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Full Length Article

Morphological and Molecular Characterization of Large-spored *Alternaria* Species Associated with Potato and Tomato Early Blight in Egypt

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Abstract

Early blight incurs huge losses to solanaceous crops at both pre- and post-harvest stages by reducing yields up to 35–78%. Large-spored *Alternaria* spp. were isolated and characterized from the symptomatic tomato and potato plants. Morphological characterization revealed heterogeneity in the different traits of the isolates, which were subsequently confirmed by molecular characterization and two different *Alternaria* spp. *viz. A. solani* and *A. linariae* were characterized. A multi-locus phylogenetic analysis was conducted to infer the taxonomic position of the isolates within *Alternaria* spp. The five conserved genome regions used to infer phylogenetic lineage and evolutionary relationship were the internal transcribed spacer (ITS), the translation elongation factor (*TEF*), the glyceraldehyde-3-phosphate dehydrogenase (*GADPH*), the major allergen Alt a1 (*Alt a1*) and the RNA-polymerase 2 (*RPB2*) genes. All *A. linariae* isolates were closely related and formed a clade to *A. linariae* isolated from the United States. Similarly, *A. solani* isolates were closely related to each other and formed an independent clade indicating their unique nature. Koch's postulates were fulfilled for all the isolates. Among six different tested fungicides, aveet (azoxystrobin 5% + mancozeb 70%) inhibited the mycelial growth the most and saver (cymoxanil 5% + chlorothalonil 37%) the least. All the isolates infected tomato and potato leaves with varying severity except *A. linariae* isolate Egy-T2 was non-pathogenic on potato leaves, while the rest of *A. linariae* isolates were more aggressive. Here, we demonstrated that early blight in Egypt is caused by two different species of fungal pathogens, *A. linariae* and *A. solani*, and reported isolation of *A. linariae* from Egypt. © 2021 Friends Science Publishers

Keywords: *Alternaria linariae; Alternaria solani*; Early blight; Multi-locus phylogeny; Potato; Tomato

Introduction

Potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) are the most important vegetable cash crops in the world. Potato is the world's third most-produced crop with an annual production of 368.17 million metric tons (MMT) (FAOSTAT 2018) and is grown on all continents except Antarctica (Birch *et al*. 2012). Tomato is the world's largest produced vegetable crop with 182.26 MMT annual production (FAOSTAT 2018) and its cultivation area has increased by 164% in the last 40 years with an increase of 314% in its consumption (Nicola *et al.* 2009). Potato is Egypt's second most cultivated crop with an annual production of about 5 million tons (FOASTAT 2018), while tomato is Egypt's top-ranked horticulture crop in terms of production and the area under cultivation (ca 161702 hectares) (FAOSTAT 2018). Globally, Egypt is the 5th largest producer of tomatoes in the world with an annual production of 6.62 MMT (Siam and Abdelhakim 2018; Abdel-Motaal *et al.* 2020a). Both potatoes and tomatoes are Egypt's important sources of food, feed, livelihood and foreign exchange, with annual exports (both fresh and processed) amounting to 90,000 million tonns and 674,480 tons, respectively (Siam and Abdelhakim 2018). Nonetheless, the production of tomatoes and potatoes in Egypt is threatened by several biotic stresses, among which early blight disease is a leading threat to potatoes and tomatoes production. The disease is responsible for considerable yield losses worldwide and accounts for up to a 78% reduction in yields (Gannibal *et al.* 2014; Adhikari *et al.* 2017). Early blight disease can infer losses of up to 20% to potatoes production and 78% to tomatoes production

(Rotem 1994; Gannibal *et al.* 2014). Early blight induces characteristics symptoms of dark-colored spots, which are necrotic in the middle with a unique pattern of concentric rings, ridges and lesions on the leaves but can also spread to stem, twigs and fruits (Grigolli *et al*. 2011). The onset of symptoms initially appears on the basal and mature leaves as small, irregular or circular brown zonate lesions. These symptoms progress gradually to the upper leaves and then turn into dark colored spots with a characteristic "target-spot appearance" (Weber and Halterman 2012; Zheng *et al*. 2015). Severe infection can lead to twig drying, defoliation and premature fruit falling under favourable conditions (Khan *et al.* 2018) and the severity of infection usually turns into an epidemic in semi-arid areas with frequent and prolonged night dew (Rotem and Reichert 1964).

Alternaria solani was initially considered the sole species inducing early blight disease in solanaceous crops (Chaerani and Voorrips 2006; Gannibal *et al.* 2014). However, a range of physiological characteristics such as pathogenicity, cultural characteristics, phenetic and genetic polymorphisms observed within *A. solani* populations (Weber and Halterman 2012) led to the taxonomic revision of *Alternaria* species from Solanaceous hosts (Simmons 2000). Consequently, several new species related to early blight disease of potatoes and tomatoes were described (Simmons 2000, 2007). In addition, small-spored *Alternaria* spp. associated with foliar disease of potatoes and tomatoes were demonstrated as *A. alternata*, *A. arborescens* and *A. tenuissima* (Simmons 2000). Nonetheless, besides sharing striking similarities, *A. solani* isolated from tomato was referred to as *A. tomatophila* (Simmons 2000). Recently, isolates belonging to this species have been recategorized to a new species and referred to as *A. linariae* (Woudenberg *et al*. 2014).

Most of the studies demonstrating fungal characterization relied on morphological identification coupled to the molecular characterization. Using morphological identification along with multi-locus phylogenetic analysis is considered as a robust approach for fungal characterization. Several studies encompassing the molecular characterization of *Alternaria* species have demonstrated different genetic loci to infer the genetic and evolutionary relationship (Lawrence *et al.* 2013; Woudenberg *et al.* 2013; Javaid *et al.* 2016). The most widely used genetic loci include ITS, *SSU rDNA*, *TEF*, *GADPH*, *RPB2* and *Alt a1* (Woudenberg *et al*. 2014, *et al.* 2015; Javaid *et al.* 2018).

