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Research Papers

Distribution of large-spored *Alternaria* species associated with early blight of potato and tomato in Algeria

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Abstract. Potato and tomato are important crops in Algerian agriculture, and both are threatened by abiotic and biotic stresses, and early blight is a major disease affecting both crops. Surveys carried out from 2012 to 2015 in 12 major growing regions for these crops yielded a total of 247 *Alternaria* isolates having morphological and cultural characteristics of sections *Alternaria* and *Porri*. Since early blight symptoms and morphological characteristics of the isolates did not allow sharp distinction between the different large-spored species of *Alternaria*, the isolates in section *Porri*, often considered primary causes of the diseases, were selected for molecular characterization by diagnostic PCR using specific primers. This allowed species identification of 147 *Alternaria* isolates as *A. solani*, *A. protenta, A. grandis* or *A. linariae*. These species were present on potato and tomato crops at varying frequencies, depending on the hosts and on bioclimatic locations. Pathogenicity tests for the four species, on detached leaflets and whole seedlings, showed that all were pathogenic to potato and tomato, with varying virulence. These results suggest that parasitic specialization of these *Alternaria* species on solanaceous plants should be reconsidered.

Keywords. Solanaceous crops, parasitic specialization, prevalence.

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INTRODUCTION

Potato (*Solanum tuberosum* L.) and tomato (*S. lycopersicum* L.) are important crops grown in Algeria. They are distributed differently in the various bioclimatic zones of the country, and are grown with different cultural practices. Potato crops occupy significant area in several regions of Algeria, from north (Ain Defla) to south (El Oued), and from east (Skikda, Guelma) to west (Mostaganem, Mascara). Tomato, however, is mainly cultivated in the northern part of the country, less extensively and mostly in plastic houses, as in the Biskra region. Both crops are often cultivated side by side or follow one another in rotations for several years, in many coastal regions of the

country. Intensification of these two crops without compliance to good agricultural practices led to the development of important diseases caused by fungi and oomycetes, including early blight and late blight.

The first reference mentioning *A. solani* Sorauer (= *Macrosporium solani* Ellis & G. Martin) as a pathogen causing potato leaf blight was that of Galloway (1891) in Australia. Chester (1892) then reported this pathogen in the United States of America, other solanaceous cultivated plants (Van der Waals *et al.*, 2001; Odilbekov, 2015). A few years later, *A. solani* and *A. alternata* (Fr.) Keissl. were identified on potato leaves but *A. alternata* was considered first as saprophytic (Jones and Grout, 1897). It was not until 1984 that *A. alternata* was recognized as pathogenic on several solanaceous plants (Droby *et al.* 1984). The name "early blight" for the disease caused by *A. solani* was coined by Jones (1893) to distinguish this from late blight. The symptoms of the early blight appear on the leaves as dark, elongated or circular lesions each with concentric rings surrounded by a yellow halo.

On the basis of cultural techniques and morphological characteristics of the conidia (size and beak length), Simmons (2000, 2007) described several new *Alternaria* species on *Solanaceae*, that may be responsible for early blight on potato and tomato. Thus, new species were described, which are morphologically very similar to *A. solani*. These include *A. grandis* E.G. Simmons on *S. tuberosum*, *A. tomatophila* E.G. Simmons on *S. lycopersicum*, *A. cretica* E.G. Simmons & Vakal. On *S. lycopersicum* var. *esculentum*, and *A. subcylindrica* E.G. Simmons & R.G. Roberts on *S. lycopersicum* var. *cerasiforme*.

More recently, molecular and genomics techniques applied to *Alternaria* allowed redefinition of various species with large conidia commonly isolated from *Solanaceae*, and considered as the primary causes of early blight. Woudenberg *et al.* (2014), by synonymizing *A. linariae* (Neerg.) E.G. Simmons with *A. cretica* E.G. Simmons & Vakal., *A. cucumericola* E.G. Simmons & C.F. Hill, *A. subcylindrica*, *A. tabasco* E.G. Simmons & R.G. Roberts, and *A. tomatophila*, have expanded the host range of *A. linariae* to *Solanaceae*, *Cucurbitaceae*, and *Scrophulariaceae*. *Alternaria linariae*, a common pathogen of tomato, was also reported on potato during surveys carried out in Algeria (Ayad *et al.*, 2018). *Alternaria Grandis*, which was confused for a long time with *A. solani*, has been regularly reported in many countries on potato (Lourenço *et al.,* 2009; Cardoso, 2014; Bessadat *et al.*, 2016; Landschoot *et al.*, 2017). *Alternaria grandis* was also recently isolated from tomato (Bessadat *et al.*, 2017) in the northwest of Algeria. *Alternaria protenta*, which is closely related to *A. solani* and whose host range was extended to *Asteraceae*, *Euphorbiaceae*, *Gramineae* and *Solanaceae* by Woudenberg *et al.* (2014), was recently detected on potato in Belgium (Landschoot *et al.*, 2017) and in Algeria (Ayad *et al.*, 2017).

