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ORIGINAL ARTICLE

Alternaria species causing pomegranate and citrus fruit rots in Albania

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Abstract
The fungal genus *Alternaria* is a relevant pathogen for several commodities including citrus and pomegranate fruits. On citrus, it mainly causes brown spots on fruits and leaves, whereas on pomegranate, it mostly causes a fruit heart rot. In the present study the presence of *Alternaria* rots on citrus and pomegranate fruits cultivated in Albania was assessed. Representative fruits were collected from different regions. Nineteen and thirteen *Alternaria* spp. isolates were obtained from pomegranate and citrus samples, respectively. The isolates were identified at species and morphotype level. Micro and macroscopic features separated isolates into four morphotypes. BLAST and phylogenetic analysis using the SCAR Marker OPA1-3 confirmed the isolate identity. All 32 isolates proved to be *Alternaria alternata* and belonged mainly to morphotype *alternata*, followed by *limoniasperae* and *tenuissima*. All *Alternaria* strains proved to possess the *pksI* gene of alternariol biosynthesis. Citrus isolates were tested for the presence of genes of the biosynthesis of the phytotoxins ACT and ACR, but none of them proved to possess them. Concluding, *Alternaria* spp. might represent a treat to pomegranate and citrus production in Albania, and thus effective control means are needed.

Keywords Fungal rots · Brown spot · Black heart · Phytotoxin · Mycotoxins

Introduction

Although pomegranate (*Punica granatum* L.) has an ancient origin, it is considered an emerging crop because of the beneficial effects that its fruit might have on consumers' health, given its high content in polyphenols with potential antioxidant, anti-inflammatory, and antiproliferative effects (Zarfeshany et al. 2014). Moreover, pomegranate tree is adaptive to a wide range of climate and soil conditions, and this has facilitated the spreading of its cultivation (Chandra et al. 2010). In Albania, pomegranate is mostly sold as fresh fruit in the local markets. The most widespread local cultivars are Devedishe, Tivaresh, and Majoshe, which are cultivated almost all over the country (Xhuveli 2012). However, recently, even some commercial cultivars, as Wonderful and its clone Wonderful One, started to be cultivated.

Another relevant fruit crop for Albania is citrus (FAOSTAT 2019). Although citrus is native to East Asia, citriculture has expanded in tropical, subtropical, and Mediterranean regions too (Ollitrault and Navarro 2011). Albania has a strong tradition in the production of citrus fruits, which is concentrated in coastal areas (Skreli and Imami 2019). Even citrus fruits are known for their high nutritional, therapeutic, and ornamental value (Chandra et al. 2010; Ollitrault and Navarro 2011).

Traditionally, Mediterranean climate is not particularly conducive to epidemic outbreaks of fungal diseases. However, in the last years, the Mediterranean areas are experiencing the emergence or re-emergence of new and endemic diseases by fungal phytopathogens, such as *Alternaria* spp. This is reasonably due to climate change, circulation of people and goods among countries, and cross-contaminations among crops (Gilardi et al. 2018). The genus *Alternaria* is distributed worldwide and includes saprophytic, endophytic, and pathogenic species (Woudenberg et al. 2013). Species of *Alternaria* can cause significant decreases in crop yields and therefore considerable economic losses. According to the

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classification system proposed by Woudenberg et al. (2015), the *Alternaria* section *Alternaria* counts 11 small-spored species including the type-species *Alternaria alternata*, and one species complex (*Alternaria arborescens*). The former species were reclassified as morphotypes, and morphologically indistinguishable isolates of a species infecting specific hosts as *forma specialis* (*f. sp.*) (*e.g. A. alternata f. sp. mali* or *A. alternata f. sp. citri*).

The relevance of this genus is also due to the production of more than 70 secondary metabolites that are toxic to plants (phytotoxins), some of which have been chemically characterized (Pavon et al. 2012). Furthermore, *Alternaria* spp. produce secondary metabolites classified as mycotoxins, such as tenuazonic acid, alternariol, alternariol monomethyl ether, altenuene, and tentoxin, which are considered a threat to humans and animals (Escrivá et al. 2017), hence the recommendation of monitoring by EFSA (EFSA 2011, 2016).

Alternaria brown spot (ABS) is one of the most important diseases of citrus (Garganese et al. 2016), associated with the production of host-specific toxins (HSTs), as ACT-toxin (ACTT) on mandarins and tangerines and ACR-toxin (ACRT) on rough lemon, which induce necrotic lesions on fruit and young leaves, as well as fruit drop in susceptible genotypes (Meena et al. 2019). *Alternaria* is also relevant to pomegranate fruit production, being the primary cause of an inner rot called black heart or *Alternaria* rot, in which the internal part of the fruit shows brown to black decay of the arils and connective tissues. In fact, airborne spores of *Alternaria* spp. can cause infections of flowers during bloom, remaining eventually latent for most of the growing season (Luo et al. 2017).

The general objective of this study was to assess the presence of *Alternaria* infections on citrus and pomegranate fruits grown and commercialised in Albania, and eventually collect *Alternaria* spp. isolates, identifying them at species level, as a contribution to the prevention of disease spread in two economically relevant crops for the country.

Materials and methods

Sampling and isolation

Fruits with characteristic *Alternaria* rot symptoms were collected in open fields and local markets in different regions of Albania. Symptomatic fruits were surface sterilized by dipping in 2% sodium hypochlorite for 2 min followed by 1 min in sterile water, or by spraying a 70% ethanol solution, if severely rotted. Then, they were aseptically air-dried on clean bench paper.

