged latency periods. These fungi may become active during host stress or increased age.

Some fungi (e.g. *Fusarium solani* and *F. proliferatum*) have the potential to exhibit both endophytic and pathogenic phases (Padhi *et al*., 2015). Similarly, *A. alternata* can be a pathogen and an endophyte in various

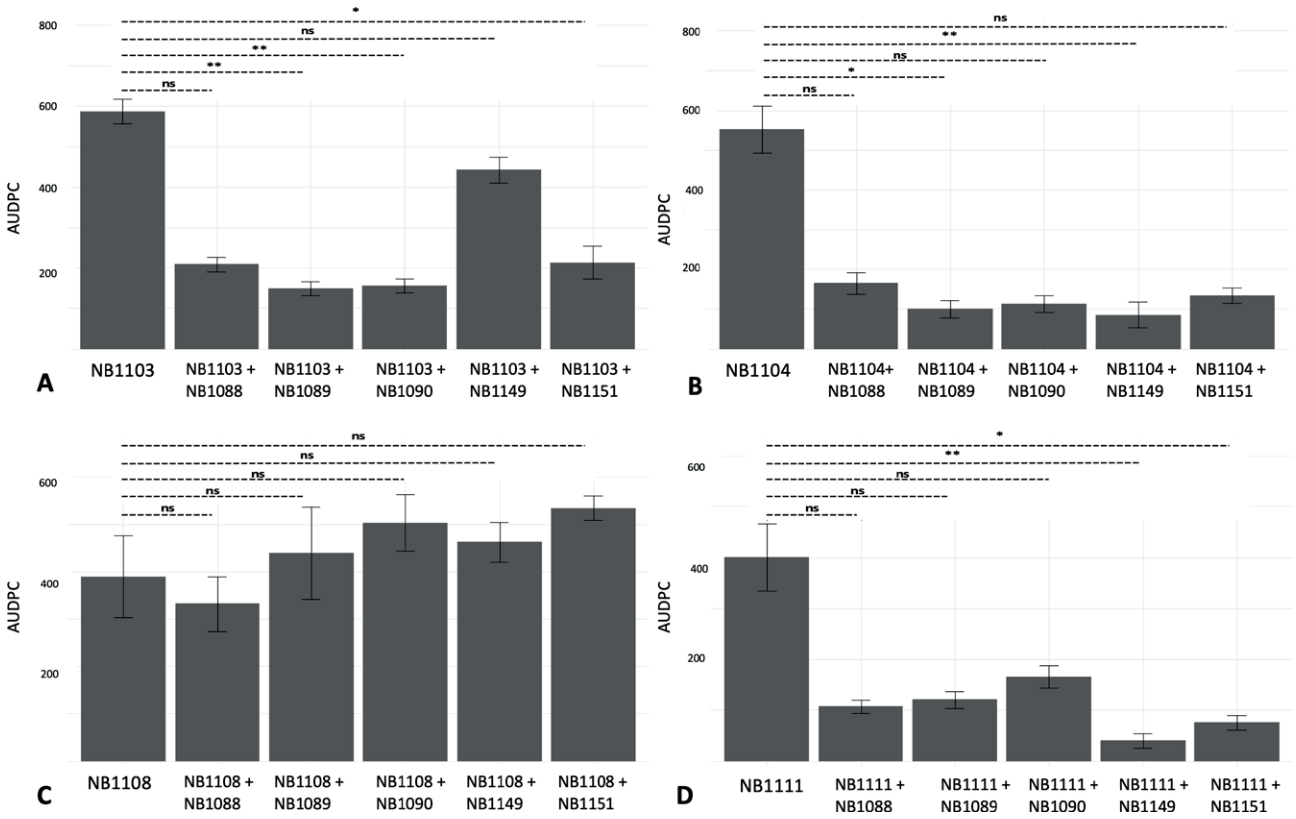

Figure 8. Mean areas under disease progress curves (AUDPCs) on romaine lettuce plants after individual (*Alternaria* **or** *Stemphylium* spp.) or cross (*Alternaria* **plus** *Stemphylium* spp.) inoculations. **(A and B)** *S. lycopersici*; **(C)** *S. gracilariae*; and **(D)** *S. eturmiunum* after 7, 14 and 21 d. Bars indicate standard errors of means. The stars indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) between the individual and cross inoculations, as shown by Kruskal Wallis tests and Dunn posthoc tests with Bonferroni corrections.

plants (De Mers, 2022), and has been described in lettuce as endophytic (D'Amico *et al*., 2008) and pathogenic (Guo *et al*., 2018). In contrast, two *Stemphylium* isolates (*S. gracilariae* and *S. lycopersici*) were found to be highly pathogenic, one (*S. eturmiunum*) was moderately pathogenic to lettuce. While some *Stemphylium* species have been reported infecting single hosts, some can infect diverse hosts (Brahamanage *et al*., 2018). *Stemphylium lycopersici* has a broad host range, infecting leaves from more than 30 host genera in at least eight plant families: *Amaranthaceae*, *Araceae*, *Asteraceae*, *Fabaceae*, *Malvaceae*, *Plantaginaceae*, *Rosaceae* and *Solanaceae* (Farr and Rossman, 2017; Manaaki Whenua, 2023; Spawton and du Toit, 2024). Nasehi *et al*. (2014) showed that *S. lycopersici* was pathogenic to lettuce, regardless of its original host, and that isolates of this fungus could genetically segregate due to host plant rather than geographic region. As *S. lycopersici* has been isolated from diseased tomato in Algeria (Bessadat *et al*., 2022), phylogenetic segregation by host could be investigated for Algerian isolates of this fungus.