The aims of the present study were, firstly, to identify at the species level the large-spored *Alternaria* isolates obtained from surveys carried out during three cropping years on different potato and tomato fields across the main bioclimatic areas of Algeria. Since early blight symptoms and the morphological characteristics of the isolates do not allow distinction between the different large-spored *Alternaria* spp., specific identification of the isolates was based on PCR techniques. The second objective was to assess the aggressiveness of isolates on their respective potato and tomato hosts, and to clarify the parasitic specialization of replace by large-spored *Alternaria* species by artificial cross inoculations *in vitro* and under conditions similar to those occurring in the field.

MATERIALS AND METHODS

Surveys and sampling

Surveys were carried in 60 locations across 12 regions in Algeria, with different cropping systems (fields and greenhouses). Three to four plots were sampled in each region during three successive cropping years: 2012–2013, 2013–2014 and 2014–2015. The majority of the localities were selected from the main potato- and tomato-producing regions represented by different bioclimatic areas of Algeria: Mediterranean climate (Algiers, Tipaza, Guelma and Skikda), semi-arid climate (Mostaganem, Chlef, Mascara, Ain Defla and Bouira), and arid climate (Laghouat, Biskra and El Oued) (Figure 1). Sampling was carried out from potato and tomato plant organs showing the typical symptoms of early blight: 164 isolates were collected from potato leaves essentially and 83 from leaves, stems and fruits of tomato.

Isolation, purification and conservation of isolates

Isolations were performed according to the method of Van der Waals *et al.* (2004). Small tissue pieces (3 to 4 mm^2) were cut from individual lesion edges, disinfected in 1% active NaOCl for 3 min, washed twice in sterile distilled water, and then dried with absorbent sterile paper. Four pieces of tissues were plated on individual Petri plates containing Potato Dextrose Agar (PDA), and these were incubated at 22°C under continuous light for 1 to 2 weeks. The purification of the *Alternaria* spp. isolates as single conidium cultures was car-

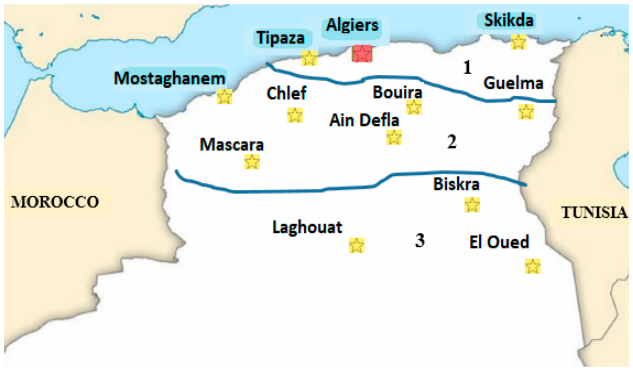


Figure 1. Geographic localities in the different bioclimatic areas: 1 = Mediterranean climate, 2 = semi-arid climate, 3 = arid climate

ried out between the 3rd and the 5th day of incubation. Plates containing the single conidium cultures were then placed under near-UV (12h dark and 12h light) to induce sporulation and allow further morphological characterization.

Single conidium cultures for short-term use were grown in Petri dishes containing a PDA for 12 d then stored at 4°C. For medium term use, 12-d-old PDA cultures in test tubes were stored at 4°C. For long term use, mycelium/agar plugs (8 mm diam.) were stored at 80°C in cryotubes containing sterile glycerol (30% w/w).

