Food Control 172 (2025) 111204

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Qualitative and relative abundance analysis of *Alternaria alternata* metabolites in pomegranate (*Punica granatum* L.) cultivars affected by Heart Rot using UHPLC-Q-TOF-MS

Mario Riolo [a,*], Carlos Luz [c], Cristian Bua [a], Salvatore Barreca [b], Maria Catena Tambè [a], Jorge Calpe [c], Marco Masi [d], Alessio Cimmino [d], Gaetano Tirrò [a], Giuseppe Meca [c], David Ezra [e], Santa Olga Cacciola [a]

[a] Department of Agriculture, Food and Environment, University of Catania, 95123, Catania, Italy
[b] Department of Chemical Sciences, University of Catania, Viale A. Doria 6, Catania, 95100, Italy
[c] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Burjassot, Spain
[d] Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario Monte S. Angelo, Via Cintia 4, 80126, Napoli, Italy
[e] Department of Plant Pathology and Weed Research, Agricultural Research Organization, ARO, The Volcani Institute, P.O. Box 15159, Rishon LeZion, 7528809, Israel

ARTICLE INFO

Keywords:
Punica granatum
Alternaria alternata
Mycotoxins
UHPLC-Q-TOF-MS
Heart rot
Punicic acid
Biomarkers

ABSTRACT

Secondary metabolites of *Alternaria alternata* in juice extracted from fruit of pomegranate (*Punica granatum*) 'Acco' and 'Wonderful' affected by heart rot were identified using the UHPLC-Q-TOF-MS analytical method. Juice from healthy and infected fruit was analyzed comparatively with particular focus on mycotoxin content. Overall, 22 metabolites were detected, including the mycotoxins tenuazonic acid, alternariol, alternariol monomethyl ether (AME) and altenuene (ALT). Multivariate analyses, including Principal Component Analysis and Random Forest models, clearly separated the metabolic profiles of healthy and infected fruit. Symptomatic fruits, especially those of 'Wonderful', exhibited markedly higher levels of mycotoxins, such as alternarlactone A and altertoxin, suggesting that these metabolites are potential biomarkers for early detection of heart rot. In contrast, they were characterized by a substantial lower level of punicic acid, indicating heart rot infection affected negatively the nutraceutical value of juice due to the depletion of the content in this omega−5 fatty acid. This study provides better insights into the effects of *A. alternata* infection on the quality of pomegranate juice as well as new evidences in favour of a regulation of the mycotoxin level in pomegranate juice.

1. Introduction

Pomegranate (*Punica granatum* L.) is an ancient fruit-bearing shrub native to central Asia. Over the centuries, it has spread widely due to its nutritional and medicinal qualities and is now cultivated commercially in various Mediterranean countries, including Italy, Israel, Spain, Turkey, Greece, Cyprus and North African countries (Aloi et al., 2021; Berbegal et al., 2014; El Barnossi et al., 2021; Faedda et al., 2015; Holland & Bar-Ya'akov, 2018; Tziros et al., 2008). In recent years, Italy has seen a rapid expansion in pomegranate cultivation, growing from approximately 130 ha in 2013 to over 1200 ha by 2019 (Aloi et al., 2021; Faedda et al., 2015). Among the imported cultivars, 'Wonderful' whose origin as a cultivar is somewhat controversial (USA/Israel) and 'Acco', of Israeli origin, have become two of the most popular varieties in Italy due to their high suitability for fresh consumption and juice production (Aloi et al., 2021; Tirrò et al., 2024). The growing interest in pomegranate stems also from its rich content in bioactive compounds, including polyphenols, anthocyanins, and other antioxidants with recognized anti-inflammatory effects (Valero-Mendoza et al., 2023). However, pomegranate cultivation faces challenges from *Alternaria* species, responsible for economically relevant diseases of this crop, such as Alternaria black spot and heart rot, the latter also known as Alternaria heart rot or black heart (Aloi et al., 2021; Rovetto et al., 2024; Tirrò et al., 2024). Diverse species and species complex of *Alternaria* are associated with heart rot disease, with *Alternaria alternata* (Fr.) Keissel being the prevalent one (Aloi et al., 2021; Ezra et al., 2015, 2019; Faedda et al., 2015; Gat et al., 2012; Luo et al., 2017; Mincuzzi et al., 2022). Typical symptoms of pomegranate heart rot are internal while

* Corresponding author.
E-mail address: mario.riolo@unict.it (M. Riolo).