For the preparation of potato dextrose agar (PDA), 200 g of sliced potatoes were boiled in 1 L of distilled water for 30 min. Then, the potato infusion was filtered through cheesecloth, and added with 20 g L^{-1} of dextrose (DIFCO Laboratories, Detroit, USA) and 20 g L^{-1} agar (Fisher BioReagents, Basingstoke, Hampshire, England). The medium was autoclaved at 121 °C for 20 min, and once the temperature dropped to 50 °C, it was amended with 250 mg L^{-1} of both ampicillin and streptomycin (Appli-Chem GmbH, Darmstadt, Germany) before pouring to prevent bacterial growth.

A sterilized scalpel was used to cut 3 × 3 mm pieces at the edge of the lesions. In case of fruit with severe and liquid rot, a sterile lancet was used to collect some rotted tissue or fungal spores. Then, rotted tissues or spores were then plated on the amended PDA plates, and incubated in the dark at 24 ± 1 °C for 3–5 days. Fungal colonies were then transferred to new PDA plates, purified as required, and deposited in the Fungal Collection of the Agricultural University of Tirana (Albania) in their monoconidial form. To obtain single-spored colonies, a conidial suspension of each isolate was streaked on water agar medium (20 g agar in 1 L distilled water) and then incubated at 24 ± 1 °C for 48 h.

Morphological identification

To identify isolates by morphology, one germinated conidium was transferred to Potato Carrot Agar (PCA; 20 g potatoes, 20 g carrots, 20 g agar in 1 L of distilled water) for micro-morphology and PDA for macro-morphology, following indications by Simmons (2007) and Pryor and Michailides (2002), respectively. Macroscopic features were observed after 10 days of incubation at 22 °C in the dark, whereas for micromorphology, after 5 days of incubation in the dark, fungal cultures were exposed to daylight for 24 h and then returned to the dark until day 10. After the period of incubation, PDA cultures were examined for colony growth (diameter, mm), colour, margin, texture, and the production of pigments or crystals in the agar medium. Whereas fungal structures from PCA cultures were examined at 40× magnification and substage illumination by a microscope (OPTECH Microscope Service, Thame, Oxfordshire, UK) for characteristics of the sporulation apparatus, including conidia size, presence and number of septa, length of conidial chains, presence of elongated secondary conidiophores, and manner by which branching of conidial chains (if present) occurred.

Molecular identification

Selected pomegranate ($n = 19$) and citrus isolates ($n = 13$), representing the range of morphological groups, were molecularly identified following Garganese et al. (2016).

For DNA extraction, the representative isolates were grown in Potato Dextrose Broth (PDB; 200 g potatoes and 20 g dextrose in 1 L of distilled water) at 25 ± 1 °C in the dark on an orbital shaker (150 rpm) for 7 days. The mycelium was collected, air-dried, and stored at − 20 °C until use. DNA extraction was performed from 100 mg of mycelium grinded in a mortar with liquid nitrogen by Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp, Thorold, ON, Canada) following manufacturer recommendations. Sample concentration and purity were determined by a spectrophotometer (VWR International srl, Milan, Italy). The purified genomic DNA was diluted at 50 ng μl^{-1} and stored at − 20 °C until use. PCR amplification of the SCAR marker OPA1-3 was performed for sequencing purposes (Table 1). Each reaction mixture contained 50 ng of template DNA, 0.2 µM of both forward and reverse primer, 1 × DreamTaq™ Hot Start Green PCR Master Mix (ThermoFisher Scientific, Milan, Italy) in a 50 µl reaction volume. PCR reactions were performed in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) according to the following conditions: 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and 72 °C for 7 min. Amplicons were resolved in 1.7% agarose gel in TAE buffer (1×), pre-stained with GelRed® (Biotium, Landing Parkway Fremont, CA, USA), and visualized by Gel Doc™EZ System (Bio-Rad). Primers were synthesised by Macrogen Europe (Amsterdam, The Netherlands), which also purified and sequenced amplicons. The nucleotide sequences were submitted to the online BLAST search engine of the National Centre for Biotechnology Information (NCBI) and deposited in GenBank database.

Assessment of toxigenicity potential

The extracted DNA of the selected strains was assayed for the presence of *pksI*, key gene of the biosynthetic pathway of the mycotoxin alternariol, *act1* and *act2* genes of ACT phytotoxin biosynthesis, and *acr1* and *acr2* genes of ACR phytotoxin biosynthesis. Primers used and synthesised by Macrogen Europe are reported in Table 1. PCR reaction mix and conditions, and amplicon resolution and visualization were arranged as reported above.

Pathogenicity assay

To fulfil Koch's postulates, one representative strain of the most common morphotype for both citrus and pomegranate was tested on its host of isolation. Conidial suspensions were prepared by flooding plates with 5 ml sterile 0.02% Tween20 solution and gently rubbing the colony surface with a sterile spatula. For pomegranate 'Wonderful' fruits, the conidial suspension (100 µL of 10^4 conidia ml^{-1}) was injected by a syringe into one side of a fruit (10 fruits per isolate). Control fruits were injected with sterile distilled water. For citrus fruit, each clementine fruit was wounded with a sterile nail (3 × 3 mm) in two equidistant points on the equatorial surface. A drop (10 µl) of conidial suspension (10^5 conidia ml^{-1}) was applied into the wounds of 10 fruit. Samples were incubated at 24 ± 1 °C and high relative humidity (85–95%) for 10 days. Pomegranate fruits were cut open longitudinally into two halves to observe heart rot symptoms.

