



Phylogenetic, toxigenic and virulence profiles of *Alternaria* species causing leaf blight of tomato in Egypt

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Abstract

Species of *Alternaria* are serious plant pathogens, causing major losses on a wide range of crops. Leaf blight symptoms were observed on tomato leaves, and samples were collected from various regions. Isolation was done from symptomatic tomato leaves, and 15 representatives were selected from a collection of 65 isolates of *Alternaria* species. The virulence of *Alternaria* isolates was investigated on detached leaves (DL) and whole plants of tomato cv. Super strain B. A phylogenetic analysis was performed based on three partial gene regions, the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), the RNA polymerase second largest subunit (*RPB2*) and the *Alternaria* major allergen gene (*Alt a 1*). The potentiality of *Alternaria* isolates to produce toxins was also investigated on the basis of thin-layer chromatography (TLC). Our investigations revealed that *Alternaria* isolates showed different levels of virulence either on tomato plants or DL. Based on the phylogeny of three genes, *Alternaria* isolates encompassed two species of small-spored morphospecies: *A. alternata* (14 isolates) and *A. arborescens* (single isolate). The produced toxins varied among *Alternaria* isolates with tenuazonic acid (TeA) being the most abundant mycotoxin produced by most isolates. This study highlighted on other *Alternaria* species in Egypt that might represent a serious concern for tomato producers as causal agents of leaf blight over other species, i.e. *A. solani*.

Keywords *Alternaria* · Leaf blight · Mycotoxin · Pathogenicity · Tomato

Introduction

The genus *Alternaria* consists of approximately 280 species that are well-known plant pathogens on a wide range of plant hosts (Rotem 1994; Simmons 2007). Several *Alternaria* species have been associated with diseases of tomato and potato (Simmons 2000). The large-spored *Alternaria solani* Sorauer was described in 1896 as the causative agent of early blight (EB) on potato (*Solanum tuberosum* L.) (Sorauer 1896). The

subsequent study of Rands (1917) had suggested that this species was also a causal agent of EB on tomato (*Solanum lycopersicum* L.) and causes a foliar disease on eggplant (*Solanum melongena* L.). However, Simmons (2000) stated that the large-spored *A. cretica*, *A. tomatophila* and *A. subcylindrica*, the former species of (*A. linariae*), are considered as causal agents of EB of tomato in several parts of the world, i.e. the USA, Australia, New Zealand and Venezuela. *Alternaria grandis* is also reported to cause EB on tomato and potato in Brazil (Rodrigues et al. 2010). The small-spored species *A. alternata* is also known as a causative of stem canker of tomato (Grogan et al. 1975; Malathrakakis 1983), leaf spot and other diseases on over 100 host species of plants (Rotem 1994). Additionally, it was also reported to cause EB of tomato in Pakistan (Akhtar et al. 2004) and affect American potato production (Nolte 2008). Also, small-spored *A. arborescens* is reported to cause stem canker of tomato (Simmons 2000; Woudenberg et al. 2015) and potato (Tymon et al. 2016). Recently, *A. alternata* has been reported to cause potato EB in Hebei province of China (Fan et al. 2013).

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The molecular taxonomy and the phylogenetic relationship among *Alternaria* species have been extensively reported (Hong et al. 2005; Andrew et al. 2009; Lourenco et al. 2009; Ozkilinc et al. 2017, 2018). The genetic markers, ITS and SSU rDNA regions poorly resolved the *Alternaria* species into groups (Pryor and Gilbertson 2000). On the other hand, the protein-coding gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is considered as more informative than ITS and mtSSU regions (Hong et al. 2005). However, one of the most informative genes is *Alt a 1* gene, which encodes the major allergen produced by *A. alternata* and in other species of the genus *Alternaria* (Hong et al. 2005). For *Alternaria* species, the *Alt a 1* and *GAPDH* sequences supported the separation of groups of *Alternaria* spp. and related taxa into several species-groups, but closely related species were not fully resolved and other polymorphic genomic regions need to be analyzed (Hong et al. 2005). However, the latest molecular revisions of Lawrence et al. (2013) introduced two new species groups, *A. panax* and *A. gypsophilae*, and elevated eight species-groups to sections within *Alternaria*. In a further study, Woudenberg et al. (2015) revised the small-spored *Alternaria* species complex, i.e. *A. arborescens*, *A. alternata* and *A. tenuissima* groups and synonymised 35 morphospecies under *A. alternata*. Recent studies of Stewart et al. (2014) and Huang et al. (2015) presented also a novel phylogenetic reconstruction of *A. alternata*. These contemporary studies demonstrated the power of phylogenetic approaches to explore divergence among populations of fungal plant pathogens that lack a known sexual stage and may discover cryptic species, independently evolving lineages (Ozkilinc et al. 2018).

Species of *Alternaria* are well known for the production of a variety of about 70 toxic secondary metabolites (Loggrieco et al. 2009). Some of these metabolites are; the dibenzo- α -pyrones altenuene (AE), alternariol (AOH), alternariol monomethyl ether (AME) and a derivate of tetramic acid and tenuazonic acid (TA) (Loggrieco et al. 2003; Ostry 2008). A number of different analytical methods have been applied to mycotoxin analysis due to their varied structures. These include thin-layer chromatography (TLC), gas chromatography (GC) and liquid chromatography (LC) (Fernández-Cruz et al. 2010). However, high-performance liquid chromatography (HPLC) is the most widely used method for the detection of *Alternaria* toxins (Patriarca et al. 2007; Ostry 2008). Atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), LC–mass spectrometry (MS) and LC–MS/MS have been also applied for the detection of AOH and AME in apple juices and other fruit beverages (Lau et al. 2003).

Based on the literature search, previous studies reported that *A. alternata* causes diseases on different hosts in Egypt, i.e. leaf blight of water hyacinth (Shabana et al. 1995) and leaf blight of jojoba (Ibrahim et al. 2017). It was also isolated from black sand (Sharaf 2005), and from Red Sea in Egypt (Hawasa et al. 2015), and reported to be a principal pathogen of

potatoes in Egypt (Droby et al. 1984). To date, the only study found in the literature of Gherbawy et al. (2018) elucidated the genetic diversity of *A. alternata* population isolated from Upper Egyptian tomato fruits. However, this study did not include the toxigenic profile of the isolated *A. alternata*. To our knowledge, this paper describes the first attempt to study the genetic identity and toxigenic profile of *Alternaria* species causing leaf blight of tomato in Egypt.

Therefore, the aim of the present study was to determine the genetic identity of *Alternaria* species isolated from tomato leaf blight samples based on the sequences of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), RNA polymerase second largest subunit (*RPB2*) and the *Alternaria* major allergen gene (*Alt a 1*). The present study supplemented with investigations of virulence, phenotypic and toxigenic profiles of isolated *Alternaria* species.