https://doi.org/10.1016/j.foodcont.2025.111204
Received 10 December 2024; Received in revised form 5 February 2025; Accepted 6 February 2025
Available online 10 February 2025

external symptoms on the fruit peel, if any, are extremely difficult to recognize (Aloi et al., 2021; Ezra et al., 2015). As a consequence, affected fruit may go undetected and escape selection, especially in the initial stages of infection (Munera et al., 2025; Zhang & McCarthy, 2012). *Alternaria* is one of the major mycotoxigenic fungal genera (Escrivá et al., 2017; Logrieco et al., 2009; Masiello et al., 2020; Pinto & Patriarca, 2017; Prelle et al., 2013; Rovetto et al., 2023; Siciliano et al., 2015). Overall, more than 70 toxic metabolites produced by *Alternaria* species have been identified in agricultural products, food and beverages, including among others tenuazonic acid, alternariol, and alternariol monomethyl ether (AME) (Carbonell-Rozas et al., 2024; Saleh et al., 2024). These compounds, in particular, are of concern for food security due to their acute and chronic toxic effects in humans (Escrivá et al., 2017; EFSA on Contaminants in the Food Chain (CONTAM), 2011). Moreover, they are heat-stable and resist industrial processes, meaning they can be found both in fresh fruits and processed products, including fruit juices (Ali et al., 2023; Bullerman & Bianchini, 2007; Carballo et al., 2018; Carbonell-Rozas et al., 2024; Pallarés et al., 2019; Sun et al., 2023). Levels of contamination of plant products by mycotoxins may vary depending on several factors, including among others cultivar susceptibility to fungal infections, climate, storage condition and mechanical injures due to improper handling or weather events (Carballo et al., 2018; Casu et al., 2024; Leggieri et al., 2021; Oteiza et al., 2017; Rovetto et al., 2023). In general, the ability of fungi to produce secondary metabolites is influenced by environmental conditions and in particular by the type of substrate (Elhamouly et al., 2022; Nawrot-Chorabik et al., 2022; Pusztahelyi et al., 2015). The European Union, through Recommendation (EU) 2022/553 (EUR-Lex, 2022), has highlighted the importance of monitoring *Alternaria* toxins, such as alternariol, alternariol monomethyl ether (AME), and tenuazonic acid, in food products such as tomato paste, fruit juices, and other processed products. While these mycotoxins are not subject to mandatory safety limits, they are monitored through indicative thresholds, aimed at guiding research and data collection on contamination levels, especially in organic and concentrated food forms. Previous studies demonstrated that *Alternaria* species responsible for heart rot of pomegranate produce mycotoxins *in vitro,* but their ability to produce mycotoxins in infected fruit has been poorly investigated (Aloi et al., 2021). The detection of mycotoxins in food and feed has been performed with various analytical techniques (Alshannaq & Yu, 2017; Carballo et al., 2018; Shi et al., 2018; Wang et al., 2022). Commonly used methods include Enzyme-Linked Immunosorbent Assay (ELISA), High-Performance Liquid Chromatography (HPLC), Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), Gas Chromatography (GC), Thin-Layer Chromatography (TLC), and Ultra-Performance Liquid Chromatography (UPLC) (Alshannaq & Yu, 2017; Shi et al., 2018; Wang et al., 2022). Among these, Liquid Chromatography (LC) combined with Mass Spectrometry (MS) is widely regarded as a rapid, cost-effective, and precise approach for detecting multiple mycotoxins in complex matrices (Carballo et al., 2018; Shi et al., 2018). In recent years, High-Performance Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-Q-TOF-MS) has emerged as a highly efficient analytical tool due to its exceptional sensitivity, precision, and resolution, along with its ability to provide rapid detection of multiple metabolites, including mycotoxins, in complex matrices (Allen & McWhinney, 2019; Bang et al., 2023; Castillo et al., 2016; Conti Taguali et al., 2024; Dasí-Navarro et al., 2022; Dopazo et al., 2021, 2022; He et al., 2023; Huang et al., 2022; Khan & Ali, 2015; Masiá et al., 2014; Riolo et al., 2023; Rovetto et al., 2023; Silva et al., 2019; Sun et al., 2023; Vijayalakshmi et al., 2023). This technique has been used to explore changes in fungal metabolomics profiles induced by biopreservative microorganisms (Dopazo et al., 2021, 2022; La Bella et al., 2024) and analyze secondary metabolites produced by several fungal pathogens in citrus fruit (Rovetto et al., 2023). In the present study, UHPLC-Q-TOF-MS analysis was employed to characterize the secondary metabolites of *Alternaria* in juice extracted from fruit of pomegranate 'Acco' and 'Wonderful' affected by heart rot, as a preliminary step toward evaluating the risk of contamination by mycotoxins.