Identification of the large-spored Alternaria species using molecular markers

On the basis of the cultural and the morphological characteristics, 155 isolates with large conidia (out of a total 247 isolates) were selected for molecular characterization by PCR using specific primers, for analysis of genomic DNA by PCR/RFLP, using double enzymatic digestion of a portion of the calmodulin gene and sequencing the calmodulin and the RPB2 genes. Isolates were grown on PDA medium at room temperature for 10 d. Total genomic DNA from fungal samples was extracted according to the method described by Goodwin and Lee (1993). The DNA extracts of the 155 isolates were amplified using two sets of specific primer pairs to differentiate between *A. linariae* and *A. solani* and related species (*A. grandis* and *A. protenta*) (Gannibal *et al.*, 2014). The primer pair OasF7 and OasR6 amplified a 164 bp fragment from the Alt a1 gene of *A. solani*, *A. grandis* and *A. protenta*, and the primer pair OatF4 and OatR2 amplified a 438 pb fragment from the calmodulin encoding gene of *A. linareae*. The PCR conditions were as described by Gannibal *et al.* (2014). To differentiate between *A. grandis* and *A. solani sensu lato* (i.e. including *A. protenta*), a portion of the calmodulin gene was amplified from the isolates that each gave a positive signal with the primer pair OasF7 – OasR6 using the primer pair CALDF1 / CALDR1 (Lawrence *et al.*, 2013). The resulting PCR products were double digested with the restriction enzymes *Rsa*I and *Hae*II. The digestion products were then separated by electrophoresis in 2% agarose gels and visualized under UV light after staining with ethidium bromide. Predicted restriction patterns mainly differed by the size of the larger fragment, i.e. 420 bp for *A. solani sensu lato* and 292 bp for *A. grandis* (Figure 2). Identification of the *Alternaria* species was validated by sequencing the fragment of the calmodulin locus amplified as described above from 42 isolates. To confirm the presence of *A. protenta*, a portion of the RPB2 locus was amplified from the isolates identified as *A. solani* based on their calmodulin sequence, using the primer pair RPB2-5F2 (Sung *et al.*, 2007) and RPB2-7cR (Liu *et al.*, 1999), and the resulting PCR product was sequenced. Sanger sequencing was performed by GATC Biotech. Sequence analyses were carried out using the Phylogeny.fr web service (Dereeper *et al.*, 2008). Multiple sequence alignments were generated with MUSCLE and curated using the Gblocks algorithm. Maximum likelihood (ML) analyses were performed with PhyML. The robustness of the ML topologies was evaluated using the Shimodaira-Hasegawa (SH)-like test for branches.

Pathogenicity tests of large-spored Alternaria species on their respective hosts

To confirm pathogenicity of isolates, inoculations onto detached leaflets and whole plants were performed on susceptible varieties of tomato (cv. Marmande) and

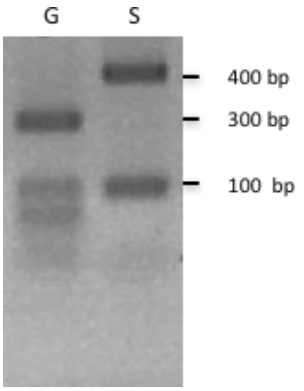


Figure 2. Typical restriction patterns obtained by double digestion of PCR product corresponding to a portion of the calmodulin gene from *A. solani* and *A. grandis*. Size of the larger fragment, 420 bp for *A. solani sensu lato* (S) and 292 bp for *A. grandis* (G)

potato (cv. Spunta). These isolates were selected for their ability to produce conidia. Conidium suspensions were prepared from 15-d-old cultures. The final concentration of the suspensions was adjusted to 10^4 conidia mL^{-1}. The inoculations were performed by depositing drops (20 µL each) on detached leaflets or by spraying suspensions onto whole plants. Disease severity was evaluated at 15 d –post-inoculation, using a visual rating scale from 1 to 9 expressing the extension of necrosis CIP (International Potato Center) (2008).

RESULTS

Characterization of the large-spored Alternaria species according to early blight symptoms on potato and tomato

Isolation from plant samples showing typical early blight symptoms yielded 247 isolates (164 from potato and 83 from tomato). On the basis of the cultural and morphological characteristics of the colonies and conidia, the isolates were divided in three groups. The first (Group A: 92 isolates) included all the isolates with abundant conidium production and small catenulate conidia and identified as species belonging to section *Alternaria*. The second (Group B: 92 isolates) contained all isolates producing large solitary beaked conidia, related to species belonging to the section *Porri*. The third (Group C: 63 isolates) consisted of large-spored isolates that only formed sterile mycelium after culture purification. In relation to the host plants, 89% of the isolates belonging to the section *Porri* originated from potato, and 73% of the non-sporulating isolates originated from tomato. No correlation was found between the presence of yellow halos on symptomatic leaves and the production of a diffusible pigment on growth medium by the isolates from the diseased samples. Similarly, the various forms of symptoms generated colonies producing either large or small conidia. Thus, there was no relationships between necrosis size (small, medium or large necrotic lesions), the types of the colonies in culture medium (sporulating or non-sporulating) and the conidium types (large solitary or small catenulate conidia).