The most common measure to control early blight disease is the application of fungicides. The most commonly used fungicides belong to the Quinone inhibitor group. Different fungicides such as azoxystrobin, mancozeb, difenoconazole, chlorothalonil and carbendazim have been reported to curb early blight disease (Rosenzweig *et al*. 2008; Horsfield *et al*. 2010; Odilbekov *et al*. 2019). However, these fungicides have a very specific mode of action that can lead to the development of fungicide resistance (Leiminger *et al*. 2014; Odilbekov *et al*. 2019), and therefore, it is recommended that alternative fungicides be used or combined with other fungicides having different modes of action.

Several *Alternaria* spp. have been found infecting potatoes and tomatoes in Egypt and the growing diversity of the *Alternaria* spp. has changed the paradigm. The study demonstrated here was therefore aimed at deciphering the EB pathogen associated with potato and tomato plants by their morphological and molecular characterization. Our results, to our best knowledge and literature surveyed, demonstrated the isolation of *A. linariae* from tomato plants that have not yet been reported earlier in Egypt. However, a complete survey and an epidemiological study is a prerequisite to evaluate the threat associated with *A. linariae* to tomatoes and potatoes production in Egypt.

Materials and Methods

Sampling and isolation of *Alternaria* species

Tomato leaves, fruits and potato tubers exhibiting early blight symptoms, like dark-colored necrotic spots with a unique pattern of concentric rings, were surveyed. A total of 140 symptomatic samples, 20 from each location, were collected during the 2018–2019 crop seasons from major crop growing areas *viz*., Aswan, Beheira, Beni-suef, Giza, Kafr El-Sheik, Qena and Sharqia of Egypt (Table 1). The collected samples were sliced into small pieces (approx.5×5 mm) around the lesion and then surface sterilized for 2 min (min) in sodium hypochlorite (2%) followed by excessive rinsing (2–3 times) with sterile distilled water (SDW). The cuttings were shifted onto Potato Dextrose Agar (PDA; Difco, Montreal, Canada) media supplemented with 100 mg L^{-1} streptomycin sulphate and incubated at 25 ± 1°C for 7 days. Growing colonies were observed on the isolated cuttings and, subsequently, colonies were sub-cultured onto PDA plates to yield purified culture. Pure culture of each grown isolate was obtained using the hyphal tip method and maintained at 4°C on PDA slants for subsequent study.

Morphological characterization of the large-spored *Alternaria* species

Purified fungal isolates were identified based on their morphological characters, including spore size, growth pattern and spore chain formation (Simmons 2007). Colony morphology (color and colony texture) was assessed on corn meal agar (CMA), malt extract agar (MEA), PDA and V8 juice agar (V8JA) media at 25°C for 10 days. To examine the spores, purified fungal isolates were grown on V8JA plates at 25°C for 7 days with 16 h of photoperiod. Aerial mycelia were removed and the colony surface was gently wounded to allow mycelia to produce spores and after 48 h, spores were examined (Langsdorf *et al.* 1990). A total of 50 fungal conidia were observed under a light

microscope (Leica, CME Microscope Model 1349522X, U.S.A.) using differential interference contrast illumination, photographs were recorded and edited in the Adobe Illustrator (v. 23.0.5, 2019).

DNA extraction, PCR amplification and sequencing

Total genomic DNA extraction from seven fungal isolates was performed after following an amended Dellaporta extraction method (Dellaporta *et al.* 1983). About 100 mg (fresh weight) of fungal mycelia were harvested by scraping the surface of the colony to extract the DNA. The quality and quantity of the extracted DNAs were assessed spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific, DE, U.S.A.) and adjusted to a concentration of 40 ng μL^{-1} in ultrapure water. The extracted DNA was aliquoted and stored at –80°C until further use.

Five partial regions of the isolated fungal genome, the internal transcribed spacer region (ITS) was amplified with ITS4 and ITS5 primers, the translation elongation factor 1-α gene (*Tef1*) with EF1-688F and EF1-1251R primers, the glyceraldehyde 3-phosphate (*GADPH*) with Gpd_F and Gpd_R primers, RNA-polymerase 2 (*RPB2*) with RPB2-6F/RPB2-7cR primers and the Alta1 region (*Alta 1*) with Alt-al-for and Alt-al-rev primers (Table 2). PCR amplification was achieved separately for each genomic region in a 25 μL reaction containing 1 μL of the fungal DNA extract (40 ng μL^{-1} of total DNA), 2.5 of 10 X PCR buffer (Biomatic, Life Technologies, U.S.A.), 2 m*M* $MgCl_2$, 1.5 μL of 10 μM of each primer, 2.5 μL of 10 m*M* dNTPs, 0.3 μL of 5U Taq DNA Polymerase and the reaction was completed to 25 μL with Nuclease-free water. All the PCR amplicons were completely sequenced (Macrogen Inc., South Korea).

Phylogenetic analysis

To infer the phylogenetic lineage and evolutionary relationship of the isolates, all the five genes (ITS, *TEF*, *GAPDH*, *Alta1* and *RPB2*) were initially BLASTn searched (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and *Alternaria* spp. showing the highest sequence similarity to the five genes were retrieved from GenBank for subsequent analysis (Table 1). Multiple sequence alignment was conducted using MUSCLE (Edgar 2004) and the evolutionary relationship was inferred by using the maximum likelihood (ML) algorithm after selecting the best fit Tamura-Nei (TN93+G+I) model (Tamura and Nei 1993) in MEGA X (Kumar *et al.* 2018). The phylogenetic dendrogram (1000 replicates of bootstrap) was constructed with an optimal tree having the highest log-likelihood -6928.26.