Table 1 Primers used for sequencing barcoding regions and detecting toxin biosynthetic gene

Gene/region	Primer name	Sequence (5'–3')	Product size (bp)	Annealing T (°C)	Source
OPA1-3	OPA1-3L	CAGGCCCTTCCAATCCAT	900	56	Peever et al. (2004)
	OPA1-3R	AGGCCCTTCAAGCTCTCTTC			
pksI	pksI-F	CCTCTCTATCCCAAACTCCACAC	249	58	Sanzani et al. (2021)
	pksI-R	CACAGATTATGGCAAGGTTC			
act1	HACT-1F	ATGCGCGAGATTTTCTGACC	197	60	Garganese et al. (2016)
	HACT-1R	CTGTCTCCCCGGTACAAAGT			
act2	HACT-2F	TGACATTACGACGTAGGACGC	186		
	HACT-2R	GCTCCTGATATCGTCCTGTGA			
acr1	ACR1-F	TCGCTGTACCCCGTATCTTC	188	58	This study
	ACR1-R	GACATGGACGTCGTTGATGG			
acr2	ACR2-F	GCGGATTTTCTGGAGTCGAC	230		
	ACR2R	CTTGATGTCGGCGAATCGTT			

Gene/region, primer name and sequence, reaction information and bibliographic source are reported

Data analysis

The evolutionary history of isolates was inferred by using the Maximum Likelihood method and Tamura–Nei model (1993). Sequences were aligned using ClustalW (Higgins and Sharp 1988). Analyses were performed with 1000 bootstrap replications. The percentage of trees in which the associated taxa clustered together was reported next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). The Markov chain Monte Carlo (MCMC) algorithm was performed to generate phylogenetic trees with Bayesian posterior probabilities for the alignment using MrBayes 3.2.6 (Ronquist et al. 2012). Four MCMC chains were run simultaneously for random trees for 1,000,000 generations and sampled every 500 generations. The first 25% of trees were discarded as burn-in phase of each analysis and posterior probabilities were determined from the remaining trees.

Results and discussion

Sampling and isolate collection

During the inspections in the main pomegranate and citrus production areas of Albania, characteristic symptoms of *Alternaria* rot were observed. Infected pomegranate fruits were characterized by a brown to black, soft to dry rot of the arils, visible when the fruit was cut open. Typically, the rot was confined to some aril compartments and did not affect the peel and the septa. The epicarp showed non-specific symptoms in correspondence of the internal rot, such as a dark-red discoloration and wrinkling. Heavily affected fruits were asymmetric and lighter in weight. Thirty-five representative pomegranate samples were collected from Tirane, Durres, Fier, Shkoder, Berat, and Gjirokaster areas, and taken to the laboratory for fungal isolation. They belonged to local (Majoshe, Devedishe, and Tivaresh) and commercial (Wonderful One) cultivars. From the above-mentioned pomegranate samples, 19 *Alternaria* isolates (AP) were obtained (Table 2).

Citrus fruit symptoms varied from light brown, slightly depressed spots, to circular and dark brown areas on the external surface, often surrounded by a yellow halo. Infected young fruits often fell from the tree and the mature fruits were unmarketable due to lesions. Thirty-nine representative samples of different citrus species, including sweet oranges (*Citrus sinensis*, cv. Valencia Olinda), lemons (*Citrus limon,* cv. Cerza), and clementines (*Citrus clementina,* cv. Comune ISA), were collected in Vlore, Durres, Tirane, Elbasan, and Berat areas. From citrus samples, 13 *Alternaria* isolates (AC) were obtained (Table 2).

Morphological characterization of isolates

Since only a polyphasic approach could allow the precise identification of the *Alternaria* genus (Andersen et al. 2008; Brun et al. 2013), both morphological and molecular approaches were used. Regarding morphological features, PCA and PDA were used as recommended by Simmons (2007) and Pryor and Michailides (2002). Macro and microscopic characteristics of the colonies of the 32 selected isolates were evaluated. Ten isolates from pomegranate (AP2, AP3, AP9, AP10, AP14, AP15, AP16, AP17, AP18, and AP19) and ten isolates from citrus (AC21, AC22, AC23, AC26, AC27, AC28, AC30, AC31, AC32, and AC33) exhibited colonies that were flat, woolly, and with colours ranging from brown to black. The colonies had a sporulation pattern with a single sub-erect conidiophore and the production of olivaceous-dark brown conidia arranged in branched chains. Conidia appeared oval-ellipsoidal with 3–5 transverse septa. These features matched those of *A. alternata* morphotype *alternata*. Furthermore, isolate AC24 from citrus presented a different sporulation pattern with broadly ovate, subovate, or oblong-elliptical, beakless conidia resembling that of *ex A. citri* now *A. alternata* morphotype *alternata f. sp. citri* (Woudenberg et al. 2015).

Three isolates from pomegranate (AP5, AP6, and AP8) and one isolate from citrus (AC25) were characterized by greenish colonies with white margins; conidia appeared elongated with a long-tapered beak and with short chains of terminal sharp-beaked conidia. This characteristic sporulation pattern matched that of *A. alternata* morphotype *tenuissima*.