Molecular characterization and identification of large-spored Alternaria species

Given the very similar morphological characteristics of the conidia, and the difficulty to distinguish the different *Alternaria* species within the section *Porri*, molecular markers and DNA-sequencing were used for their identification. The small-spored isolates (Group A) were not included in this examination. The three molecular approaches allowed clear species identification for 147 of 155 isolates from Groups B and C, while eight isolates could not be identified due to problems in DNA extraction and amplification at the selected loci (Table 1). Preliminary tests were carried out for the identification of the 147 isolates using two pairs of specific primers that differentiate *A. solani*, *A. protenta* and *A. grandis* (OasF7/OasR6) from *A. linariae* (OATF4/OATR2). All the tested isolates generated positive signals with one of the two primer pairs, confirming the presence of these species on potato and tomato crops in Algeria. In this analysis, 37 isolates including seven from potato and 30 from tomato, were identified as *A. linariae* by amplification of the 438 pb from the gene encoding for calmodulin (Table 1). A PCR product corresponding to a fragment of the Alt a1 gene (164 bp) was amplified from the remaining 110 isolates. These isolates, of which 86 were isolated from potato and 24 from tomato, could be assigned to either *A. solani* or to phylogenetically related-species, i.e. *A. grandis* and *A. protenta*. Further identification was conducted in order to differentiate between *A. solani* and *A. grandis* isolates using PCR/RFLP, by amplifying the calmodulin locus followed by restriction enzyme digestion (*Hae*II and *Rsa*I). Based on the characteristic electrophoretic profiles of the restricted PCR products, *A. solani* and *A. protenta* were separated from *A. grandis*. Thus, of the 110 isolates that gave positive signals with the OasF7/OasR6 primer pair, 93 were *A. solani*, 12 were *A. grandis* and five showed abnormal enzymatic restriction digestion patterns that may be due to partial digestion with at least one of the restriction enzymes. These five isolates, as well as 37 other representative isolates obtained from the two host plants collected in nine of the twelve surveyed Algerian regions and representing the three identified species (*A. linariae*, *A. grandis* and *A. solani*), were selected for species confirmation by DNA sequencing which was firstly performed at the calmodulin locus. Phylogenetic analyses of sequences derived from the calmodulin locus of the 42 isolates (Figure 3) separated the isolates into two clades: the first (A) contained the isolates previously identified by PCR with specific primers as *A. linariae*, and the second (B) included two sub clades (B1 and B2) regrouping isolates previously identified as *A. grandis* (sub clade B1) and *A. solani* (sub clade B2). DNA sequencing at the RPB2 locus then carried out on the isolates of the sub clade B2 gave the phylogenetic tree shown in Figure 4, in which among the 17 isolates of *A. solani*, three were identified as *A. protenta*, two of which were isolated from the potato and one from tomato.

Table 1. Identification of large-spored of *Alternaria* spp isolates.