Pathogenicity test on detached leaves

Pathogenicity of the seven isolates of large-spored *Alternaria* spp. was evaluated under *in vitro* conditions by inoculating the detached leaflets of 30-days-old tomato (cv. Super Strain B) and potato (cv. Spunta) plants. Fully expanded disease-free young leaflets of tomato and potato plants grown under greenhouse conditions were used after surface sterilization with sodium hypochlorite (2%) and then washing with SDW. The spore suspension was prepared by pouring 10 mL of SDW into the sporulated isolates growing on V8JA plates, and the spores count was adjusted to 1 x 10^6 conidia/mL using a haemocytometer, then 20 μL droplet was inoculated on the adaxial sides of the leaflets, while 20 μL SDW droplet was used as a negative control. Each inoculation was replicated seven times and the whole experiment was repeated twice. Inoculated leaflets were placed on sterilized Petri dishes harboring wet sterilized tissue papers and incubated at 25°C and 80% relative humidity with 12 h of photoperiod in the growth chamber. The inoculated leaflets were observed on daily basis and the symptoms on the leaves were recorded after seven days. Eventually, the re-isolation of *Alternaria* spp. from the inoculated leaves was carried out on PDA media to confirm the Koch's postulate (Sharma *et al.* 2004). A 0-5-point disease severity scale was followed (Vakalounakis 1983) and the disease severity index (DSI) was calculated as "DSI% = [100 × sum of all rating scales]/(N × maximum disease scale), where n is the number of the infected leaflets and N is the total number of leaves.

Evaluation of selected fungicides on the isolated *Alternaria* spp.

The poisoned food technique (Nene and Thapliyal 1993) was used to assess the efficacy of six different commercially available fungicides against the isolated *Alternaria* spp. (Table 3). Recommended/standard quantity of each fungicide was added aseptically to the molten PDA agar (50°C) and control plates were devoid of any fungicide. The plates were inoculated aseptically with ten-day-old 5 mm mycelial disc and incubated at 25 ± 2°C until the mycelial growth of the tested isolate covered the entire media in the control plates. Four replicates of each inoculation were used and the experiment was repeated twice. The diameter of the colonial radial growth was recorded for each treatment, a mean of four replicates was calculated, compared to the control and percent inhibition of mycelial growth was determined using the formula given by (Vincent 1947):

$$I\ \%=[(C-T)/C]\ 100$$

Where; I = Inhibition (%),
C = the mean of colony growth (mm) of the tested isolate in untreated control plates
T = the mean of colony growth (mm) of the tested isolate in treated plates

Data analysis

To evaluate the pathogenicity of the fungal isolates and the effect of different fungicides, a completely randomized design was employed. ANOVA and least significant

Table 1: Details of the *Alternaria* spp. isolates from Egypt with reference isolates and their accession numbers used in the study

Fungus	Isolate Code	Host, plant organ	Origin	Collection date	GenBank accession No.				
					ITS	*Tef-1*	*GAPDH*	*Alt a 1*	*RPB2*
A. linariae	Egy-T1	*Solanum lycopersicum*, fruit	Giza- Egypt	2018	MT996270	MT996277	MT996256	MT996249	MT996263
A. linariae	Egy-T2	*S. lycopersicum*, leaf	Kafr el-sheikh	2018	MT996271	MT996278	MT996257	MT996250	MT996264
A. linariae	Egy-T3	*S. lycopersicum*, leaf	Aswan- Egypt	2018	MT996272	MT996279	MT996258	MT996251	MT996265
A. solani	Egy-T4	*S. lycopersicum*, leaf	Qena- Egypt	2018	MT996273	MT996280	MT996259	MT996252	MT996266
A. linariae	Egy-T5	*S. lycopersicum*, leaf	Sharqia- Egypt	2019	MT996274	MT996281	MT996260	MT996253	MT996267
A. linariae	Egy-T6	*S. lycopersicum*, leaf	Beheira- Egypt	2019	MT996275	MT996282	MT996261	MT996254	MT996268
A. solani	Egy-P1	*Solanum tuberosum*, tuber	Giza- Egypt	2019	MT996276	MT996283	MT996262	MT996255	MT996269
A. allii	CBS 107.28	*Allium cepa*, leaf spot	Puerto Rico	-	KJ718100	KJ718449	KJ717954	KJ718620	KJ718274
A. allii	CBS 225.76	*Allium porrum*, leaf	Italy	-	KJ718102	KJ718451	KJ717956	KJ718622	KJ718276
A. grandis	CBS 109158	S. *tuberosum*, leaf	U.S.A., Pennsylvania	-	KJ718239	EU130547	JQ646341	JQ646425	KJ718414
A. grandis	CBS 116695	S. *tuberosum*, leaf	U.S.A., Pennsylvania	-	KJ718241	KJ718587	KJ718070	KJ718748	KJ718416
A. limicola	CBS 483.90	*Citrus aurantiifolia*, leaf	Mexico, Colima	-	KJ718178	KJ718526	JQ646329	JQ646413	KJ718351
A. limicola	CBS 117360	*Citrus* spp.	Mexico, Jalisco	-	KJ718179	KJ718527	KJ718023	-	KJ718352
A. linariae	CBS 109156	*Solanum lycopersicum*, leaf	U.S.A., Indiana	-	KJ718183	KJ718531	JQ646347	JQ646431	KJ718356
A. multirostrata	CBS 712.68	*Richardia scabra*, floral bract	U.S.A., Georgia	-	KJ718195	EU130546	JQ646362	KJ718704	KJ718368
A. multirostrata	CBS 713.68	*Richardia scabra*, floral bract	U.S.A., Georgia	-	KJ718196	KJ718542	KJ718034	KJ718705	KJ718369
A. passiflorae	CBS 113.38	*Passiflora edulis*	Australia, South Queensland	-	KJ718207	KJ718553	JQ646353	JQ646437	KJ718380
A. passiflorae	CBS 166.77	*Capsicum frutescens*, leaf	New Zealand	-	KJ718208	KJ718554	KJ718044	KJ718716	KJ718381
A. solani	CBS 111.41	*Solanum aviculare*, leaf		-	KJ718237	KJ718583	KJ718067	KJ718744	KJ718411
A. solani	CBS 116442	*Vicia faba*	New Zealand	-	KJ718240	KJ718586	KJ718069	KJ718747	KJ718415
A. solani	CBS 109157	*Solanum tuberosum*	U.S.A.	-	KJ718238	KJ718585	GQ180080	KJ718746	KJ718413
A. brassicicola	CBS 118699	*Brassica oleracea*	U.S.A.	-	KC584259	KC584642	KC584103		KC584383