Finally, six isolates from pomegranate (AP1, AP4, AP7, AP11, AP12, and AP20) and one isolate from citrus (AC29) showed a colony that was pale brown, flat, granulated with undulating edges. Conidia appeared long and ellipsoidal with 1–3 transverse septa. The sporulation pattern resembled that of *A. alternata* morphotype *limoniasperae*. The finding of morphotype *limoniasperae*, generally associated with citrus (Simmons 2007), among strains of pomegranate fruit, is not surprising as often pomegranate and citrus fields where sampling was conducted, were bordering (M. Cara, personal communication), thus cross-contamination might have occurred.

Table 2 *Alternaria* strain, host, cultivar, location of isolation, and GenBank accession numbers

Strain	Host	Cultivar	Location	Accession no	*pksI*
AP1	*Punica granatum*	Majoshe	Tirane (Ndroq)	OM283829	+
AP2	*Punica granatum*	Majoshe	Tirane (Ndroq)	OM283830	+
AP3	*Punica granatum*	Majoshe	Tirane (Ndroq)	OM283831	+
AP4	*Punica granatum*	Majoshe	Tirane (Mullet)	OM283832	+
AP5	*Punica granatum*	Devedishe	Fier (Lushnje)	OM283833	+
AP6	*Punica granatum*	Devedishe	Fier (Lushnje)	OM283834	+
AP7	*Punica granatum*	Majoshe	Fier (Lushnje)	OM283835	+
AP8	*Punica granatum*	Tivaresh	Shkoder (Stajke)	OM283836	+
AP9	*Punica granatum*	Devedishe	Berat (Syzez)	OM283837	+
AP10	*Punica granatum*	Devedishe	Berat (Syzez)	OM283838	+
AP11	*Punica granatum*	Wonderful one	Tirane (Laknas)	OM283839	+
AP12	*Punica granatum*	Devedishe	Tirane (Laknas)	OM283840	+
AP14	*Punica granatum*	Wonderful one	Berat (Banaj)	OM283841	+
AP15	*Punica granatum*	Wonderful one	Berat (Banaj)	OM283842	+
AP16	*Punica granatum*	Wonderful one	Fier (Divjake)	OM283843	+
AP17	*Punica granatum*	Devedishe	Durres (Kallm)	OM283844	+
AP18	*Punica granatum*	Devedishe	Durres (Kallm)	OM283845	+
AP19	*Punica granatum*	Wonderful one	Gjirokaster	OM283846	+
AP20	*Punica granatum*	Majoshe	Durres (Shkallnur)	OM283847	+
AC21	*Citrus limon*	Cerza	Elbasan (Librazhd)	OM283848	+
AC22	*Citrus sinensis*	Valencia olinda	Vlore (Konispol)	OM283849	+
AC23	*Citrus clementina*	Comune ISA	Vlore (Mursi)	OM283850	+
AC24	*Citrus clementina*	Comune ISA	Vlore (Mursi)	OM283851	+
AC25	*Citrus clementina*	Comune ISA	Durres (Manze)	OM283852	+
AC26	*Citrus sinensis*	Valencia olinda	Durres (Manze)	OM283853	+
AC27	*Citrus limon*	Cerza	Berat (Syzeze)	OM283854	+
AC28	*Citrus sinensis*	Valencia olinda	Tirane (Kavaje)	OM283855	+
AC29	*Citrus clementina*	Comune ISA	Tirane (Kavaje)	OM283856	+
AC30	*Citrus sinensis*	Valencia olinda	Vlore (Xarre)	OM283857	+
AC31	*Citrus limon*	Cerza	Vlore	OM283858	+
AC32	*Citrus clementina*	Comune ISA	Vlore	OM283859	+
AC33	*Citrus clementina*	Comune ISA	Vlore	OM283860	+

Molecular characterization of isolates

For all *Alternaria* isolates, sequencing of the barcoding region OPA1-3 was conducted. Sequences were deposited in GenBank database (Table 2) and underwent BLAST analysis. They showed 99–100% identity with NCBI reference sequences (data not shown). Moreover, the phylogenetic analysis, conducted including CBS and reference strains for comparison or as outgroups (Table 3), allowed to confirm that all 32 isolates belonged to species *A. alternata* (Figs. 1, 2).

Table 3 Reference strains used in phylogenetic analyses and their GenBank accession numbers

Strain	Accession no
Alternaria alternata morphotype limoniasperae strain A42	KU933223
Alternaria alternata morphotype alternata f.sp. citri strain CBS10727	MG063726
Alternaria alternata morphotype alternata strain A65	KU933229
Alternaria alternata morphotype alternata strain CBS 112,249	MG063725
Alternaria alternata morphotype limoniasperae CBS 102.595	MG063729
Alternaria alternata morphotype tenuissima isolate 37FrB	JQ800561
Alternaria solani	KY561993