Isolate	Species[a]	Host	Geographic origin	PCR OATF4/ OATR2	PCR OAsF7/OAsR6	PCR RFLP Profile[b]	CAL	Rpb2
DA001	***A. linariae***	Potato	Tipaza	+	-	ND	MH243795	_
DA002	A. linariae	Potato	Tipaza	+	-	ND	MH243769	_
DA003	***A. linariae***	Potato	Tipaza	+	-	ND	MH243793	_
DA005	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA006	***A. linariae***	Potato	Alger	+	-	ND	MH243789	_
DA007	***A. linariae***	Potato	Alger	+	-	ND	MH243794	_
DA008	***A. solani***	Potato	Alger	-	+	S	MH243805	MH243818
DA009	***A. grandis***	Potato	Alger	-	+	?	MH243790	_
DA010	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA011	***A. solani***	Potato	Alger	-	+	S	MH243806	MH243820
DA012	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA013	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA014	*A. solani s.l*	Potato	Alger	-	+	S	MH243796	_
DA015	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA016	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA017	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA018	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA019	***A. solani***	Potato	Bouira	-	+	S	MH243808	MH243822
DA020	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA021	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA022	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA025	***A. solani***	Potato	Bouira	-	+	S	MH243786	MH243823
DA026	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA028	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA029	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA030	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA031	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA032	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA033	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA034	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA035	*A. solani s.l*	Potato	Mascara	-	+	S	_	_
DA036	*A. solani s.l*	Potato	Mascara	-	+	S	_	_
DA037	*A. linariae*	Tomato	Mostaganem	+	-	ND	_	_
DA038	***A. grandis***	Potato	Alger	-	+	G	MH243770	_
DA039	*A. linariae*	Potato	Alger	+	-	ND	_	_
DA040	***A. linariae***	Tomato	Mascara	+	-	ND	MH243792	_
DA041	*A. linariae*	Tomato	Mostaganem	+	-	ND	_	_
DA042	*A. linariae*	Tomato	Mostaganem	+	-	ND	_	_
DA043	*A. solani s.l*	Potato	Mostaganem	-	+	S	_	_
DA045	*A. solani s.l*	Potato	Mascara	-	+	S	_	_
DA046	*A. solani s.l*	Potato	Mostaganem	-	+	S	_	_
DA047	***A. grandis***	Potato	Mostaganem	-	+	G	MH243771	_
DA048	*A. solani s.l*	Potato	Mostaganem	-	+	S	_	_
DA049	*A. solani s.l*	Potato	Chlef	-	+	S	_	_
DA050	***A. solani***	Potato	Chlef	-	+	S	MH243772	MH243809
DA051	***A. grandis***	Potato	Chlef	-	+	G	_	_
DA052	***A. grandis***	Potato	Chlef	-	+	G	MH243773	_

(Continued)

Table 1. (Continued).

Isolate	Species[a]	Host	Geographic origin	PCR OATF4/ OATR2	PCR OAsF7/OAsR6	PCR RFLP Profile[b]	CAL	Rpb2
DA053	*A. solani s.l*	Potato	Chlef	-	+	S	_	_
DA054	***A. grandis***	Potato	Mostaganem	-	+	G	MH243774	_
DA055	***A. grandis***	Potato	Mostaganem	-	+	G	_	_
DA056	*A. solani s.l*	Potato	Mostaganem	-	+	S	_	_
DA057	***A. solani***	Potato	Mascara	-	+	?	MH243791	MH243819
DA058	*A. solani s.l*	Potato	Mascara	-	+	S	_	_
DA059	*A. solani s.l*	Potato	Mostaganem	-	+	S	_	_
DA060	***A. grandis***	Potato	Mostaganem	-	+	G	MH243775	_
DA061	***A. grandis***	Potato	Mostaganem	-	+	G	_	_
DA062	***A. protenta***	Tomato	Mostaganem	-	+	S	MH243797	MH243810
DA063	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA064	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA065	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA066	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA067	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA068	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA069	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA070	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA071	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA072	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA073	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA074	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA075	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA076	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA077	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA078	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA079	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA080	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA081	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA082	***A. protenta***	Potato	El Oued	-	+	?	KX870505	KX870507
DA083	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA084	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA085	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA086	***A. protenta***	Potato	El Oued	-	+	S	KX870506	KX870508
DA087	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA088	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA089	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA090	***A. solani***	Potato	El Oued	-	+	S	MH243776	MH243811
DA091	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA092	*A. solani s.l*	Potato	El Oued	-	+	S	_	_
DA093	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA094	***A. solani***	Potato	Alger	-	+	S	MH243798	MH243812
DA096	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA097	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA098	*A. solani s.l*	Potato	Alger	-	+	S	_	_
DA099	***A. grandis***	Tomato	Alger	-	+	G	MH243777	_
DA100	***A. linariae***	Tomato	Alger	+	-	ND	MH243807	_

(Continued)

Table 1. (Continued).