Table 2: Nucleotide sequence of the primers used in the study

Locus Name	Primer*	Sequence (5'-3')	Reference
ITS region	ITS4	TCCTCCGCTTATTGATATGC	White *et al.* (1990)
	ITS5	GGAAGTAAAAGTCGTAACAAGG	
Tef-1	EF1-688F	CGGTCACTTGATCTACAAGTGC	Alves *et al.* (2008)
	EF1-1251R	CCTCGAACTCACCAGTACCG	
GADPH	Gpd_F	CAACGGCTTCGGTCGCATTG	Berbee *et al.* (1999)
	Gpd_R	GCCAAGCAGTTGGTTGTGC	
	Alt-al-for	ATG CAG TTC ACC ACC ATC GC	Hong *et al.* (2005)
Alt a 1	Alt-a1-rev	ACG AGG GTG AYG TAG GCG TC	
RPB2	RPB2-6F	TGG GGK WTG GTY TGY CCT GC	Liu *et al.* (1999).
	FRPB2-7cR	CCC ATR GCT TGY TTR CCC AT	

*F for the forward primer and R for the reverse primer

Table 3: Details of the used fungicides, their active ingredients and standard concentrations in *in vitro* evaluation

Fungicides No.	Trade name	Active ingredient	Conc. *
A	Aveet 75% WG	Azoxystrobin5% + mancozeb 70%	1 g L^{-1}
D	Decent plus 32% SC	Difenoconazole 12.5% + Azoxystrobin 20%	0.4 mL L^{-1}
C	Cuprofix CM	Cymoxanil 2.5% + Metalic copper 15%	1.5 g L^{-1}
R	Ridomil Gold R 26.89%WG	Metalaxyl M2% + Metalic copper 14.19%	3.3 g L^{-1}
P	Prevex-N72% SL	Propamocarb hydrochloride	3 mL L^{-1}
S	Saver 42% SC	Cymoxanil 5% + Chlorothalonil 37%	3 mL L^{-1}

* recommended dose

difference (LSD) tests were conducted to determine the statistical significance at $P < 0.05$. The collected data were statistically analyzed using the MSTAT-C program (v 2.10).

Results

Isolation and identification of *Alternaria* spp.

Microscopic examination of 63 purified isolates of *Alternaria* spp. revealed that 56 isolates were small spored with abundant sporulation on the PDA media and were morphologically assigned to *A. alternata* and *A. tenuissima* while 7 *Alternaria* isolates failed to produce spores on the PDA media. All the small-spored *Alternaria* isolates were excluded in this study, whereas different media including V8JA, CMA and MEA were used to yield spores from the remaining 7 *Alternaria* spp. Among the tested media, V8JA increased the mycelial growth followed by MEA, PDA and CMA, respectively, and sporulation was observed only on V8JA two days post mycelium removal. Whereas PDA and MEA media were best suited to induce colony pigmentation. Detailed microscopic examination of morphological characters on V8JA like sporulation patterns, conidia size and shape, and beak length revealed the presence of two groups of large-spored *Alternaria* spp. The identification of the purified isolates was achieved after following the set-criteria for the sporulation of the *Alternaria* genus. The spores of *Alternaria* isolates in this

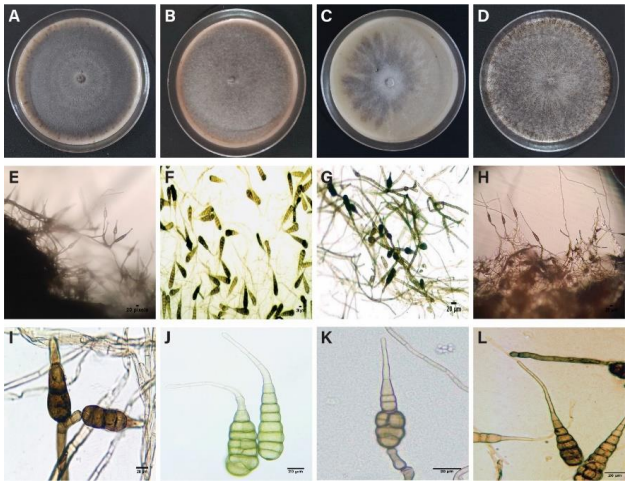

Fig. 1: Colonies, sporulation patterns and conidia of four *Alternaria* spp. isolates. Colonies of Egy-P1 (**A**), Egy-T1 (**B**), Egy-T3 (**C**), and Egy-T4 (**D**) observed on V8J agar plates; their sporulation patterns (**E-H**), and their conidia (**I-L**)