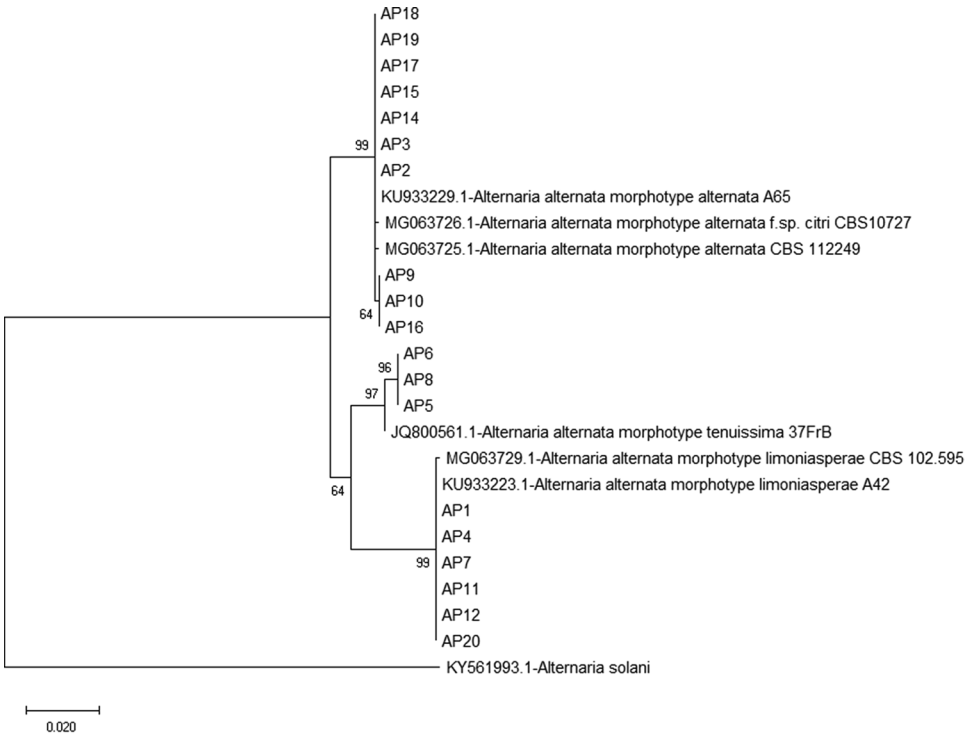

Fig. 1 Phylogenetic tree for pomegranate *Alternaria* strains based on SCAR Marker OPA1-3. Numbers on nodes represent the Maximum Likelihood bootstrap percentages. Branch lengths are proportional to the numbers of nucleotide substitutions and are measured using the bar scale (0.02)

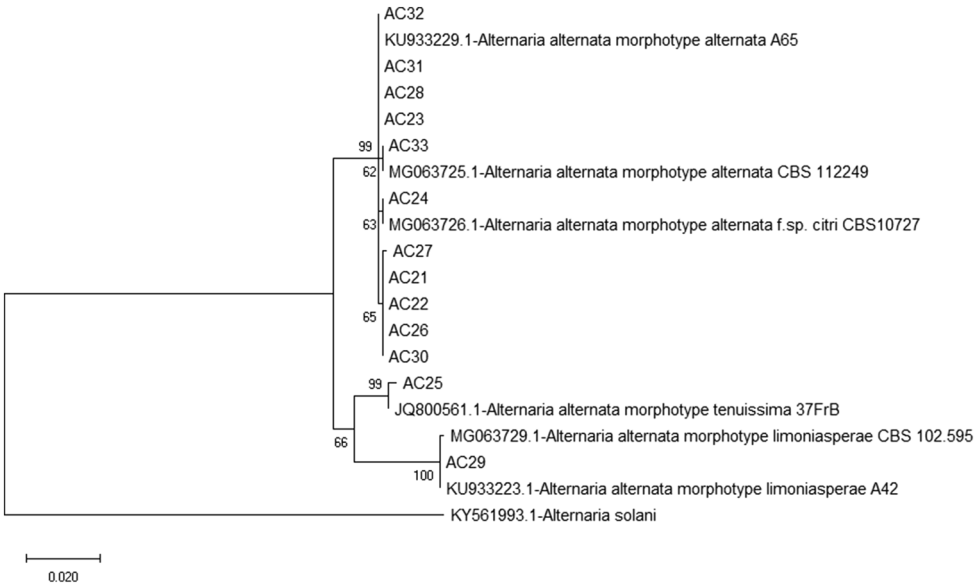

Fig. 2 Phylogenetic tree for citrus *Alternaria* strains based on SCAR Marker OPA1-3. Numbers on nodes represent the Maximum Likelihood bootstrap percentages. Branch lengths are proportional to the numbers of nucleotide substitutions and are measured using the bar scale (0.02)

For pomegranate batch of isolates (19), sequence alignment displayed 865 characters, of which 45 were parsimony informative. Five haplotypes were identified using the genetic variation, with a pairwise identity of 97.40% (Table 4). The 45 Single Nucleotide Polymorphisms (SNPs) corresponded to transitions (29) and transversions (16). There were insertions at nucleotides 227–237 (AP5, AP6, and AP8) and 792 (AP10), and deletions at 524 (AP1, AP4, AP7, AP11, AP12, and AP20). For those isolates, the tree with the highest log likelihood (− 1747.91) was reported (Fig. 1). This analysis involved 26 nucleotide sequences including reference strains. Ten isolates (AP19, AP18, AP17, AP15, AP14, AP3, AP2, AP9, AP10, and AP16) confirmed to belong to morphotype *alternata*, three (AP5, AP6, and AP8) to morphotype *tenuissima*, and six (AP1, AP4, AP7, AP11, AP12, and AP20) to morphotype *limoniasperae*. A similar *Alternaria* population was reported on tomato (Sanzani et al. 2021).