Materials and methods

Isolate collection and maintenance

Symptoms were observed on tomato leaves at the beginning as pale, brown to olivaceous brown spots surrounded by halo-chlorotic tissues. The spots gradually increased in size in a concentric manner and often coalesced, leading to a blighted appearance (Fig. 1). Symptomatic leaf blight samples from various regions and cultivars were collected during field survey trips conducted in the beginning of spring and summer seasons of 2015 and 2016. Samples were surface-sterilized by sequential immersion in 5% sodium hypochlorite and sterilized distilled water, then plated on PDA amended with 0.5 g streptopenicide, and incubated at 25 °C. Developing colonies were then picked up and sub-cultured on PDA for single-spore purification. The putative isolates resembling the morphology of the genus *Alternaria* were maintained as single-spore colonies and then initially identified according to the description of Simmons (2007). Cultures were then conserved on PDA in slant tubes for further studies.

Pathogenicity tests

Preparation of inoculum

Isolates of *Alternaria* were obtained from naturally infected tomato leaves, maintained on V-8 juice agar, and grown at ~ 25 °C under natural daylight/dark photoperiod 12 h each. After 6 days of growth, the conidia were dislodged from the agar surface by repeatedly flooding the plates with sterile distilled water containing five drops of Tween 20 per 100 ml. The resulting suspension was passed through double layers of cheesecloth into a flask. The concentration of the conidial

Fig. 1 Leaf blight symptoms on tomato leaves appeared at the beginning as small brown spots surrounded by yellow halo and gradually increased in size in a concentric manner and often coalesce, leading to a blighted appearance



suspension was adjusted to 2×10^4 spores/ml using a hemocytometer.

Greenhouse (GH) experiment

Tomato seedlings cv. Super strain B, 30 days old, were grown in plastic pots filled with sterilized soil. Three plants were used for each replicate, and four replicates were used per isolate. A 100-ml spore suspension (2×10^4 spores/ml) was sprayed and swabbed with a moist cotton pad onto leaves. The inoculated plants were then covered with polyethylene bags to maintain high (80%) relative humidity (RH). After 48 h, polyethylene bags were removed and the plants were kept in the greenhouse at 24 ± 2 °C. Control plants were sprayed with only sterile distilled water. Symptom development was monitored weekly. Re-isolations were made from infected plants for each isolate tested, and the cultures were compared with the original ones to confirm the identity of the pathogen. The experiment was conducted twice. The disease severity (DS) of leaf blight and the area under disease progress curve (AUDPC) were calculated by recording the data observed over time using a 0–5 modified scale of Saharan (1997) as follows:

- 0 No symptoms on leaf
- 1 Small, round, black dots covering up to 1% of the leaf area
- 2 Grey circular spots containing concentric rings to cover 1.1–10% of the leaf area
- 3 Lesions enlarge grey circular spots with concentric rings and black border to cover 10.1–25% of the leaf area
- 4 Lesions enlarge grey circular spots with concentric rings and black border to cover 25.1–50% of the leaf area

- 5 Lesions coalesced with concentric rings and black border to cover 50% of the leaf area

Detached-leaflets (DL) experiment

Fully expanded, apparently healthy young leaflets of tomato plants cv. Super strain B was detached and placed in an elevated polyhardware plastic mesh screen fitted in the moist plastic box. Each plastic box was $20 \times 10 \times 12$ cm with a lid that accommodated two leaflets from one plant. For each plastic box, 15 ml of sterilized distilled water was added to maintain high RH. Two leaflets were used as one replicate, and four replicates were used for each isolate. Leaflets were laid down and the pedicels were inserted into the water. A 50- μ l droplet of spore suspension was placed on the upper surface of each leaflet. The inoculated leaflets were incubated at room temperature (~ 25 °C under natural daylight/dark photoperiod 12 h each). The data were recorded starting on day 4; lesion size (length and width on perpendicular directions) on each leaflet was measured three times. The trial ended on day 10, and final radius of each lesion was calculated as one half of the mean of the two perpendicular diameters.

Data analysis

For the GH experiments, DS and AUDPC were calculated. For the DL experiment, the lesion expansion (mm) and chlorosis expansion (mm) for each isolate were also calculated. The data of AUDPC, DS, lesion radius and chlorosis were subjected to analysis of variance (ANOVA) using WASP

software, and the differences between means were compared using Fisher's least significant differences test (LSD).

Phenotyping analysis

Morphological descriptions were made for isolates grown on potato carrot agar (PCA) medium. Cultures were incubated at moderate temperatures (~25 °C) under cool white fluorescent light with 8-h/16-h photoperiod of light/dark for 7 days (Andersen et al. 2001). The sell tape technique was used for making slide preparations according to Crous et al. (2009) with Shear's medium as mounting fluid. The characteristic structures, i.e. conidia, conidiophores and beak, were measured for some representative isolates with a dissecting microscope according to Simmons (2007) and Tymon et al. (2016).

Molecular characterization

Isolate culturing and DNA extraction

Isolates were grown in 250-ml conical flasks containing 100 ml of Wickerman's medium (glucose 4%, malt extract 0.3%, yeast extract 0.3 and peptone 0.5%) and incubated at 25 °C under shaking (120 rpm). Two days later, liquid cultures were filtered on Whatman No. 4 filter paper, washed with distilled water, frozen and lyophilized. Approximately 15 mg of powdered lyophilized mycelia was used for DNA extraction using the Wizard Magnetic DNA Purification System for Food (Promega Corp., Madison, WI) following the manufacturer's protocol. Quantity and quality of DNA were checked using Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Fisher Scientific, Nepean, ON, Canada).

PCR amplification

The amplification of the *GAPDH* region was done with *gpd1* and *gpd2* (Berbee et al. 1999), the *RPB2* region with *RPB2–5F2* (Sung et al. 2007) and *FRPB2–7cR* (Liu et al. 1999), and *Alt a1* gene region was amplified with primers *alt-for* and *alt-rev* (Hong et al. 2005). The PCR mix and amplification conditions were done as described by Woudenberg et al. (2015). The PCRs were performed in a MyCycler™ Thermal Cycler in a total volume of 12.5 µl. Amplified PCR products were electrophoresed on 1.5% agarose gel for 50 min at 80 V in Tris-borate-EDTA (TBE) buffer and visualized via UV illumination.

DNA sequencing

PCR products were sequenced in both directions using the PCR primers and the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations, and analyzed with an ABI Prism 3730XL Sequencer

(Applied Biosystems, Foster City, USA) according to the manufacturer's instructions. Sequencing was done by the authors in the lab of the Institute of Sciences of Food Production, National Research Council, Bari, Italy.

Phylogenetic analysis

Consensus sequences were computed from forward and reverse sequences using the BioNumerics v. 4.61 software package (Applied Maths, St-Martens-Latem, Belgium). All generated sequences were deposited in GenBank (Table 1). The aligned sequences were manually checked and corrected where necessary, and ambiguous ends were excluded. The relatives of the sequences were determined with standard nucleotide BLASTn searches, with the *GAPDH*, *RPB2* and *Alt a1* reference sequences, against the nucleotide database in GenBank. Sequences for each gene region were individually analysed for conflict using 70% reciprocal NJ (neighbour-joining) bootstrap analysis, and the topology of the resulting trees was compared visually for inconsistency (Mason-Gamer and Kellogg 1996; Gueidan et al. 2007).