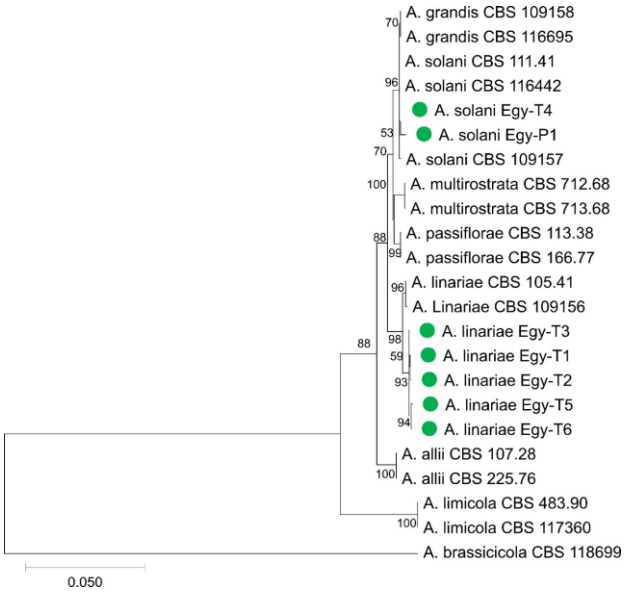

Fig. 2: Phylogenetic tree illustrating the relationship between *A. solani* and *A. linariae* isolates (highlighted with a green circle) and other *Alternaria* spp. The phylogenetic tree is based on the nucleotide sequences of five genes (ITS, *TEF*, *GAPDH*, *ALT* and *RPB2*) and was produced in MEGAX using the maximum-likelihood method and the best-fit model TN93+G+I. Only bootstrap values greater than 50% are shown

study exhibited a high-level of resemblance to the large-spored *Alternaria* spp. associated with tomato and potato groups and accepted as *A. solani* and *A. linariae*. The isolates produced olive green and brown conidia with conidium ellipsus body shape and different numbers of cells per conidium (Table 4). Conidia were generally individual, rarely in short chains, straight or slightly curved, or with conidium ellipsus body tapering to an average beak length 27.91–135.01 μm. Conidia of *A. linariae* produced beak length ranged from 64.09 to 135.01 μm, while body length ranged from 33.64 to 136.22 μm with a width of 17.20 to 28.51 μm (Fig. 1 and Table 4). While two isolates of *A. solani* beak length ranged from 27.91 to 116.06 μm, with a conidium, the total length of 70.63 to 77.61 μm and width of 16.94–25.05 μm (Table 4). Colonies were thick grey to black with scattered aerial mycelium, often branched and simple conidiophores with multi-septate conidia constitute 0–7 transverse septa and 2–10 longitudinal septa.

Molecular characterization of the large-spored *Alternaria* spp.

To characterize two different types of conidia, all isolates were subjected to molecular characterization by DNA-sequencing. PCR amplicons of highly-variable to moderately variable genes, ITS (~550 bp), *Tef-1* (~220 bp), *GADPH* (~580 bp), *Alt a1* (~450 bp) and *RPB2* (~760 bp) were achieved from all isolates and then completely sequenced. All sequences were submitted to NCBI GenBank database (accession numbers mentioned in Table 1) and BLASTn searched for the most closely related sequences. To infer the phylogenetic lineage and evolutionary relationship of the isolates, a multi-locus phylogeny was performed and sequences of only those *Alternaria* strains, whose all five genes sequences were available at GenBank, were included in the analysis, while *A. brassicola* (CBS 118699) was used as an outlier to validate the tree (Fig. 2). Neighbor-joining (NJ) and maximum likelihood (ML) algorithms produced similar tree topologies but ML produced clades with higher bootstrap values, therefore ML tree is shown. The isolates namely *A. solani* (Egy-P1 and Egy-T4) were closely related and grouped with *A. solani* CBS 116442 (an isolate from Netherland). The other isolates namely *A. linariae* (Egy-T1, Egy-T2, Egy-T3, Egy-T5 and Egy-T6) formed a multiphyletic group together and were closely related to other isolates of *A. linariae* (CBS 109156 [isolated from the United States] and CBS 105.41[isolated from Netherland]). Within the *A. linariae* group, a clear split was observed that formed 3 subgroups with moderate-to-high bootstrap values (Fig. 2).

Pathogenicity tests on detached leaves

All the tested isolates of *A. linariae* and *A. solani* produced early blight symptoms on tomato and potato detached leaves irrespective of their host plant origin. While control leaflets, inoculated with sterile distilled water remained asymptomatic (Fig. 3 and 4). However, a wide range of pathogenesis was observed when symptoms were evaluated (Table 5). Initially, the symptoms appeared as yellow flecks on the 4th day of inoculation, which then increased in size and became sunken lesion. Subsequently, the lesions

Table 4: Morphological characteristics of *A. linariae* and *A. solani* isolates

Isolate Code	species	Colony colour*	Body**		Beak	Septa	
			Length µm	width µm	length µm	Long.	Tran.
Egy-T1	*A. linariae*	Greyish to black with red pigment	98.84	22.46	111.82	6-10	1-7
Egy-T2	*A. linariae*	Greyish-white, - No pigmentation	106.13	20.93	104.76	4-9	0-4
Egy-T3	*A. linariae*	dark grey to black with pale pigment	33.64	17.20	64.09	3-5	1-2
Egy-T4	*A. solani*	dark grey to black with dark pigment	77.61	16.94	116.06	5-11	3-7
Egy-T5	*A. linariae*	Greyish to black with red pigment	113.18	21.27	117.77	4-7	0-4
Egy-T6	*A. linariae*	Greyish to black with red pigment	136.22	28.51	135.01	6-10	2-6
Egy-P1	*A. solani*	Black with pale red pigment	70.63	25.05	27.91	2-5	0-3

* colony colour was recorded on fungal culture grown on V8JA at 25+ 2°C for 10 days
** measurement of conidial structure

Table 5: Pathogenicity test of *A. solani* and *A. linariae* isolates on potato and tomato detached leaves under *in vitro* conditions