Table 4 Genetic information of the sequences produced

	Isolates no	Haplotypes no	Alignment length	Sites			
				Conserved	Variable	Parsimony-informative	Singletons
Pomegranate	19	5	865	819/865	45/865	45/865	0/865
Citrus	13	8	864	808/864	48/864	14/864	34/864
Combined	32	9	865	816/865	48/865	45/865	3/865

Whereas, for citrus isolates (13), sequence alignment displayed 864 characters, of which 14 were parsimony informative. Eight haplotypes were identified using the genetic variation, with a pairwise identity of 98.70% (Table 4). The 48 SNPs corresponded to transitions (30) and transversion (18). There was an insertion at nucleotides 229–236 (AC25) and a deletion at nucleotide 524 (AC29). The tree with the highest log likelihood (− 1748.46) was reported (Fig. 2). This analysis involved 20 nucleotide sequences including reference strains. Eleven isolates (AC32, AC31 AC28 AC23, AC33, AC27, AC21, AC22, AC26, and AC30) belonged to morphotype *alternata*, of which one (AC24) clustered with the CBS representative of *f. sp. citri*, one (AC26) to morphotype *tenuissima*, and one (AC29) to morphotype *limoniasperae*. The presence of a supposed *f. sp. citri* strain was of interest since generally considered of higher virulence because of the putative production of a phytotoxin (Meena et al. 2017). For example, it has been reported that the citrus leaf may be killed by the host-selective ACTT even without tissue colonization by the fungus (Timmer et al. 2003). However, to confirm the belonging of an isolate to a *forma specialis*, the presence of biosynthetic genes or the actual production of the relevant phytotoxin should be assessed.

Considering the evolutionary analysis of the whole batch of isolates (32 nucleotide sequences), sequence alignment displayed 865 characters, of which 48 were parsimony informative. Nine haplotypes were identified using the genetic variation, with a pairwise identity of 97.80% (Table 4). The tree with the highest log likelihood (− 1758.10) was reported (Fig. 3). This analysis involved 39 nucleotide sequences including reference strains. Pomegranate and citrus *Alternaria* strains belonging to the same morphotypes clustered readily and consistently together, without differences according to host (citrus or pomegranate) or geographical provenience (Fig. 3). This finding seems to further support the cross-infectivity hypothesis related to the neighbouring fields of the two commodities where sampling was conducted.

All strains from citrus and pomegranate included in the study proved to match with *A. alternata*, which is the most widespread *Alternaria* species across plants, seasons, and geographical regions, and that includes host-specific pathogenic strains, as well as opportunistic and saprophytic forms causing the spoilage of freshly harvested crops (Huang et al. 2015). Within this species, in the present study, four morphotypes were identified according to colony and sporulation apparatus features, as well as sequence similarity and phylogenesis analysis with CBS and reference strains included for comparative purposes; minor differences among isolates within each morphotype were recorded. Besides, independently from geographical or tissue origin, the most abundant morphotype proved to be *alternata*. This finding agrees with other reports on the same and other hosts (Garganese et al. 2016; Sanzani et al 2019, 2021; Aloi et al. 2021).

Assessment of toxigenicity potential

Additionally, strains were tested for the presence of *pksI*, a key gene of the biosynthetic pathway of the mycotoxin alternariol, by a specific PCR assay. All 32 isolates confirmed the presence of *pksI* (Table 2). Although specific evaluation of the actual production of alternariol should be conducted, this finding confirmed the genetic ability of the strains to produce the toxin. This ability represents a risk for consumers, as although flavedo seems to work as a barrier for such substance (Magnani et al. 2007), when rot reaches the pulp or when the disease starts as heart rot, the edible parts might contain the main *Alternaria* toxins alternariol, alternatiol monomethyl ether and tenuazonic acid (Logrieco et al. 2003). Similarly, in 2014–2015, the presence of *Alternaria* toxins was detected in 65.2 and 64.0% of maize and wheat samples, respectively, analysed in Albania (Topi et al. 2019). Furthermore, the contamination might have a significance from a disease control perspective, as the role of alternariol as pathogenicity factor has been recently highlighted (Wenderoth et al. 2019).

On the contrary, none of them proved to possess *act1* and *act2* (key genes of ACTT biosynthesis) or *acr1* and *acr2* (key genes of ACRT biosynthesis) (data not shown). Thus, none of them could be classified as *f. sp. citri* according to recent bibliography (Woudenberg et al. 2015). In particular, the ability to produce ACTT would have characterised the *f. sp. citri* strains as belonging to pathovar tangerine, whereas the ACRT as *f. sp. citri* pathovar rough lemon. This discrepancy between identification of AC24 strain as *f. sp. citri* and actual genetic ability to produce the phytotoxin is not surprising as the genes coding for the biosynthesis of phytotoxins are contained on Conditionally Dispensable