The maximum likelihood (ML) analysis was carried out with MEGA v.6 (Tamura et al. 2013). The optimal ML tree search was using the default algorithm of the program from a random starting tree for each run. The final tree was selected among suboptimal trees from each run by comparing likelihood scores under the GTR+GAMMA substitution model. The robustness of trees was evaluated by 500 bootstrap replications. Suitable models for Bayesian analysis were first selected using MrModeltest v. 2.2 (Nylander 2004) and included for each gene partition. Bayesian analyses were performed with MrBayes v. 3.1.1 (Huelsenbeck and Ronquist 2001) using the Markov chain Monte Carlo (MCMC) (Larget and Simon 1999) algorithm to generate trees with Bayesian probability values. Four chains were run simultaneously starting from random trees set at 100,000 generations, sampled every 100 generations, and 25% of trees were discarded as the burn-in phase. The temperature value was reduced to 0.1, burn-in was set to 0.2 and then the run was automatically stopped as soon as the average standard deviation of split frequencies reached below 0.01.

Mycotoxins analysis

The toxigenic potential of the 15 *Alternaria* spp. was investigated in vitro on liquid medium. The extraction and identification of mycotoxins were carried out following the method of Paterson and Bridge (1994). The 15 *Alternaria* isolates were transferred into a 250-ml Erlenmeyer flask containing 100 ml of potato-dextrose broth (PDB) medium and incubated for 21 days at 24 ± 2 °C. Cultural filtrates of isolates were collected and extracted three times with equal volume of chloroform to methanol (2:1). The extracts were evaporated until dryness and residues were dissolved in 1 ml of chloroform to

Table 1 Sequences of *Alternaria* species retrieved from GenBank and sequences obtained in this study

Species name	Strain number	Country	Host	Genbank accession numbers		
				GAPDH	RPB2	<i>Alt a 1</i>
<i>A. alstroemeriae</i>	CBS118808	USA	<i>Alstroemeria</i> sp.	KP124153	KP124764	KP123845
<i>A. alternantherae</i>	CBS124392	China	<i>Solanum melongena</i>	KC584096	KC584374	KP123846
<i>A. alternata</i>	CBS107.27	USA	<i>Citrus limonium</i>	KP124157	KP124768	KP123849
<i>A. alternata</i>	CBS127671	USA	<i>Stanleya pinnata</i>	KP124233	KP124851	KP123929
<i>A. alternata</i>	ALT-9	Egypt	<i>Lycopersicon esculentum</i>	MH054467	MH054482	MH054452
<i>A. alternata</i>	ALT-11	Egypt	<i>Lycopersicon esculentum</i>	MH054468	MH054483	MH054453
<i>A. alternata</i>	ALT-14	Egypt	<i>Lycopersicon esculentum</i>	MH054454	MH054469	MH054439
<i>A. alternata</i>	ALT-18	Egypt	<i>Lycopersicon esculentum</i>	MH054455	MH054470	MH054440
<i>A. alternata</i>	ALT-19	Egypt	<i>Lycopersicon esculentum</i>	MH054456	MH054471	MH054441
<i>A. alternata</i>	ALT-21	Egypt	<i>Lycopersicon esculentum</i>	MH054457	MH054472	MH054442
<i>A. alternata</i>	ALT-33	Egypt	<i>Lycopersicon esculentum</i>	MH054458	MH054473	MH054443
<i>A. alternata</i>	ALT-34	Egypt	<i>Lycopersicon esculentum</i>	MH054459	MH054474	MH054444
<i>A. alternata</i>	ALT-38	Egypt	<i>Lycopersicon esculentum</i>	MH054460	MH054475	MH054445
<i>A. alternata</i>	ALT-44	Egypt	<i>Lycopersicon esculentum</i>	MH054461	MH054476	MH054446
<i>A. alternata</i>	ALT-146	Egypt	<i>Lycopersicon esculentum</i>	MH054462	MH054477	MH054447
<i>A. alternata</i>	ALT-148	Egypt	<i>Lycopersicon esculentum</i>	MH054463	MH054478	MH054448
<i>A. alternata</i>	ALT-154	Egypt	<i>Lycopersicon esculentum</i>	MH054464	MH054479	MH054449
<i>A. alternata</i>	ALT-160	Egypt	<i>Lycopersicon esculentum</i>	MH054465	MH054480	MH054450
<i>Alternaria</i> sp.	ALT-164	Egypt	<i>Lycopersicon esculentum</i>	MH054466	MH054481	MH054451
<i>A. arborescens</i>	CBS112633	South Africa	<i>Malus domestica</i>	KP124252	KP124870	KP123947
<i>A. astragali</i>	CBS127672	USA	<i>Astragalus bisulcatus</i>	KP124234	KP124852	KP123930
<i>A. betae-kenyensis</i>	CBS118810	Kenya	<i>Beta vulgaris</i> var. <i>cicla</i>	KP124270	KP124888	KP123966
<i>A. brassicinae</i>	CBS118811	USA	<i>Brassica oleracea</i>	KP124210	KP124824	KP123904
<i>A. broussonetiae</i>	CBS121455	China	<i>Broussonetia papyrifera</i>	KP124220	KP124838	KP123916
<i>A. burnsii</i>	CBS110.50	Mozambique	<i>Gossypium</i> sp.	KP124271	KP124890	KP123968
<i>A. caudata</i>	CBS121544	USA	<i>Cucumis sativus</i>	KP124223	KP124841	KP123919
<i>A. cerealis</i>	EGS43-072	New Zealand	<i>Avena sativa</i>	JQ646321	KP124878	KP123955
<i>A. cinerariae</i>	CBS612.72	Germany	<i>Senecio cineraria</i>	KP124165	KP124777	KP123861
<i>A. citri</i>	CBS192.81	Egypt	<i>Citrus sinensis</i>	KP124170	KP124782	KP123867
<i>A. citriarbusti</i>	CBS102598	USA	<i>Minneola tangelo</i>	KP124184	KP124797	KP123878
<i>A. citricancri</i>	CBS119543	USA	<i>Citrus paradisi</i>	KP124215	KP124831	KP123911
<i>A. citrimacularis</i>	CBS102596	USA	<i>Citrus jambhiri</i>	KP124183	KP124796	KP123877
<i>A. daucifolii</i>	CBS118812	USA	<i>Daucus carota</i>	KC584112	KC584393	KP123905
<i>A. destruens</i>	CBS121454	USA	<i>Cuscuta gronovii</i>	AY278812	KP124837	JQ646402
<i>A. dumosa</i>	CBS109455	Canada	Human arm tissue	KP124189	KP124803	KP123883
<i>A. eichhorniae</i>	CBS489.92	India	<i>Eichhornia crassipes</i>	KP124276	KP124895	KP123973
<i>A. gaisen</i>	CBS118389	Japan	<i>Pyrus pyrifolia</i>	KP124259	KP124877	KP123954
<i>A. geophila</i>	CBS101.13	Switzerland	Peat soil	KP124244	KP124862	KP123940
<i>A. godetiae</i>	CBS117.44	Denmark	<i>Godetia</i> sp.	KP124160	KP124772	KP123854
<i>A. gossypina</i>	CBS100.23	Mozambique	<i>Gossypium</i> sp.	KP124271	KP124891	KP123969
<i>A. grisea</i>	CBS107.36	Indonesia	Soil	JQ646310	KP124901	JQ646393
<i>A. interrupta</i>	CBS102603	Israel	<i>Minneola tangelo</i>	KP124188	KP124801	KP123882
<i>A. iridialustralis</i>	CBS118404	New Zealand	<i>Iris</i> sp.	KP124283	KP124904	KP123980
<i>A. jacinthicola</i>	CBS878.95	Mauritius	<i>Arachis hypogaea</i>	KP124286	KP124907	KP123983
<i>A. limoniasperae</i>	BMP0316	USA	<i>Citrus jambhiri</i>	AY562411	KC584408	AY563306
<i>A. lini</i>	CBS106.34	Unknown	<i>Linum usitatissimum</i>	JQ646308	KP124771	KP123853
<i>A. longipes</i>	CBS113.35	Unknown	<i>Nicotiana tabacum</i>	KP124289	KP124910	KP123986