Isolate	Species[a]	Host	Geographic origin	PCR OATF4/ OATR2	PCR OAsF7/OAsR6	PCR RFLP Profile[b]	CAL	Rpb2
DA101	***A. linariae***	Tomato	Alger	+	-	ND	MH243778	_
DA102	*A. linariae*	Tomato	Alger	+	-	ND	_	_
DA103	*A. linariae*	Tomato	Alger	+	-	ND	_	_
DA104	*A. linariae*	Tomato	Alger	+	-	ND	_	_
DA107	*A. linariae*	Tomato	Alger	+	-	ND	_	_
DA108	*A. solani s.l*	Potato	Guelma	-	+	S	_	_
DA109	***A. linariae***	Tomato	Tipaza	+	-	ND	MH243779	_
DA110	*A. linariae*	Tomato	Tipaza	+	-	ND	_	_
DA111	***A.linariae***	Tomato	Tipaza	+	-	ND	MH243780	_
DA112	*A. linariae*	Potato	Skikda	+	-	ND	_	_
DA113	***A. solani***	Tomato	Guelma	-	+	S	MH243799	MH243821
DA114	***A. solani***	Potato	Guelma	-	+	?	MH243788	MH243813
DA115	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA116	*A. solani s.l*	Potato	Bouira	-	+	S	_	_
DA117	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA118	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA119	***A. linariae***	Tomato	Biskra	+	-	ND	MH243800	_
DA120	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA121	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA122	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA123	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA124	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA125	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA126	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA127	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA128	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA129	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA130	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA131	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA132	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA133	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA134	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA135	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA136	***A. solani***	Tomato	Biskra	-	+	S	MH243801	MH243814
DA137	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA138	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA139	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA140	***A. solani***	Tomato	Biskra	-	+	S	MH243802	MH243815
DA141	***A. solani***	Tomato	Biskra	-	+	S	MH243803	MH243816
DA142	***A. solani***	Tomato	Biskra	-	+	S	MH243804	MH243817
DA143	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA144	*A. linariae*	Tomato	Biskra	+	-	ND	_	_
DA145	*A. solani s.l*	Tomato	Biskra	-	+	S	_	_
DA146	*A. solani s.l*	Potato	Tipaza	-	+	S	_	_
DA147	***A. linariae***	Tomato	Tipaza	+	-	ND	MH243781	_
DA148	*A. linariae*	Tomato	Mostaganem	+	-	ND	_	_
DA149	*A .linariae*	Tomato	Mostaganem	+	-	ND	_	_

(Continued)

Table 1. (Continued).

Isolate	Species[a]	Host	Geographic origin	PCR OATF4/ OATR2	PCR OAsF7/OAsR6	PCR RFLP Profile[b]	CAL	Rpb2
DA150	*A. linariae*	Tomato	Mostaganem	+	-	ND	_	_
DA152	***A. grandis***	Tomato	Alger	-	+	G	MH243782	_
DA153	***A. linariae***	Tomato	Alger	+	-	ND	MH243783	_
DA154	***A .grandis***	Tomato	Alger	-	+	?	MH243787	_
DA155	***A. grandis***	Tomato	Alger	-	+	G	MH243784	_
DA156	***A. grandis***	Tomato	Alger	-	+	G	MH243785	_

[a] Species names in bold characters were supported by sequence data; *A. solani s.l* stands for *A. solani sensu lato* (i.e. including *A. protenta*). [b] PCR-RFLP profiles: S = typical of *S. solani*; G = typical of *A. grandis*; ND = not determined; ? = atypical restriction profile



Figure 3. Phylogenetic tree reconstructed by the maximum likelihood method from the alignment of calmodulin sequences of 42 isolates belonging to the *Alternaria* section *Porri*. Bootstrap support values greater than 80 % are indicated by arrows. The calmodulin sequences of the following strains were included as references: *A. linariae* CBS 109156 (GenBank number JQ646257), *A. protenta* CBS 116696 (GenBank number JQ646236), *A. solani* CBS 109157 (GenBank number KJ397981), *A.grandis* CBS 109158 (GenBank number JQ646249). The calmodulin sequence from *A. alternata* EGS 34016 (Genbank number JQ646208) was used to root the tree.

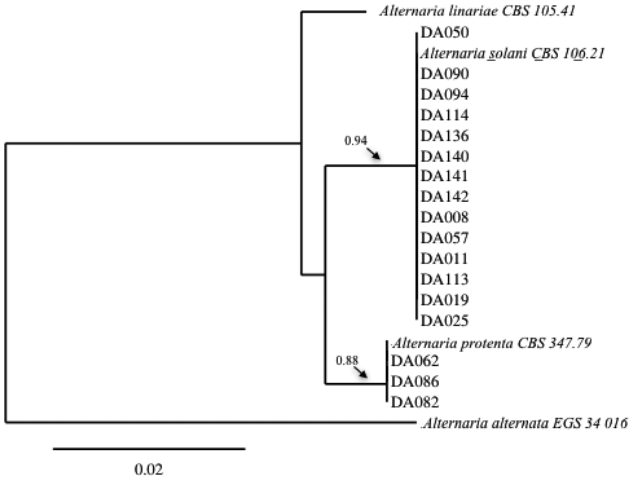


Figure 4. Phylogenetic tree reconstructed by the maximum likelihood method from the alignment of RPB2 sequences of 17 isolates belonging to cluster B2 in the calmodulin phylogeny. Bootstrap support values greater than 80 % are indicated by arrows. The RPB2 sequences of the following strains were included as references: *A. linariae* CBS 105.41 (GenBank number KJ718353), *A. solani* CBS 106.21 (GenBank number KJ718410) *A. protenta* CBS 347.79 (GenBank number KJ718392). The RPB2 sequence from *A. alternata* EGS 34016 (Genbank number JQ646490) was used to root the tree.