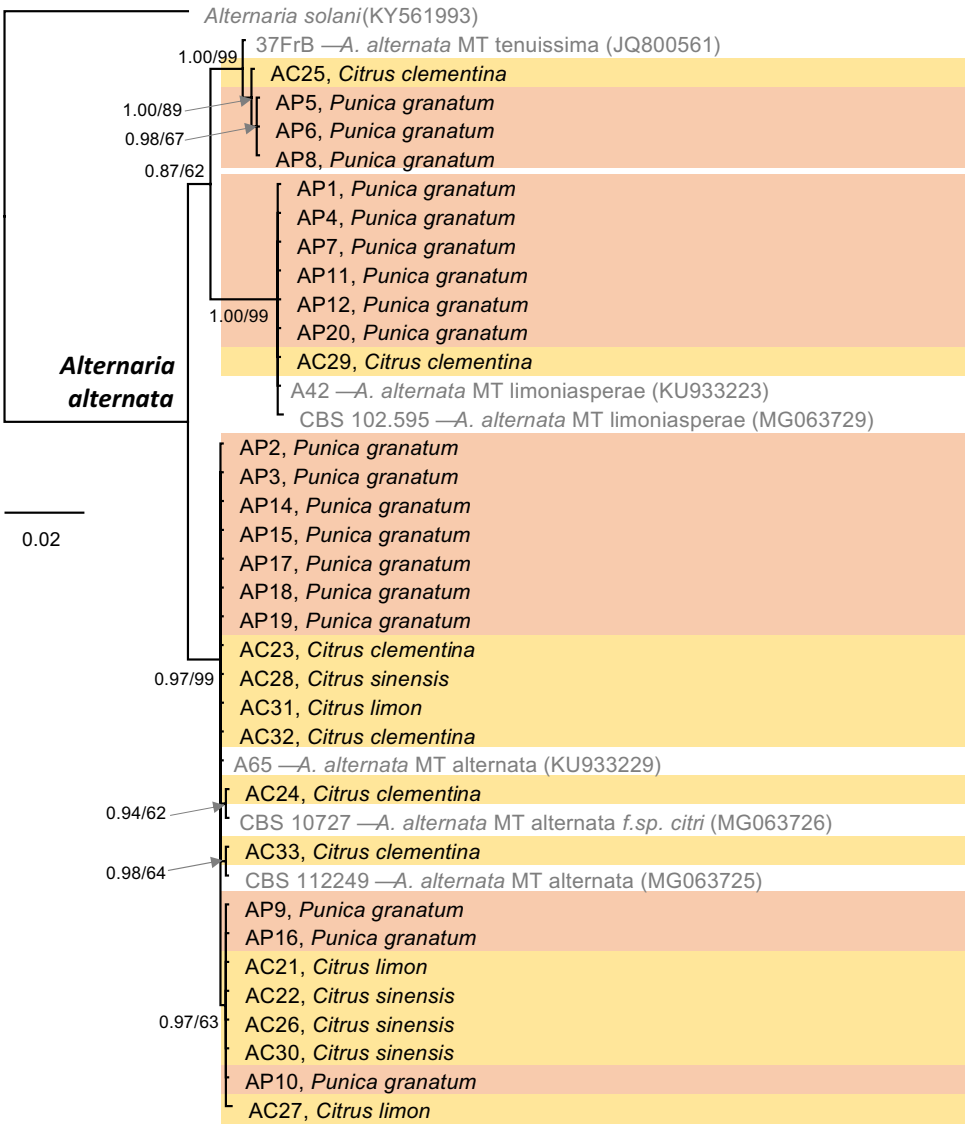

Fig. 3 Phylogenetic tree for pomegranate (in red) and citrus (in yellow) *Alternaria* strains based on SCAR Marker OPA1-3. Numbers on nodes represent Bayesian posterior probabilities and the Maximum Likelihood bootstrap percentages. Branch lengths are proportional to the numbers of nucleotide substitutions and are measured using the bar scale (0.02)

Chromosomes (CDC) that may be gained or lost by the fungal strain (Meena et al. 2017).

Pathogenicity assays

The selected strain of *A. alternata* from citrus (AC28) caused necrosis around the inoculation points of all inoculated clementine fruit, expanding into surrounding tissues. The lesions were fast-growing, almost circular, brown water-soaked spots, quickly turning into dark brown, necrotic spots. No symptoms were observed on control fruit. The fungus was reisolated and identified.

Similarly, the representative strain of *A. alternata* from pomegranate (AP18) induced typical symptoms of heart rot on artificially inoculated pomegranate fruits and was reisolated from rotten arils. In each symptomatic fruit, the rot extended to about half of the entire longitudinal section. No symptoms were observed on control fruit. Those symptoms were those observed on naturally contaminated fruits and associated with *Alternaria* spp. infections in similar studies on citrus and pomegranate fruits (Garganese et al. 2016; Aloi et al. 2021).

Conclusions

The presented findings highlight the presence of the genus *Alternaria* as pathogen of citrus and pomegranate fruits cultivated and marketed in Albania with a certain geographical distribution. To the best of our knowledge, no previous reports exist in Albania. Although further large-scale studies would be needed to further evaluate the conditions that favour the disease in Albania, this study provides a first indication of its relevance. The strains could compromise

fruit quality and safety being genetically able to produce the mycotoxin alternariol. As such, attention should be paid to its detection and control by national governing authorities.

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References

Aloi F, Riolo M, Sanzani SM, Mincuzzi A, Ippolito A, Siciliano I, Pane A, Gullino ML, Cacciola SO (2021) Characterization of *Alternaria* species associated with heart rot of pomegranate fruit. J Fungi 7(3):172

Andersen B, Dongo A, Pryor BM (2008) Secondary metabolite profiling of *Alternaria dauci*, A. *porri*, A. *solani*, and A. *tomatophila*. Mycol Res 112(2):241–250

Brun S, Madrid H, Gerrits Van Den Ende B, Andersen B, Marinach-Patrice C, Mazier D, De Hoog GS (2013) Multilocus phylogeny and MALDI-TOF analysis of the plant pathogenic species *Alternaria dauci* and relatives. Fungal Biol 117(1):32–40

Chandra R, Babu KD, Jadhav VT, Teixeira da Silva JA (2010) Origin, history and domestication of pomegranate. Fruit Veg Cereal Sci Biotechnol 4(2):1–6