Table 1 (continued)

Species name	Strain number	Country	Host	Genbank accession numbers		
				GAPDH	RPB2	<i>Alt a 1</i>
<i>A. mali</i>	CBS106.24	USA	<i>Malus sylvestris</i>	KP124155	KP124766	KP123847
<i>A. nobilis</i>	CBS826.68	Germany	<i>Lolium</i> sp.	KP124164	KP124776	KP123860
<i>A. palandui</i>	CNU131062	USA	<i>Allium</i> sp.	KJ862255	KP124833	KJ862259
<i>A. pellucida</i>	CBS479.90	Japan	<i>Citrus unshiu</i>	KP124174	KP124787	KP123870
<i>A. perangusta</i>	CBS102602	Turkey	<i>Minneola tangelo</i>	KP124187	KP124800	KP123881
<i>A. platycodonis</i>	CBS121348	China	<i>Platycodon grandiflorus</i>	KP124219	KP124836	KP123915
<i>A. postmessia</i>	CBS119399	USA	<i>Minneola tangelo</i>	JQ646328	KP124829	KP124829
<i>A. pulvinifungicola</i>	CBS194.86	USA	<i>Quercus</i> sp.	KP124172	KP124784	KP123869
<i>A. sanguisorbae</i>	CBS121456	China	<i>Sanguisorba officinalis</i>	KP124221	KP124839	KP123917
<i>A. senecionicola</i>	CBS119545	New Zealand	<i>Senecio skirrhodon</i>	KP124260	KP124879	KP123956
<i>A. septoriodies</i>	CBS175.80	Italy	Unknown	JQ646324	KP124781	KP123866
<i>A. soliaegyptiaca</i>	CBS103.33	Egypt	Soil	KP124159	KP124770	KP123852
<i>A. tenuis</i>	CBS126910	USA	Soil	KP124231	KP124849	KP123927
<i>A. tenuissima</i>	CBS620.83	USA	<i>Nicotiana tabacum</i>	KP124171	KP124783	KP123868
<i>A. tenuissima</i>	CBS686.68	Sahara	Desert sand	KP124163	KP124775	KP123859
<i>A. tomaticola</i>	CBS118814	USA	<i>Solanum lycopersicum</i>	KP124211	KP124825	KP123906
<i>A. tomato</i>	CBS103.30	Unknown	<i>Solanum lycopersicum</i>	KP124294	KP124915	KP123991
<i>A. toxicogenica</i>	CBS102600	USA	<i>Citrus reticulata</i>	KP124186	KP124799	KP123880
<i>A. turkisafria</i>	CBS102599	Turkey	<i>Minneola tangelo</i>	KP124185	KP124798	KP123879
<i>A. vaccinii</i>	CBS118818	USA	<i>Vaccinium</i> sp.	KP124213	KP124827	KP123908
<i>A. yali-inficiens</i>	CBS121547	China	<i>Pyrus bretschneideri</i>	KP124224	KP124842	KP123920

methanol (2:1). TLC plates 20 × 20 cm (Merck aluminum sheet, silica gel 60 F₂₅₄, layer thickness 0.2 mm) were used by spotting each isolate at the same distance from the base and 2 cm apart from edges. The plates were developed for 17 cm in toluene to ethyl acetate to 90% formic acid (5:4:1) (TEF) in a solvent-saturated atmosphere using saturation pads. The *R_f* values of greater intensity spots and its color were recorded when the TLC plate was exposed to UV light (365 nm) (Betina 1993). Griseofulvin was developed with all TLC plates for adjustment of the *R_f* values of all fractions. On the other hand, altenuene standard was used for calculating the relative quantity of the isolated compounds in addition to the intensity of spots. The TLC plates were scanned at 254 and 365 nm using GAMAG TLC Scanner 3. Distinct peaks of separated metabolites were observed at 365 nm for isolates of *A. alternata*. Peak area (representing the quantity of each spot) for standard and metabolites was measured, and quantities of metabolites were obtained according to amount and peak area of standard (altenuene).

The method of Maiero et al. (1991) was followed for detecting alternaric acid by spraying the TLC plate with 3 g vanillin dissolved in 100 ml ethanol + 3 ml sulfuric acid, then heating at 120 °C for 10 min until violet-blue spots are detected under white light. In case of botcinolide, the TLC plate was sprayed with the detector anisaldehyde at 0.5% (v/v) in

methanol to acetic acid to concentrated sulphuric acid (17:2:1), then heated at 100 °C for 8 min for detecting deep-blue spots under white light (Cutler et al. 1996).

Results

Isolates

During the experimental course of isolation, 65 isolates were collected and identified according to their morphological features to the genus level. Isolates were collected from different locations in Egypt, i.e. Ismailia, Sharqia, Beheira, Kafr El-Shiehk, Fayum, Menofia and Kaliobya during the growing seasons 2015–2016. Only 15 isolates were selected for further studies. These 15 isolates represented different geographical areas, different colony morphologies and different tomato cultivars. They all belonged to the genus *Alternaria*, and the identification was further confirmed based on multiple gene analysis and phenotypic criteria.

Phenotyping analysis

After sporulation on PCA medium, close microscopic observation was made of morphological structures such as

conidiophores, conidia and beak. The illustrated conidia (Fig. 5) were obclavate or ellipsoid septate, dark brown, varied in size and bearing a short beak. Conidiophores were straight or curved, short to long, simple or branched. The morphological structures of each *Alternaria* isolate are shown in Table 2.