Alternaria species	Isolates Code	Disease severity index (DSI%)	
		Tomato leaves	Potato leaves
A. linariae	Egy-T1	54.3	37.1
A. linariae	Egy-T2	68.6	0.0
A. linariae	Egy-T3	45.7	11.4
A. solani	Egy-T4	22.9	17.1
A. linariae	Egy-T5	51.4	51.4
A. linariae	Egy-T6	65.7	42.9
A. solani	Egy-P1	37.1	45.7
LSD		0.685	0.52

Table 6: Effect of different fungicides on the mycelial growth of *A. linariae* and *A. solani* isolates

Isolate Code	Fungicides													
	Inhibition of mycelial growth (%)													
	(A) Aveet		(D) Decent plus		(C) Cuprofix CM		(R) Ridomil gold R		(P) Prevex		(S) Saver		Control	
	G* mm	I%**	G mm	I%	G mm	I%	G mm	I%	G mm	I%	G mm	I%	G mm	I%
Egy-T1	0	100	0	100	29	67.8	38	57.8	31	65.6	81	10	90	0
Egy-T2	5	94.4	12	86.7	43	52.2	18	80	70	22.2	68	24.4	90	0
Egy-T3	0	100	0	100	46	48.9	40	55.6	78	13.3	90	0	90	0
Egy-T4	0	100	0	100	53	41.1	27	70	79	12.2	88	2.2	90	0
Egy-T5	0	100	19	78.9	60	33.3	28	68.9	50	44.4	74	17.8	90	0
Egy-T6	12	86.7	12	86.7	50	44.4	25	72.2	82	8.9	90	0	90	0
Egy-P1	0	100	17	81.1	12	86.7	23	74.4	90	0	85	5.6	90	0
LSD	Fungi= 4.853		fungicide =4.292		Fungi X fungicide= 10.51									

* growth of *Alternaria* isolates (mm) on PDA media (mean of four replicates)
**Inhibition of the mycelial growth (%)

progressed to a grayish tint in the center surrounded by a yellow halo. All five isolates of *A. linariae* were virulent to tomato leaves with the DSI ranged from 51.4 to 68.6% and 11.4 to 51.4% on potato leaves (Table 5). *A. linariae* isolate Egy-T2 was found to be the most aggressive isolate on tomato leaves as it induced more severe symptoms with the highest disease severity index 68.6%, while it did not exhibit any pathogenicity on potato leaves and the inoculated leaves remained asymptomatic. Two of the isolates of *A. solani* (Egy-T4 and Egy-P1) produced typical early blight symptoms on both tomato and potato leaves. Egy-P1 was more severe on both tomato and potato leaves than Egy-T4 whereas DSI of Egy-T4 was 22.9% on tomato leaves and 17.1% on potato leaves, while DSI of Egy-P1 was 37.1% on tomato leaves and 45.7% on potato leaves. Generally, two different patterns of symptoms were induced by *A. solani* and *A. linariae* isolates. For instance, *A. linariae* induced wide and irregular lesions that covered most of the expanded area of the inoculated leaves, while *A. solani* produced limited lesions around the inoculated areas (Fig. 4). All *Alternaria* isolates were re-isolated successfully from all leaf lesions to fulfill Koch's postulate. Generally, all *A. linariae* isolates were more aggressive on tomato than potato leaves as compared to *A. solani* isolates.

Evaluation of the inhibitory effect of selected fungicides on the isolates

The inhibitory effect of different commercially available fungicides (Aveet, Decent plus, Cuprofix CM, Ridomil Gold R, Prevex, Saver) on the hyphal extension and colony growth of seven *Alternaria* isolates was investigated (Table 6). All the tested fungicides had significant delaying or inhibiting effect on the mycelial growth of *Alternaria* spp. However, Aveet 75% WG (azoxystrobin 5% + mancozeb 70%) had strong inhibitory effect and was able to reduce the growth of all the isolates ranging from 86.7 to 100%, followed by Decent plus (difenoconazole 12.5% + azoxystrobin 20%) whose inhibitory effect ranged from 78.9 to 100%, Ridomil Gold R (metalaxyl M 2% + metalic copper 14.19%) inhibited from 55.6 to 80.0% and Cuprofix CM (cymoxanil 2.5% + metalic copper 15%)

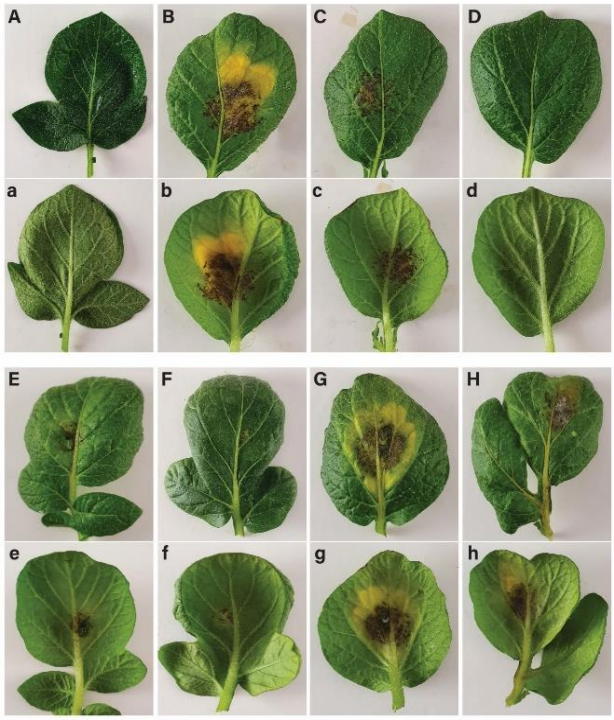

Fig. 3: Early blight symptoms exhibited by all the isolates on potato detached leaves. Both sides of the leaves, adaxial sides (mentioned in capital letters) and abaxial sides (mentioned in small letters) are shown. The leaves were either non-inoculated (**A**, **a**) or inoculated with Egy-P1 (**B**, **b**), Egy-T1 (**C**, **c**), Egy-T2 (**D**, **d**), Egy-T3 (**E**, **e**), Egy-T4 (**F**, **f**), Egy-T5 (**G**, **g**), and Egy-T6 (**H**, **h**). The experiment was conducted using detached of 30-day-old plants of potato (cv. Spunta). Leaves were photographed at 7 days post-inoculation and disease severity (DS) was scored on a 0-5-point rating system