2. Material and methods

2.1. Pomegranate fruit sampling

On September 10, 2024, mature fruits from two pomegranate cultivars, 'Wonderful' and 'Acco', were collected from two 9-year-old commercial organic-farming orchards located in two distinct production zones (around 6 km from each other), in the province of Catania (eastern Sicily). Two distinct types of fruit were selected for each pomegranate: fruits exhibiting typical internal symptoms of heart rot (selected preliminarily on the basis of a slight external peel discoloration and confirmed upon cutting in the laboratory), and asymptomatic fruits with no visible internal symptoms (Fig. 1). Fruits with wounds, scars or peel russeting were not sampled. In each orchard, fruits were collected from four trees of each cultivar (one to three fruits per tree), randomly selected across the two diagonals of the orchard among trees bearing both heart rot-affected and asymptomatic fruits. In total, 72 fruits of 'Acco' and 72 fruits of 'Wonderful', at stages 81 and 85 of the BBCH scale (Melgarejo et al., 1997), were sampled from the two orchards. The following was performed for fruits of each cultivar separately: 18 of the 36 heart rot-affected fruits were used for fungal isolations. The other 18 were pooled together to be processed for UHPLC-Q-TOF-MS analysis. The same was done with the asymptomatic fruit. Each of these four composite subsamples (Table 1) was divided into three replicates, each consisting of six fruits, which were processed and analyzed separately. Fruit samples were placed into separate plastic bags, inside a polystyrene container refrigerated with dry ice, and immediately shipped via express courier to the Laboratory of Food Toxicology at the Department of Preventive Medicine, Nutrition, and Food Science at the University of Valencia, Spain. Upon arrival (within 48 h) the fruits, including those affected by heart rot, did not show external symptoms of decay. Samples were then stored at 4 °C until fungal isolation or processing for UHPLC-Q-TOF-MS analysis. To this purpose, juice was extracted from the fruit.

2.2. Fungal isolation

Isolation procedures were performed from pomegranate fruits following the method of Ezra et al. (2015) with slight modifications. Fruits were preliminary washed with tap water for few seconds, blotted dry, surface-sterilized using a 3% sodium hypochlorite solution for 1 min, followed by two rinses with sterile distilled water, each lasting 30 s. Then they were sectioned using a sterile scalpel, dividing them into two groups: fruits showing internal symptoms of heart rot (Fig. 1) and asymptomatic fruits. For heart-rot affected fruits, small pieces (5 × 5 mm) of rotten aril tissue were carefully taken from the discolored areas inside the fruit. For asymptomatic fruits, tissue pieces were collected randomly from the arils. Tissue samples were blotted dry with sterile filter paper and placed in Petri dishes (three dishes per fruit) containing Potato Dextrose Agar (PDA; Oxoid Ltd., Basingstoke, UK) amended with 150 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MA, USA). Dishes were incubated in the dark at 25 °C for 5–7 days. Pure cultures of each fungal isolates were obtained by single-hypha subcultures on PDA. The isolates were stored in the culture collection of the Laboratory of Molecular Plant Pathology of the Department of Agriculture, Food, and Environment (Di3A) at the University of Catania.

2.3. Molecular characterization

The fungal isolates recovered from symptomatic pomegranate fruits were cultured on Potato Dextrose Agar (PDA) for 7 days at 25 ± 1 °C. Afterward, mycelium from each isolate was carefully collected using a sterile scalpel, and genomic DNA was extracted using a PowerPlant® Pro