Molecular characterization

The resulting PCR products are approximately 616 bps for the *GAPDH* region, 514 bps for the *Alt a 1* region and 500 bps for the *RPB2* region. The individual BLASTn search results of the sequences *GAPDH*, *RPB2* and *Alt a 1* indicated that the isolates resided in *A. alternata* and *A. arborescens*. The final sequence alignment of the combined dataset comprising 42 taxa included 15 isolates obtained in this study, 27 *Alternaria* reference sequences retrieved from GenBank and *A. alternantherae* strain CBS 124392 as the outgroup taxon. The phylogenetic analysis using a data set of three loci (*GAPDH*, *RPB2* and *Alt a 1*) made it possible to accurately discriminate the two species. The maximum likelihood and Bayesian analyses of *Alternaria* species are illustrated in Fig. 2. The isolate namely *A. arborescens* AL-164 grouped with *A. arborescens* CBS 112633 along to sister clade-containing isolates *A. cerealis* EGS 34-072, *A. geophila* CBS 101.13, *A. gaisen* CBS 118389 and *A. senecionicola* CBS 119545 that poorly supported with ML bootstrap 66% and posterior probability (PP) 0.8. But these all formed a well-supported clade with bootstrap 100% and PP 1.0. On the other hand, the 14 *A. alternata* isolates obtained in this study formed a polyphyletic group with numerous morphospecies, which are synonymised under *A. alternata*. Within the *A. alternata* group, a clear split was obvious to form subgroups supported

with moderate to high values in both ML and PP analyses (Fig. 2).

Pathogenicity tests

GH experiment

The pathogenic status of the 15 selected isolates of *Alternaria* was evaluated over 28 days post inoculation under greenhouse conditions (Table 3). The first incubation period (7 days) of inoculated plants was long enough for the appearance of disease symptoms. Our data indicated that variations in the aggressiveness of the tested isolates of *A. alternata* could be clearly differentiated during the experimental period elapsed from seven to reach the maximum of 28 days. There were significant ($P < 0.01$) differences among isolates in terms of DS. The *Alternaria* isolates ALT-19 and ALT-154 were highly virulent isolates that exhibited DS reached to 3 and 3.1 relative to control plants that exhibited the lowest DS 0.2%. However, ALT-154 displayed AUDPC 45.9% higher than ALT-19 43.3%. According to the statistical analysis, the remaining isolates revealed no significant differences among them in DS. Isolates ALT-40 and ALT-160 exhibited also high DS, 2.7 and 2.6 respectively, but they revealed low AUDPC, 37.1% and 38.7% respectively. Furthermore, the isolate ALT-9 was also virulent which displayed DS 2.3 and high value of AUDPC 45.3%.

DL experiment

The lesions developed on detached tomato leaves were initially water-soaked spots that quickly turned into dark brown and expanded over the experiment and was surrounded by

Table 2 Measurements of morphological structures of *Alternaria* species in this study

Isolates no.	Identity	Conidia Length × width (μm)	Beak Length × width (μm)	Conidiophores Length × width (μm)
ALT-9	<i>A. alternata</i>	19.7–22.6 × 7.9–11.4	4.4–19.5 × 3.3–4.8	22.3–59.1 × 2.1–6.2
ALT-11	<i>A. alternata</i>	17.6–21.3 × 8–12.6	1.1–4.3 × 2.1–3.6	35–98 × 3.2–4.2
ALT-14	<i>A. alternata</i>	18.5–30.2 × 7.1–10	6.5–13.5 × 2.1–7.4	35.2–53.5 × 8.4–12.5
ALT-18	<i>A. alternata</i>	15.5–28.5 × 4.2–8.5	2.8–16.2 × 2.7–4.1	29.6–50.2 × 3–7.3
ALT-19	<i>A. alternata</i>	16.4–50.1 × 5.7–10.8	2.3–40.2 × 1.4–7.7	14.6–48.2 × 3.1–5.5
ALT-21	<i>A. alternata</i>	16.8–24.2 × 4.4–11.4	3–6.1 × 2.8–8.9	11.9–90.5 × 2.7–4.3
ALT-33	<i>A. alternata</i>	11.5–22.2 × 5.4–10.5	3.2–8.9 × 2.6–5.2	25–38.9 × 1.9–5
ALT-38	<i>A. alternata</i>	16.1–22.7 × 6.5–11.9	3.1–7.6 × 2.6–3.9	7.8–66.1 × 1.4–4.6
ALT-40	<i>A. alternata</i>	20.3–32.6 × 6.9–12.5	7.8–13.6 × 2–7.8	30.2–50.6 × 7.2–15.1
ALT-44	<i>A. alternata</i>	15.4–22.3 × 6.7–10.7	4.4–22.1 × 2.3–5.9	21.6–87.9 × 1.7–4.3
ALT-146	<i>A. alternata</i>	16.4–50.1 × 5.7–10.8	2.3–40.2 × 1.4–4.5	14.6–53.3 × 2.5–5
ALT-148	<i>A. alternata</i>	18.2–27.8 × 5.3–9.5	4.2–15.2 × 2.8–6.4	24.5–40.2 × 4.3–8.6
ALT-154	<i>A. alternata</i>	17.6–23.7 × 8.2–10.8	2.1–6 × 2.4–3.9	8.6–74.7 × 2.3–4.9
ALT-160	<i>A. alternata</i>	16.3–34.9 × 5.1–10	3.4–12 × 1.9–4.5	5.2–28 × 1.1–8
ALT-164	<i>A. arborescens</i>	12–21.6 × 7.4–9.7	2.6–9.4 × 1.4–4.4	20.6–61.5 × 2.9–4.3