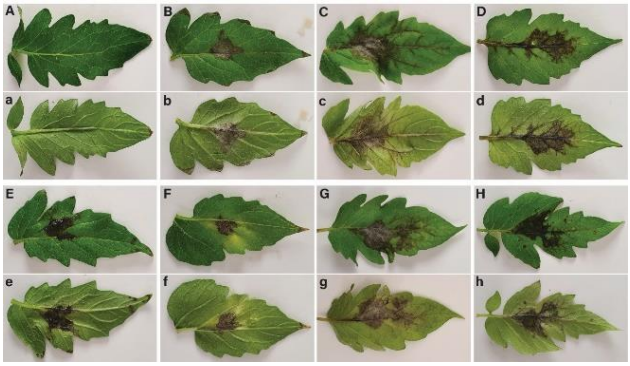

Fig. 4: Early blight symptoms exhibited by all the isolates on tomato detached leaves. Both sides of the leaves, adaxial sides (mentioned in capital letters) and abaxial sides (mentioned in small letters) are shown. The leaves were either non-inoculated (**A**, **a**) or inoculated with Egy-P1 (**B**, **b**), Egy-T1 (**C**, **c**), Egy-T2 (**D**, **d**), Egy-T3 (**E**, **e**), Egy-T4 (**F**, **f**), Egy-T5 (**G**, **g**), and Egy-T6 (H, h). The experiment was conducted using detached of 30-day-old plants of tomato (cv. Super strain B). Leaves were photographed at 7 days post-inoculation and disease severity (DS) was scored on a 0-5-point rating system

from 33.3 to 67.8%, respectively. Nonetheless, Prevex (Propamocarb hydrochloride) and Saver (cymoxanil 5% + chlorothalonil 37%) had the least effect on the mycelial growth of *Alternaria* isolates. All the tested isolates exhibited a small degree of variations in mycelial growth towards the tested fungicides.

Discussion

Alternaria spp. are not only a major limiting factor to tomatoes and potatoes production, but also causes postharvest spoilage to tomatoes and potatoes (El-Mougy and Abdel-Kader 2009; Adss *et al.* 2017). Several *Alternaria* spp. have been found infecting potatoes and tomatoes in Egypt, including *A. arborescens*, *A. alternata*, *A. phragmospora* and *A. solani* (Abada *et al.* 2008; El-Abd-Kareem *et al.* 2009; Ashour *et al.* 2012; El Gobashy *et al.* 2018; Hussein and Voigt 2019; Attia *et al.* 2020). Among these, *A. solani* is the most prevalent and leading cause of EB and *A. alternata* is the most prevalent species associated with leaf blight, whereas *A. arborescens* has been found to be the least prevalent in Egypt (El Gobashy *et al.* 2018; Hussein and Voigt 2019). A very recent study reported the first identification of *A. phragmospora* causing early blight disease in tomato plants (Abdel-Motaal *et al.* 2020a, b). The results of the study demonstrated that the population structure of large-spored *Alternaria* spp. associated with early blight disease in Egypt has a more complex structure than previously observed. The first identification of *A. linariae* from tomatoes and the identification of *A. solani* from tomatoes and potatoes suggest that the mounting scenario could be alarming. However, this is far from a conclusion, as this study was based on a few isolates, but the identification of *A. linariae* from most of the collected tomato samples from different areas demonstrated a changing paradigm. Both of these *Alternaria* spp. have previously been found associated with early blight disease of tomatoes and potatoes in Russia and Algeria (Gannibal *et al.* 2014; Ayad *et al.* 2019).

As the morphological characterization of *Alternaria* spp. is a challenging and often misleading task due to the striking similarities among the conidia of several species and the cultural features of some *Alternaria* spp., such as *A. solani*, are unstable (Rotem 1994). Similarly, at the molecular level, the use of a single gene region is insufficient to distinguish various *Alternaria* spp. or even the same group of individual isolates (Woudenberg *et al.* 2013, 2015; El Gobashy *et al.* 2018). To circumvent this problem, a multi-locus phylogeny was followed which demonstrated that the use of multiple genes, such as *GAPDH*, *ITS*, *RPB2*, *OPA10*-2, *Alt a 1*, *endoPG* and *KOG1058*, could separate all the isolates and species belonging to *Alternaria* (Woudenberg *et al.* 2014). Therefore, the same strategy was followed in this study and five genes (ITS, TEF, GAPDH, ALT and RPB2) were included which revealed a higher genotype level in *Alternaria* spp. and easily distinguish our isolates.

A comparative study with representative strains helped to ascertain that the two *Alternaria* spp., *A. linariae* and *A. solani*, are found associated with tomato and potato plants. A varying degree of coloration was exhibited by our isola *A. linariae* and *A. solani* on different media. Morphological characters of *A. solani* or *A. linariae* on V8JA media were quite close to those defined by Simmons (2007) and V8JA was the only media that induced sporulation of all the isolates. To achieve closely related morphological identification, the beak length and its branching and conidial body size were studied. Sporulation was conducted on V8JA plates to distinguish the two species, but it is important to take into account that the conidia size and shape depend on the used substratum. Compared to V8JA, both species produced two types of beaks, long and short. Similar findings have been appraised for other large-spored *Alternaria* species, for example, *A. calendulae* (Simmons 2007). Cultural characters have been used previously to identify different *Alternaria* spp. especially *A. solani* populations (Ivanuk and Palilova 1996), additionally, it is noteworthy that the cultural characters of *A. solani* are unstable (Rotem 1994).