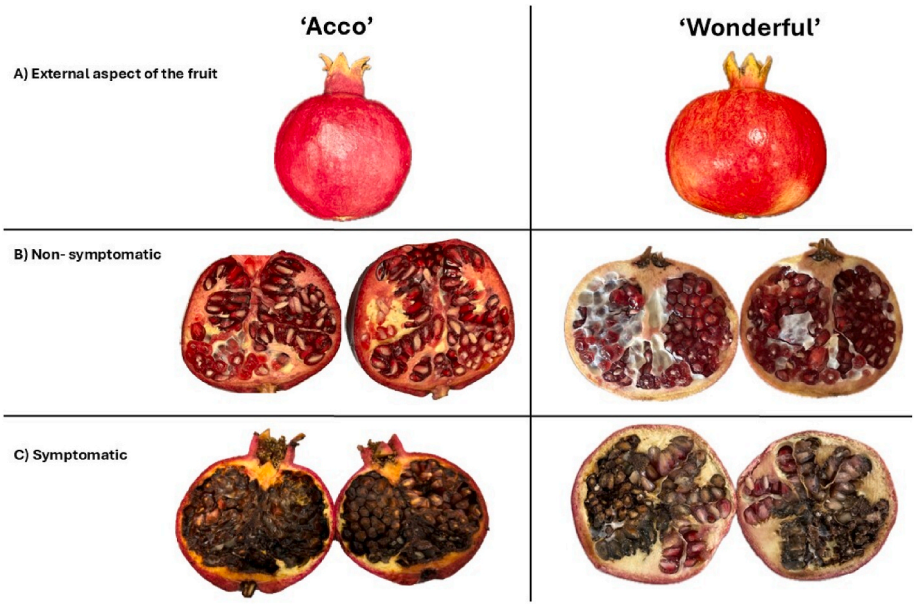

Fig. 1. Comparison of asymptomatic and heart rot-affected fruit of two pomegranate varieties, Acco (left) and Wonderful (right). (A) External aspect of the pomegranate fruit without visible differences between asymptomatic and symptomatic fruit; (B) Cross-sections of asymptomatic fruit; (C) Cross-sections of symptomatic fruit.

Table 1
Experimental design for sampling and processing to analyze the metabolite profile of juice extracted from fruits of two pomegranate cultivars.

Cluster	Number of fruits	Cultivar
Asymptomatic fruits ('Acco'_healthy)	18[a]	'Acco'
Heart rot-affected fruits ('Acco'_affected)	18	
Asymptomatic fruits ('Wonderful'_healthy)	18	'Wonderful'
Heart rot-affected fruits ('Wonderful'_affected)	18	

[a] Three replicates, each of 6 fruits.

DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's instructions. The extracted DNA was stored at −20 °C for further use.

To characterize and determine the phylogenetic relationship of the isolates from pomegranate fruits showing symptoms of heart rot, a multilocus sequence analysis was performed. Sequencing was conducted on four barcode gene regions commonly used for fungal identification: the internal transcribed spacer (ITS), translation elongation factor 1-α (EF-1α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and a specific SCAR marker (OPA10–2). The primers used for amplifying these regions were: ITS1/ITS4 for the ITS region (White et al., 1990), EF1-728 F/EF1-986 R for the EF-1α gene (Carbone & Kohn, 1999), GPD1/GPD2 for the GAPDH gene (Berbee et al., 1999) and OPA10-2R/OPA10-2 L for the OPA10-2 SCAR marker (Aloi et al., 2021; Andrew et al., 2009; Tirrò et al., 2024). PCR amplifications were carried out using the GeneAmp PCR System 9700 (Applied Biosystems, Monza-Brianza, Italy) and Taq DNA polymerase (Invitrogen™) in a total reaction volume of 25 μL. The reaction mixture included a PCR buffer (1 ×), a dNTP mix (0.2 mM), $MgCl_2$ (1.5 mM), forward and reverse primers (0.5 μM each), Taq polymerase (1 U), and 1 μL of genomic DNA. The PCR conditions included an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C (ITS), 58 °C (EF-1α), 54 °C (GAPDH), or 62 °C (OPA10-2) for 30 s, and extension at 72 °C for 30 s, with a final extension step at 72 °C for 10 min.

The amplified DNA products were visualized on a 1% agarose gel, and purified amplicons were sequenced in both forward and reverse directions by Macrogen Europe (Amsterdam, The Netherlands). Sequence data were analyzed using FinchTV v.1.4.0, and the resulting sequences were submitted to GenBank. For molecular identification, sequences were initially compared with those reported in the literature and publicly available on the NCBI BLAST platform (https://blast.ncbi.nlm.nih.gov/Blast, accessed on November 10, 2024) as well as in the Mycobank (http://www.mycobank.org, accessed on November 10, 2024) and CBS-KNAW databases (Westerdijk Fungal Biodiversity Institute, http://www.westerdijkinstitute.nl, accessed on November 10, 2024). Phylogenetic analysis was performed using sequences from this study's isolates alongside validated reference sequences from GenBank (Table 2). Prior to analysis, duplicate reference sequences were removed using Elim Dupes software. MUSCLE was used for sequence alignment, and phylogenetic trees were generated with MEGA6.

2.4. Sample preparation and extraction of pomegranate juice

Fresh pomegranate juice from each category (heart rot-affected and asymptomatic fruit) was extracted using a manual juicer. After extraction, each juice sample was filtered through qualitative grade 2 filter paper with a particle size retention of 8 μm (Whatman® Filter Paper, Sigma-Aldrich, USA) to remove aril fragments. The filtered samples were then centrifuged at 4 °C and 3000 rpm for 10 min. The resulting supernatant was carefully collected and transferred into individual conical polytetrafluoroethylene (PTFE) centrifuge tubes (15 mL). Prior to extraction, all samples were stored in polyethylene tubes at −80 °C.