The measurement of each structure was done for $n = 50$

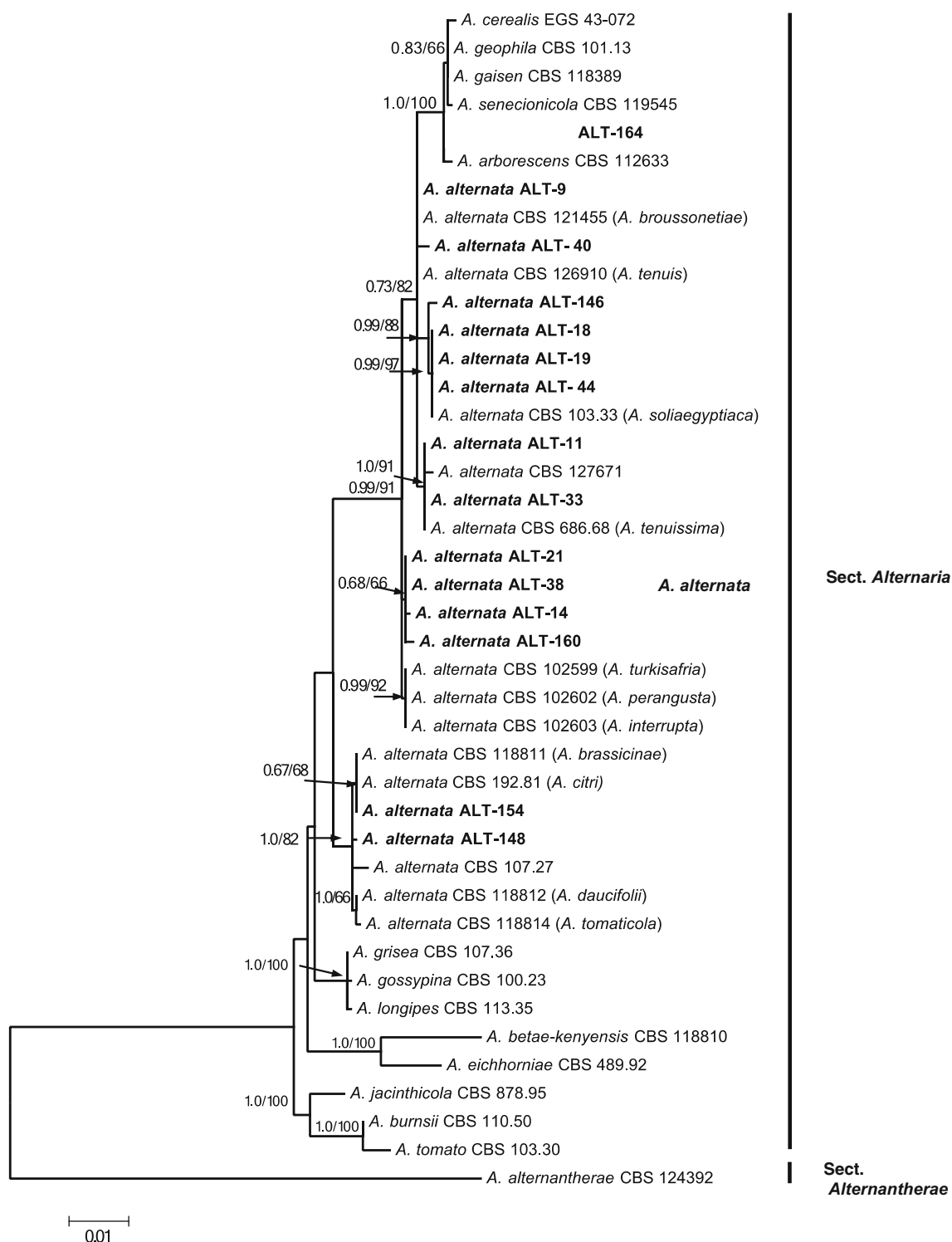


Fig. 2 Maximum likelihood tree based on the *GAPDH*, *RPB2* and *Alt a1* sequences of 42 *Alternaria* strains. The Bayesian posterior probabilities (PP) and ML bootstrap support values (ML) are given at the nodes (PP/

ML). Species names between parentheses are now synonymised under *A. alternata*. The isolates of this study are printed in bold face. The tree was rooted to *A. alternantherae* (CBS 124392)

yellowish hallows. There were no significant ($P < 0.01$) differences among isolates in final lesion size (Table 3). Isolate ALT-44 was more aggressive on detached leaves that exhibited lesion diameter 7.5 mm, followed by isolates ALT-19 and

ALT-154 that recorded final lesion size (lesion radius) 5.6 and 5.5 mm, respectively. There was no significant ($P < 0.01$) difference among the remaining isolates that exhibited close values in final lesion size (Table 3). All screened *Alternaria*

Table 3 Pathogenicity test of 15 *Alternaria* isolates on tomato cv. Super strain B under greenhouse conditions and detached-leaflet level

Isolates	Greenhouse		Detached leaves	
	DS (%)	AUDPC (%)	Lesion radius (mm)	Chlorosis radius (mm)
ALT-9	2.3 a–d	45.3	4.5 b	9.1bc
ALT-11	1.5c–f	32.4	5.0 b	9.3bc
ALT-14	1.1 def	18.4	4.6 b	16.0ab
ALT-18	1.5c–f	22.6	5.1 b	11.1abc
ALT-19	3.1 a	43.3	5.6 b	12.5abc
ALT-21	2.0 a–e	32.4	4.5 b	13.1abc
ALT-33	1.4c–f	24.7	3.8 bc	7.8c
ALT-38	1.6b–e	18.0	4.1 b	11.0bc
ALT-40	2.7abc	37.1	4.0 b	11.1abc
ALT-44	0.8 ef	17.2	7.5 a	10.6bc
ALT-146	1.4 c–f	17.7	4.5 b	12.8abc
ALT-148	2.1a–e	29.4	4.5 b	18.0a
ALT-154	3.0 ab	45.9	5.5 b	9.3bc
ALT-160	2.6abc	38.7	3.6 bc	12.1abc
ALT-164	2.3 a–d	35.8	4.3 b	12.1abc
Control	0.2 f	2.5	1.3 c	0.0d
LSD ($p < 0.01$)	1.4		0.29	0.79

isolates exhibited yellow haloes surrounding the lesions. There was no significant ($P < 0.01$) difference in chlorosis diameter (mm). *Alternaria* isolate ALT-148 exhibited the largest (18 mm) chlorotic halo, followed by ALT-14 that produced chlorotic lesion (16 mm). The remaining isolates produced chlorotic lesions that ranged from 7.9 to 13.1 mm.

Mycotoxin analysis

The chromatographic behavior of the four fractions in the TLC system is shown in Table 4 and Figs. 3 and 4. The four toxins altenuene (ALT), altertotoxin (ATX), alternariol (AOH) and tenuazonic acid (TeA) were detected in the culture filtrate extracts of 15 isolates of *Alternaria* under UV light; altenuene (ALT) developed a blue color, while altertotoxin (ATX) developed yellow-orange color. Alternariol (AOH) developed a violet-blue color and tenuazonic acid (TeA) developed green color under UV light 365 nm (Fig. 3) and UV light 254 nm (Fig. 4). The R_f values of ALT, ATX, AOH and TeA were 0.39, 69, 81 and 79, respectively. These compounds were identified based on the comparison of their R_f values with the R_f value of griseofulvin standard based on the database in TLC scanner. The first metabolites, ALT, were detected in the culture filtrate extracts of only five isolates of *A. alternata*, ALT-21, ALT-9, ALT-33, ALT-44 and ALT-40 with concentrations 3.04, 0.80, 2.72, 1.46 and 3.0 mg/100 ml, respectively. The second metabolites, ATX, were detected in the culture filtrate extracts of four isolates, ALT-18, ALT-146, ALT-164 and ALT-14 with concentrations 4.80, 0.541, 2.64 and 1.88 mg/100 ml, respectively. The third metabolites, AOH,

were detected in the culture filtrate extracts of four isolates ALT-160, ALT-44, ALT-154 and ALT-40 with concentration 0.329, 3.842, 3.370 and 4.348 mg/100 ml, respectively. The fourth metabolites, TeA, were detected in the culture filtrate extracts of all isolates of *A. alternata* excluding ALT-38 and ALT-11 (Table 4).