Pathogenicity assay and cross-inoculation of detached tomato and potato leaves revealed that *A. linariae* infected all the inoculated tomato and potato leaves except Egy-T2 isolate which was avirulent on the potato. Variation of severity between all the tested isolates was observed indicating that there are different types of aggressiveness in the Egyptian population of *Alternaria* spp. However, there was a strong infection and association with tomato plants as the induced symptoms were more severe. *A. linariae* was able to infect the leaves of the potato, but with less aggressiveness than the tomatoes. This may indicate that *A. linariae* isolates are acclimatizing rapidly under Egyptian environmental conditions. While *A. solani* also infected both tomatoes and potato leaves with a moderate degree of aggressiveness. These results are consistent with earlier reports in which *A. linariae* showed strong association and aggressiveness with tomato leaves but weak with potato leaves (Rodrigues *et al.* 2010; Gannibal *et al.* 2014). However, *A. solani* was moderately aggressive to tomato and potato leaves (Ivanuk and Palilova 1996; Gannibal *et al.* 2014). In contrast to our observation, *A. solani* and *A. linariae* have been found infecting their respective potato or tomato hosts in Algeria, but cross-inoculations on detached leaflets had shown that the aggressiveness towards the two host leaves varies depending on the isolate but not on the specific fungal species (Ayad *et al.* 2019). Thus, our results are congruent to earlier studies that both two *Alternaria* spp. can infect both tomatoes and potato plants but exhibit severe symptoms in their respective hosts.

Early blight disease is prevalent wherever potatoes and tomatoes are cultivated. The disease is considered difficult to control since only a few cultivars having a strong resistance are available (Xue *et al.* 2019). The most effective control measure is the frequent use of fungicides at the early stage of the growing season (Horsfield *et al.* 2010; Odilbekov *et al.* 2019). An effective control strategy is a key prerequisite for achieving a laconic control over early blight disease. This research was further extended to evaluate the inhibitory effects of six commercial fungicides on the growth of *A. linariae* and *A. solani* isolates under in vitro conditions. Although all of the tested fungicides significantly inhibited mycelial growth, but the systemic fungicide, Aveet 75% WG (azoxystrobin 5% + mancozeb 70%), substantially inhibited the colony growth up to 100% in five of the seven tested isolates of *A. solani* and *A. linariae*. The next most effective fungicide was Decent plus 32% SC (Difenoconazole 12.5% + Azoxystrobin 20) which inhibited colony growth ranging from 78.9 to 100%. Difenoconazole, along with boscalid and pyraclostrobin, was reported to exhibit better performance against *A. solani* in 2014 but not in 2017, later the underlying reason for poor performance was found to link with mutations and the lower infection pressure in 2017 (Odilbekov *et al.* 2019). The protective mancozeb and the systemic azoxystrobin and difenoconazole materials are widely used fungicides against *A. solani* in Solanaceous crops (Rosenzweig *et al.* 2008; Horsfield *et al.* 2010). The current finding confirms the reports of several earlier studies that reported the efficacy of Mancozeb on inhibiting the growth of *A. solani* isolated from tomato (Gondal *et al.* 2012; Singh *et al.* 2018). Wang *et al.* (2008) showed that sixty isolates of *A. solani* were sensitive to difenoconazole using the mycelial growth method. Horsfield *et al.* (2010) also reported that azoxystrobin and difenoconazole were highly effective in controlling EB under field conditions.

The symptoms induced by *A. solani* and *A. linariae* isolates on the detached leaves of potatoes and tomatoes did not differ significantly from that of the infected tomato and potato plants collected from the field. Similar results have been obtained for *A. tenuissima*, *A. alternata* and *A. solani* (Zheng *et al.* 2015). However, a few studies found contrasting results, where the symptoms induced by *A. alternata* and *A. solani* differ substantially from those of the early blight disease symptoms observed in field plants. The observed difference may be due to the detached leaf (DL) assay, as no correlation has been observed between the pathogenicity of the DLassay in greenhouse conditions and/or field conditions (Foolad *et al.* 2000; El Gobashy *et al.* 2018). However, both greenhouse and field tests have a clear correlation, suggesting that whole-plant inoculation is the best method for assessing the pathogenicity of *Alternaria* spp. associated with early blight disease. The reason behind this difference is unclear yet and requires further investigations.

Summarizing all our results, such as the aggressiveness and adaptability of *A. linariae* to tomato and potato plants along with aberrant climatic changes, we can suggest that *A. linariae*, either alone or with *A. solani*, could potentially cause substantial losses to tomato and potato production in Egypt. However, a comprehensive

disease-survey, assessment of the susceptibility of the available tomato and potato cultivars, synergistic interactions between *A. linariae* and *A. solani*, evaluation of pathogenicity under greenhouse conditions and characterization of *A. linariae* is a prerequisite to assess the mounting scenario.

Conclusion

The underlying pathogens associated with early blight disease in Egypt are found to be more diverse than previously recognized. A higher level of pathogenicity of *A. linariae* on potato and tomato plants revealed its potential to become a wide-spread and leading cause of early blight disease, either alone or combining with other *Alternaria* spp. Further studies are necessary to reveal the aggressiveness, distribution, symptoms pattern in the field and difference in fitness of the species.

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Author Contributions

SME and SEE isolated the pathogen and evaluated the fungicides. YA wrote the discussion section. ZI and SME performed molecular analysis. SME, SEE and YA drafted the first version of the manuscript. ZI draw figures and drafted the final manuscript which was approved by all the authors.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data will be made avaialble on acceptable requests to the corresponding author.

Ethics Approval

Not applicable.

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