Distribution and prevalence of the large-spored Alternaria species in the bioclimatic regions of Algeria

The surveys carried out through the different Algerian potato and tomato growing regions revealed the presence of *A. grandis*, *A. linariae* and the species complex *A. solani*-*A. protenta* at variable levels. The frequencies of these species varied according to the host plants (potato or tomato) and to the geographical locations (Table 2). *Alternaria solani sensu lato* (i.e. including *A. protenta*) was the most common species in Algeria, representing 65% of the large-spored isolates, followed by *A. linariae* (25%) and *A. grandis* with low isolation frequency (10%). Isolation frequencies of these species varied with the sampled crop, although they were all found on both potato and tomato. *Alternaria solani sensu lato* (80%) and *A. grandis* (64%) were more prevalent on potato than on tomato. Conversely, *A. linariae*

Table 2. Frequencies of the four large-spored *Alternaria* species according to the host plants (potato and tomato) and to the geographical locations

Origin	*A. solani-A protenta*	*A. grandis*	*A. linariae*
Host plant origin			
Potato	80.2[a]–82.8[b]	64.3–9.6	18.9–7.5
Tomato	19.8–35.2	35.7–9.2	81.1–55.6
Geographic origin (bioclimatic area)			
Mediterranean	16.7–39.0	50.0–17.0	48.6–44.0
Semi-arid	34.3–70.2	50.0–14.9	18.9–14.9
Arid	49.0–79.7	0–0	32.4–20.3

[a] Percentage of the total number of isolates of the same species according to the host plant or the geographic origin
[b] Percentage of the total number of isolates from the same host or from the same geographic origin



Figure 5. Symptoms on detached leaflets (a, d) and whole tomato (c, e) or potato (b, f) plants inoculated by *A. linariae* isolate DA002 (a, b and c) or by *A. grandis* isolate DA009 (d, e and f)

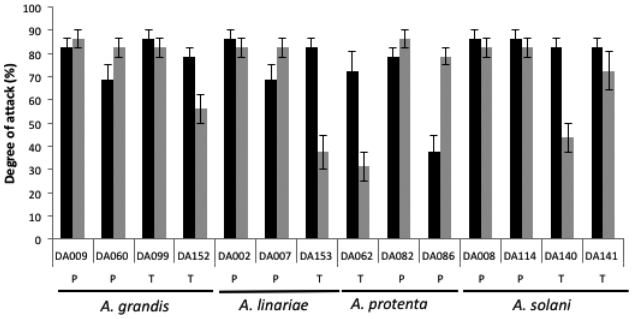


Figure 6. Disease severity evaluated using a visual rating scale (0-100%) at 15 days post-inoculation of tomato (black bars) and potato (grey bars) leaves with isolates representing the four large-spored *Alternaria* species. Letters (P or T) under the isolates identities refer to the host of origin (Potato or Tomato).

was more frequently isolated from tomato than from potato. Locations of sample collection could be divided into three temperature and precipitation zones (Mediterranean, semi-arid or arid). In Mediterranean and arid zones, isolates were almost equally distributed between potato and tomato. *Alternaria linariae* was over-represented in the Mediterranean zone (44% of the collected isolates) compared to the arid zone where it represented only 20% of the collected isolates. The reverse situation was observed for *A. solani sensu lato*. *Alternaria grandis* was not isolated from samples collected from the arid zone, although it represented 15% of isolates from the semi-arid zone and 17% of those from the Mediterranean zone. Only small-spored *Alternaria* isolates were obtained from samples in two locations, i.e. Ain Defla and Laghouat.