Discussion

This study revealed variations among *Alternaria* isolates in terms of virulence, toxin production and genetic variability based on three (*GAPDH*, *RPB2* and *Alt a 1*) gene regions. This variation might be attributed to several factors, i.e. temperature and geographical origin of isolates (Nagrle et al. 2013). Not surprisingly, that the morphotype *A. alternata* was more prevalent than *A. arborescens* independently from geographical or tissue origin. This is evidently supported by the suggestion that *A. alternata* is the most widespread species across plants, seasons and geographical regions, causing the spoilage of freshly harvested crops (Huang et al. 2015). This prevalent species can be more aggressive and of importance to tomato than the most common causal agent *A. solani* in Egypt. *Alternaria arborescens* had a low incidence in this study representing a single isolate out of 15 *Alternaria* isolates studied. Similarly, the investigation of Bessadat et al. (2014) revealed that the morphospecies *A. arborescens* only represented 1.5% of the isolates collected in northwestern Algeria. On the other hand, Pryor and Michailides (2002) and Ozkilinc et al. (2017) demonstrated also that *A. alternata* is more

Table 4 The concentrations of four mycotoxins produced by 15 isolates of *Alternaria* based on R_f values

Mycotoxins								
Isolate	Altenuene (ALT) (B)		Altertoxin (ATX) (Y–O)		Alternariol (AOH) (V–B)		Tenuazonic acid (TeA) (G)	
	R_f	Conc. mg/100 mL	R_f	Conc. mg/100 mL	R_f	Conc. mg/100 mL	R_f	Conc. mg/100 mL
ALT–9	0.36	0.80	N.D.	–	N.D.	–	0.78	1.811
ALT–11	N.D.	–	N.D.	–	N.D.	–	N.D.	–
ALT–14	N.D.	–	0.69	1.88	N.D.	–	0.77	0.954
ALT–18	N.D.	–	0.65	4.80	N.D.	–	0.74	4.163
ALT–19	N.D.	–	N.D.	–	N.D.	–	0.75	0.573
ALT–21	0.35	3.04	N.D.	–	N.D.	–	0.79	2.080
ALT–33	0.34	2.72	N.D.	–	N.D.	–	0.78	4.955
ALT–38	N.D.	–	N.D.	–	N.D.	–	N.D.	–
ALT–40	0.36	3.00	N.D.	–	0.80	4.348	0.77	5.256
ALT–44	0.37	1.46	N.D.	–	0.81	3.842	0.78	3.012
ALT–146	N.D.	–	0.68	0.541	N.D.	–	0.75	2.706
ALT–148	N.D.	–	N.D.	–	N.D.	–	0.80	2.878
ALT–154	N.D.	–	N.D.	–	0.81	3.370	0.77	2.290
ALT–160	N.D.	–	N.D.	–	0.79	0.329	0.76	1.901
ALT–164	N.D.	–	0.69	2.64	N.D.	–	0.79	6.164
Blank	N.D.	–	N.D.	–	N.D.	–	N.D.	–

*The compounds were visualized under UV light (365 and 254 nm)

**Concentration of altenuene was 5 mg/100 ml

N.D. not detected, Blank liquid media without inoculum, B blue, y–o yellow–orange, v–b violet–blue, g green

abundant as compared to *A. arborescens*, unlike Tymon et al. (2016) who demonstrated that from the surveyed potato samples, *A. arborescens* was more frequently isolated than *A. alternata*.

We investigated the potency of different isolates of *Alternaria* to infect either detached leaves or whole plants of tomato cv. Super strain B. The tested isolates exhibited symptoms distinct from EB symptoms in which the margins of spots are raised and the zonation is indistinct. However, EB is characterized by the formation of dark-coloured spots that are necrotic in the centre with a pattern of concentric rings

(van der Waals et al. 2004). Leaf blight severity is usually evaluated by recording the disease severity and calculating the area under disease progressive curve (AUDPC) using the data observed over time. All of the investigated isolates were pathogenic to both detached leaves and whole plants, although isolates proved to be more virulent on whole plants. There was no correlation observed between the pathogenicity of GH and DL experiments. Also, Foolad et al. (2000) stated that the results of detached leaflet assays did not correlate with either field or greenhouse results. However, there was a clear correlation between field and greenhouse results, suggesting the

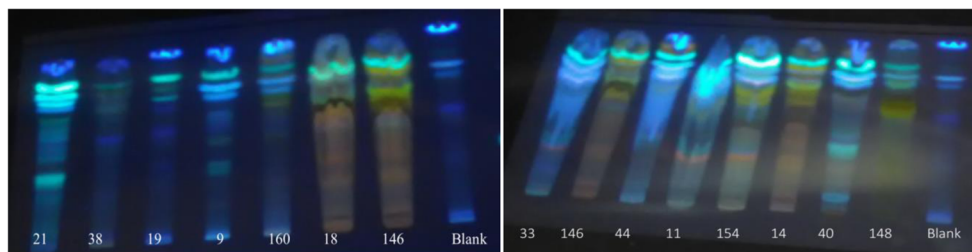


Fig. 3 TLC analysis of toxins produced by *Alternaria* displaying different colored bands on TLC plates; blue for altenuene (ALT), yellow-orange for altertoxin (ATX), violet-blue for alternariol (AOH) and green for tenuazonic acid (TeA), on the basis of their R_f values. The standards of

these toxins were also run on similar plates for comparisons of the R_f values. Fractions in culture filtrates extracts of 15 isolates *Alternaria* measured at 365 nm

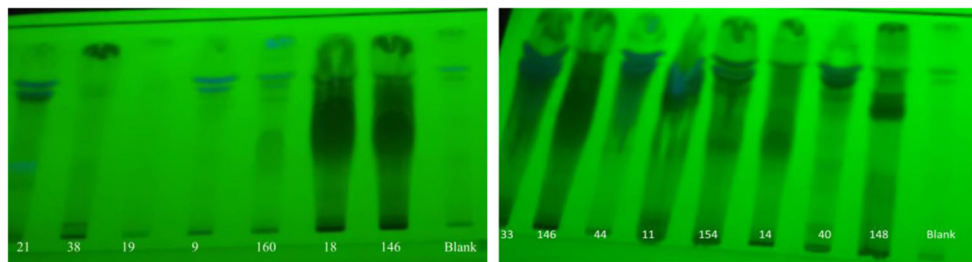


Fig. 4 TLC analysis of toxins produced by *Alternaria* displaying different colored bands on TLC plates; blue for altenuene (ALT), yellow-orange for altertoxin (ATX), violet-blue for alternariol (AOH) and green for tenuazonic acid (TeA), on the basis of their R_f values. The standards of

these toxins were also run on similar plates for comparisons of the R_f values. Fractions in culture filtrates extracts of 15 isolates *Alternaria* measured at 254 nm