EFSA (2011) Scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. EFSA J 9(10):2407

EFSA (2016) Dietary exposure assessment to *Alternaria* toxins in the European population. EFSA J 14(12):4654

Escrivá L, Oueslati S, Font G, Manyes L (2017) *Alternaria* mycotoxins in food and feed: an overview. J Food Qual 1569748:1–20

FAOSTAT (2019) http://www.fao.org/faostat/en/#home. Accessed 20 Aug 2021

Garganese F, Schena L, Siciliano I, Prigigallo MI, Spadaro D, De Grassi A, Ippolito A, Sanzani SM (2016) Characterization of citrus-associated *Alternaria* species in mediterranean areas. PLoS ONE 11(9):e0163255

Gilardi G, Garibaldi A, Gullino ML (2018) Emerging pathogens as a consequence of globalization and climate change: leafy vegetables as a case study. Phytopathol Mediterr 57(1):146–152

Higgins DG, Sharp PM (1988) CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 73(1):237–244

Huang F, Fu Y, Nie D, Stewart JE, Peever TL, Li H (2015) Identification of a novel phylogenetic lineage of *Alternaria alternata* causing citrus brown spot in China. Fungal Biol 119(5):320–330

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35(6):1547–1549. https://doi.org/10.1093/molbev/msy096

Logrieco A, Bottalico A, Mulé G, Moretti A, Perrone G (2003) Epidemiology of toxigenic fungi and their associated mycotoxins for some Mediterranean crops. Eur J Plant Pathol 109:645–667

Luo Y, Hou L, Förster H, Pryor B, Adaskaveg JE (2017) Identification of *Alternaria* species causing heart rot of pomegranates in California. Plant Dis 101(3):421–427

Magnani RF, De Souza GD, Rodrigues-Filho E (2007) Analysis of alternariol and alternariol monomethyl ether on flavedo and albedo tissues of tangerines (*Citrus reticulata*) with symptoms of *Alternaria* brown spot. J Agric Food Chem 55(13):4980–4986

Meena M, Samal S (2019) *Alternaria* host-specific (HSTs) toxins: an overview of chemical characterization, target sites, regulation and their toxic effects. Toxicol Rep 6:745–758

Meena M, Gupta SK, Swapnil P, Zehra A, Dubey MK, Upadhyay RS (2017) *Alternaria* toxins: potential virulence factors and genes related to pathogenesis. Front Microbiol 8:1451

Ollitrault P, Navarro L (2011) Citrus. In: Badenes M, Byrne D (eds) Fruit breeding: handbook of plant breeding 8. Springer, Boston, pp 623–662

Pavon MA, Gonzalez Alonso I, Martín de Santos R, García Lacarra T (2012) The importance of genus *Alternaria* in mycotoxins production and human diseases. Nutr Hosp 27(6):1772–1781

Peever TL, Su G, Carpenter-Boggs L, Timmer LW (2004) Molecular systematics of citrus-associated *Alternaria* species. Mycol 96(1):119–134

Pryor BM, Michailides TJ (2002) Morphological, pathogenic, and molecular characterization of *Alternaria* isolates associated with *Alternaria* late blight of pistachio. Phytopathol 92(4):406–416

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61(3):539–542

Sanzani SM, Gallone T, Garganese F, Caruso AG, Amenduni M, Ippolito A (2019) Contamination of fresh and dried tomato by *Alternaria* toxins in southern Italy. Food Addit Contam Part A 36(5):789–799

Sanzani SM, Djenane F, Incerti O, Admane N, Mincuzzi A, Ippolito A (2021) Mycotoxigenic fungi contaminating greenhouse-grown tomato fruit and their alternative control. Eur J Plant Pathol 160(2):287–300

Simmons EG (2007) Alternaria: an identification manual: Fully illustrated and with Catalogue Raisonné 1796–2007. CBS biodiversity Centre, Utrecht

Skreli E, Imami D (2019) Citrus sector study. https://aasf.com.al/wp-content/uploads/2019/08/Citrus-EN.pdf.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10(3):512–526

Timmer LW, Peever TL, Solel ZVI, Akimitsu K (2003) *Alternaria* diseases of citrus-novel pathosystems. Phytopathol Mediterr 42(2):99–112

Topi D, Tavčar-Kalcher G, Pavšič-Vrtač K, Babič J, Jakovac-Strajn B (2019) *Alternaria* mycotoxins in grains from Albania: alternariol, alternariol monomethyl ether, tenuazonic acid and tentoxin. World Mycotoxin J 12(1):89–99

Wenderoth M, Garganese F, Schmidt-Heydt M, Soukup ST, Ippolito A, Sanzani SM, Fischer R (2019) Alternariol as virulence and colonization factor of *Alternaria alternata* during plant infection. Mol Microbiol 112(1):131–146

Woudenberg JHC, Groenewald JZ, Binder M, Crous PW (2013) Alternaria redefined. Stud Mycol 75:171–212

Woudenberg JHC, Seidl MF, Groenewald JZ, De Vries M, Stielow JB, Thomma BPHJ, Crous PW (2015) *Alternaria* section *Alternaria*: species, *formae speciales* or pathotypes. Studies Mycol 82:1–21

Xhuveli L (2012) Albania, the domestication country for pomegranate (*Punica granatum* L.). Genetic Res Crop Evol 59(8):1605–1610

Zarfeshany A, Asgary S, Javanmard SH (2014) Potent health effects of pomegranate. Adv Biomed Res 3:100