Pathogenicity and parasitic specialization of Alternaria species on potato and tomato

Fourteen isolates representing the four large-spored *Alternaria* species were used in the pathogenicity tests. These were: *A. solani* (isolates DA008, DA114, DA140 and DA141), *A. grandis* (isolates DA009, DA060, DA099 and DA152), *A. protenta* (DA062, DA082 and DA086) and *A. linariae* (DA002, DA007 and DA153). The symptoms (necroses surrounded by yellow halos) obtained on detached leaflets in Petri dish assays were confirmed by high degrees of aggressiveness obtained on whole seedling plants of potato and tomato (Figure 5). All the tested isolates produced symptoms on both plant species irrespective of their host plant of origin (Figure 6). The degrees of aggressiveness for each host/pathogen combination varied for the isolates, however. On average, *A. solani* isolates were more aggressive on both tomato and potato, while *A. protenta* isolates were less aggressive with a marked preference for their original host plants.

DISCUSSION

Early blight has long been attributed to large-spored *Alternaria* species, i.e. *A. solani* and *A. grandis* on potato and *A. solani* and *A. linariae* on tomato. Small-spored species related to *A. alternata* are considered to be responsible for the brown spot disease (Nolte, 2008, Tymon *et al.*, 2016). The surveys carried out in Alge-

ria between 2012 and 2015 have shown the presence of several *Alternaria* species producing large conidia in samples from both hosts with typical early blight symptoms, as well as in samples with symptoms that resemble brown spot. On the other hand, many Algerian isolates of the section *Alternaria* collected by Bessadat *et al.* (2017) have caused typical early blight symptoms after artificial inoculations on tomato, and were therefore considered pathogenic but with low aggressiveness. This complicated the attribution of a given symptom to a given *Alternaria* species. Moreover, morphological similarities between species within the *Alternaria* section *Porri* precludes reliable identification at the species level based only on these criteria. In the present work, molecular tools were used to confirm the identity of large-spored isolates originating from tomato and potato leaves. The diagnostic species-specific primers developed by Gannibal *et al.* (2014) were successfully used to quickly characterized 147 isolates from a collection the 155 large-spored isolates, and to distinguish *A. linariae* from isolates that could correspond to either *A. solani*, *A. grandis* or *A. protenta*. No amplification signal was obtained for eight isolates either due to poor quality of the extracted DNA or because these isolates corresponded to another species within section *Porri*. Although never previously observed in Algeria, it has recently been reported that *A. macrospora* may be responsible for leaf spot of tomato in China (Zhang *et al.*, 2017). To discriminate between *A. grandis*, on one hand, and *A. solani* or *A. protenta*, on the other, we took advantage of sequence polymorphism within the calmodulin gene to develop a PCR-RFLP assay. Reliability of these diagnostic methods was checked by sequencing the calmodulin locus, and taken together they allowed us to accurately type more than 90% of the large-spored isolates without the need for nucleotide sequencing. Applying these tools to our isolates, we observed that *A. solani* and *A. linariae* predominate on their respective potato or tomato hosts in most of the surveyed areas. In agreement with recent observations (Bessadat *et al.*, 2017, Ayad *et al.*, 2018), the presence of *A. linariae* and *A. grandis* in Algeria, traditionally considered to be restricted, respectively, to either tomato or potato, was also confirmed on these two plant hosts. *Alternaria linariae* was found on potato and *A. grandis* on tomato in the coastal regions of Algiers and Tipaza, where these two crops often exist side by side or follow each other in the same plot during the seasons. *Alternaria grandis* has not been isolated from the Saharan regions that are characterized by warm and arid periods during each year. Analyses of nucleotidic polymorphism at the RPB2 locus has shown that *A. protenta* was recorded for the first time on tomato in the coastal region of Mostaganem (northwest of Algeria), where the climate is very different from that of the South. This species has already been reported on potato in Belgium (Landschoot *et al.,* 2017), but also in a particular region of the south of Algeria (El Oued), where the temperatures are high and the potato crops are grown under overhead irrigation (Ayad *et al.*, 2017).

Cross inoculations carried out with isolates representing the four identified large-spored *Alternaria* species (*A. solani*, *A. grandis*, *A. linariae* and *A. protenta*) on potato and tomato, showed that aggressiveness toward the two host plants varied according to the isolates but not with respect to particular fungal species. Similar experiments carried out on detached leaves with *A. linariae* and *A. grandis* (Rodrigues *et al.*, 2010; Cardoso, 2014; Gannibal *et al.*, 2014) have previously shown that these two species were able to cause disease on both plants with more severe symptoms on their respective traditional hosts. This suggests that the parasitic specialization of large-spored *Alternaria* species on solanaceous crops should be reconsidered.

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