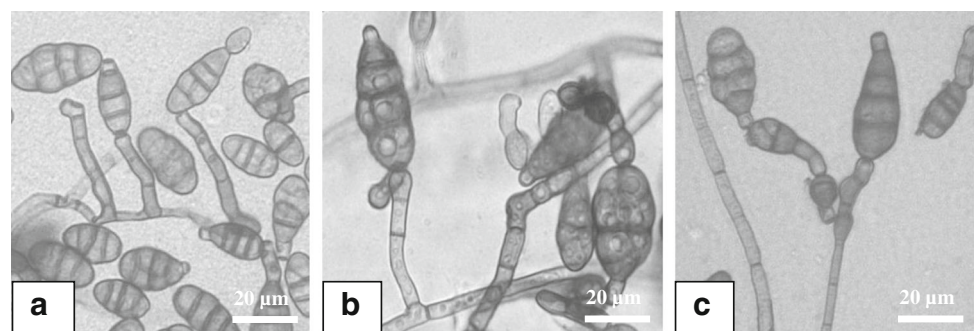
necessity of the whole plant for the evaluation of leaf blight disease. Therefore, in our study, we relied on the data of both DL and GH to determine the virulence profile of *Alternaria* species. It is well known that *A. arborescens* is a causal agent of stem canker of tomato (Caldas et al. 1994). The single isolate obtained in this study ALT-164 (*A. arborescens*) proved to be highly virulent on tomato plants as well as on DL (Fig. 5). Other studies on *Alternaria* spp. showed that isolates of *A. arborescens* can also cause diseases on apple (Rotondo et al. 2012; Hartevelde et al. 2014) and can act as postharvest pathogens on apple and citrus (Kang et al. 2002; Serdani et al. 2002).

According to the phylogenetic results, *A. alternata* is a paraphyletic group, which did not form clear phylogenetic clades. The subclades that are formed by *A. alternata* are incongruent between the three different genes sequenced (data not shown). The sequenced gene regions did not reveal the same grouping from any of the 14 isolates. This result coincided with the previous one demonstrated by Woudenberg et al. (2015). There were difficulties to distinguish species in the *Alternaria* sect. *Alternaria*, until the study of Woudenberg et al. (2015), who made a consistent taxonomic treatment to this section that led to synonymise 35 morphospecies under *A. alternata* based on the multi-gene phylogeny. Woudenberg et al. (2015) stated that *A. arborescens* cannot be separated from *A. alternata* based on the ITS region alone. Therefore, the recent descriptions based on ITS alone of *A. arborescens* as the cause of cutaneous alternariosis in a healthy person (Hu et al. 2014) and of *A. longipes* as the cause of a severe leaf spot

disease on potato (Shoaib et al. 2014) need to be re-investigated by other molecular markers. Furthermore, *GAPDH* sequence data alone could not separate the *A. arborescens* species from *A. alternata* (data not shown). This finding is supported by the statement of Woudenberg et al. (2015) who showed that the commonly used *GAPDH* sequence could distinguish all species, except the *A. arborescens* from *A. alternata*. However, Woudenberg et al. (2015) confirmed that five genes, namely *RPB2*, *OPA10-2*, *Alt a 1*, *endoPG* and *KOG1058*, could separate all species from *A. alternata*. These statements mentioned above led us to avoid the use of the ITS region and choosing other genetic markers, i.e. *RPB2* and *Alt a 1* proposed by Woudenberg et al. (2015). Furthermore, in the very recent study of Ozkilinc et al. (2018), they pointed out that 37 multilocus haplotypes were found among *A. alternata/tenuissima* isolates and 21 among *A. arborescens* isolates, revealing much higher genotypic diversity and multiple clonal lineages within taxa. However, they partially resolved species boundaries within section *Alternaria* based on the three gene regions (*Alt a 1*, ATPase and calmodulin), and there were no well-defined species boundaries.

The present study demonstrated that all isolates of *Alternaria* produced ALT, altertoxin (ATX), AOH and TeA and determined based on their retention factor (R_f) values in the presence of different solvents using TLC (Devi et al. 2010). Patel et al. (2011) reported that *A. solani* isolates produced the alternaric acid toxin in culture filtrate extract after 18 days onwards and its R_f value was 0.5 on TLC. The toxin

Fig. 5 Conidia and conidiophores of *A. alternata* (a, b) and *A. arborescens* (ALT-164) (c)



appeared as blue fluorescing spots when placed under a UV lamp at 365 nm. We have observed variations among isolates of *Alternaria* for toxin production, being isolates recovered from tomato leaves produced higher amounts of toxins when compared to isolates recovered from fruits (ALT-19) and stems (ALT-21). Meena et al. (2017) stated also that *Alternaria* TM4 (isolated from tomato leaf) was more pathogenic and produced the maximum amount of mycotoxins as compared to other isolates. They attributed this variation in toxin production to defense mechanism responded by plants or to some extent their resistance against the pathogen.

There was also a variation in the amount of four toxins produced by *Alternaria* isolates. Tenuazonic acid (TeA) was the most dominant produced toxin with variable amounts by all isolates except two isolates (ALT-38 and ALT-11). Also, Stinson et al. (1980) reported that mycotoxins produced by numerous species of *Alternaria*, being (TeA) was the main mycotoxin produced in *Alternaria*-infected tomatoes from commercial sources with concentration as high as 13.9 mg/100 g. Similarly, Garganese et al. (2016) found that tenuazonic acid (TeA) was the most abundant mycotoxin (0.2–20 mg/L) produced by 20 isolates of *Alternaria*. Bottalico and Logrieco (1998) extracted the phytotoxins from tomatoes infected with 17 isolates of *A. alternata* that produced alternariol, alternariol methyl ether, tenuazonic acid, altertoxin and altenuene at concentrations of up to 600, 100, 4750, 30 and 12.5 mg/kg, respectively. Stoessl et al. (1983) found similar differences in the production of alternaric acid and altersolanol among isolates of *A. solani* and between experiments with the same isolate. Cotty and Misaghi (1984) reported that the quantities of zinniol produced by different *Alternaria* species and by different isolates of one species were quite variable. Only *A. brassicae* and *A. zinnia* did not produce detectable amounts of zinniol in vitro. The ability of a nonpathogenic isolate of *A. zinniae* to produce the detectable level of zinniol in vitro might have been due to the loss of its pathogenicity. Stack and Prival (1986) reported that all 14 *A. alternata* cultures produced TA and ATX I, II and III, although sometimes in only trace amounts.

Conclusion

The current study represents the first case study of the diversity of *Alternaria* species associated with leaf blight of tomato in Egypt based on a three multi-locus phylogeny of *GAPDH*, *RPB2* and *Alt a 1*. We report for the first time in Egypt the potential of *A. alternata* and *A. arborescens* to cause leaf blight on tomato. Due to the geographic proximity of several tomato, potato and other solanaceous hosts, an important consideration is the occurrence of different fungal species in a given area causing leaf blight or EB diseases. Therefore, further studies on many more isolates would be required to reveal

the importance, the real distribution and the potential of *A. arborescens* or other species to incite disease either on solanaceous or other hosts in Egypt. The present study did not investigate the toxicogenic effect of each of the individual mycotoxins on tomato plants to develop disease symptoms. This point might be covered in the future work by the authors.

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