Stemphylium gracilariae also has a broad host range, and has been reported on hosts in six botanical families, including lettuce (Woudenberg *et al*., 2017; Brahmanage *et al*., 2019). Similarly, other fungi such as *S. vesicarium* and *S. eturmiunum* can infect several host species and colonize raw food ingredients (Samson *et al*., 2010; Olsen *et al*., 2018). *Stemphylium vesicarium* and *S. lycopersici* have been identified as causing leaf spot on lettuce in China (Liu *et al*., 2019).

Stemphylium amaranthi was first described from necrotic leaf spots on *Amaranthus retroflexus* (Pei *et al*., 2009), and on several host plants (Poursafar *at al*., 2018; Spawton and du Toit, 2024). The present study has shown that lettuce as a previously unrecognized host for *S. amaranthi*, and this fungus is also identified as a new addition to the recorded mycobiota of Algeria. However, under experimental conditions used in the present study, the tested isolates of *S. vesicarium* and *S. amaranthi* only produced small non-progressing necrotic spots on inoculated lettuce leaves. Although *S. solani* has also been identified as causing lettuce leaf spot in Malaysia and

China (Kim *et al*., 2004; Nasehi *et al*., 2013), none of the isolates collected on symptomatic lettuce leaves in Algeria corresponded to this species.

No synergistic effects were shown for *Alternaria* - *Stemphylium* cross inoculations, indicating that antagonism could reduce disease progression. Tao *et al*. (2021) showed that *Pleospora* was among five fungi that interact directly with pathogenic *Alternaria* on *Nicotina tobaccum* to suppress disease development. O'Neill (2019) considered *Stemphylium* and *Alternaria* as weak pathogens or secondary decay organisms on lettuce. These fungi are polyphagous and can colonize novel environments. Lamichhane and Venturi (2015) reported that co-occurrence of identical pathogens on a single host can lead to antagonistic or synergistic interactions, often determined by the order in which pathogens infect host plants. Modulation of these complex interactions could be influenced by multiple factors (Mishra *et al*., 2024).

The present study has demonstrated that isolates of *S. lycopersici* and *S. gracilariae* obtained from lettuce were primary pathogens of this host, and can cause significant damage under greenhouse conditions. The association of these two fungi is likely to be affected by several external factors, which is not uncommon in closely related pathogens (Bessadat *et al*., 2022).

Although *Stemphylium* and *Alternaria* were widespread fungi causing leaf spot disease on plants (Schlub et al. 2022), this is the first report of natural infections of lettuce plants in Algeria by *Stemphylium* and *Alternaria* fungi. These pathogens may become serious problems because of their broad host ranges and pathogenic variability. International trade of agricultural seeds has moved crops and pathogens away from their original environments, and many fungi causing diseases of leafy vegetables are seed-borne (Gullino *et al*., 2019). Rapid emergence of new diseases is often due to seed contamination, and results in severe economic losses. Greenhouse gases generated by human activities can cause shifts in rainfall patterns and influence development and spread of plant pathogens (Velásquez *et al*., 2018; Gullino *et al*., 2019; Pathak *et al*. 2023).

Foliar diseases of lettuce caused by *Alternaria* or *Stemphylium* spp. could be difficult to manage because these fungi can rapidly produce large amounts of secondary inocula under favorable environmental conditions (Grewling *et al*., 2020; González *et al*., 2024). Conidium production also depends on external factors such as light, photoperiods, pollution, presence of weed hosts, soil chemistry, agronomic practices, and crop density, which can enhance disease spread (Gossen *et al*. 2021; Miranda-Apodaca *et al*. 2023; Waheed *et al*., 2023; Lahlali *et al*., 2024). Some pathogens can infect plant leaves in the field throughout crop growing seasons, and can remain dormant until harvest. However, these pathogens have similar temperature and moisture optima for infection and development, so disease control strategies could be developed according to weather forecasts, for timing of fungicide or other chemical applications. Control measures such as foliar spray applications of fungicides combined with sodium carbonate and/or ammonium sulfate have been shown to be effective inducers of systemic protection against the lettuce pathogens *S. botryosum* and *A. alternata* (Naglaa and Safaa, 2020). Results of the present study emphasize the need for further research to evaluate influences of fungal communities and effect of agronomic practices (chemical fertilizers, pesticides, fungicides, etc.) on *S. gracilariae* and *S. lycopersici*. Additionally, lettuce breeding programmes should aim to develop lettuce cultivars with enhanced resistance to these pathogens.

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AUTHOR CONTRIBUTIONS

Conceptualization: NB and PS. Data curation: PS, JC, and NBS. Formal analysis: NB and PS. Investigation: NBS and NB. Methodology: NBS, NB, and BH. Resources: NB and BH. Supervision: PS and KM. Validation: PS. Visualization: NB. Writing: original draft manuscript; NB; review and editing; NB and PS.

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