2.5. Extraction of fungal secondary metabolites from pomegranate juice

The extraction of fungal secondary metabolites from pomegranate juice was performed following the method of Rovetto et al. (2023). Specifically, 10 mL of pomegranate juice and 20 mL of ethyl acetate (used as the extraction solvent) were added to a 50 mL PTFE centrifuge tube. The mixture was thoroughly shaken for 1 min and then centrifuged at 4000 rpm for 10 min at 5 °C using an Eppendorf centrifuge 5810 R (Eppendorf, Hamburg, Germany). The upper organic phase was carefully transferred to a separate 15 mL PTFE centrifuge tube and set aside. All collected organic phases were evaporated to dryness under a nitrogen stream using a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA). The resulting dry extract was reconstituted in 1 mL of methanol, then filtered through a 0.22-μm nylon syringe filter (13 mm, Membrane

Table 2
GenBank accession numbers of sequences of the *Alternaria* spp. isolates of different country and host origins used as references in phylogenetic analyses.

Species	Isolate	Country	Host	Source	Accession numbers			
					ITS	EF-1α	GPDH	OPA 10-2
Alternaria alternata (ex citri)	CBS 102.47	USA	*Citrus sinensis*	Woudenberg et al. (2015)	KP124304	KP125080	KP124161	KP124610
A. alternata (ex-type)	CBS 916.96	India	*Arachis hypogaea*	Woudenberg et al. (2015)	AF347031	KC584634	AY278808	KP124632
A. alstroemeriae	CBS 118808	USA	*Alstroemeria* sp.	Woudenberg et al. (2015)	KP124296	KP125071	KP124153	KP124601
A. alternata	AaMDc5b	Italy	*Punica granatum*	Aloi et al. (2021)	MW580731	MW585112	MW590490	MW590532
A. alternata	AaMDc5d	Italy	*P. granatum*	Aloi et al. (2021)	MW580755	MW585133	MW590514	MW590556
A. alternata	AaMMH6b	Italy	*P. granatum*	Aloi et al. (2021)	MW580756	MW585134	MW590515	MW590557
A. alternata	MNC2	Italy	*P. granatum*	Tirrò et al. (2024)	PP791895	PP820783	PP803580	PP803610
A. alternata	MNC4	Italy	*P. granatum*	Tirrò et al. (2024)	PP791896	PP820784	PP803581	PP803611
A. alternata	MNC10	Italy	*P. granatum*	Tirrò et al. (2024)	PP791902	PP820790	PP803587	PP803617
A. alternata	CBS 102595	USA	*C. jambhiri*	Woudenberg et al. (2015)	FJ266476	KC584666	AY562411	KP124636
A. alternata	CBS 112252	–	–	Woudenberg et al. (2015)	KP124340	KP125116	KP124194	KP124650
A. alternata	CBS 117.44	Denmark	*Godetia* sp.	Woudenberg et al. (2015)	KP124303	KP125079	KP124160	KP124609
A. alternata	CBS 916.96	India	*Arachis hypogaea*	Woudenberg et al. (2015)	AF347031	KC584634	AY278808	KP124632
A. arborescens	AaMDc1b	Italy	*P. granatum*	Aloi et al. (2021)	MW580737	MW585151	MW590496	MW590538
A. arborescens	AaMRa1	Italy	*P. granatum*	Aloi et al. (2021)	MW580759	MW585153	MW590518	MW590560
A. arborescens	CBS 108.41	–	wood	Woudenberg et al. (2015)	KP124394	KP125172	KP124246	KP124707
A. arborescens	CBS 109730	USA	*Solanum lycopersicum*	Woudenberg et al. (2015)	KP124399	KP125177	KP124251	KP124713
A. arborescens	CBS 112749	South Africa	*Malus domestica*	Woudenberg et al. (2015)	KP124401	KP125179	KP124253	KP124715
A. arborescens	CBS 115517	South Africa	*M. domestica*	Woudenberg et al. (2015)	KP124404	KP125182	KP124256	KP124718
A. arborescens	AaMDc1a	Italy	*P. granatum*	Aloi et al. (2021)	MW580736	MW585150	MW590495	MW590537
A. betae-kenyensis	CBS 118810	Kenya	*Beta vulgaris* var. *cicla*	Woudenberg et al. (2015)	KP124419	KP125197	KP124270	KP124733
A. burnsii	CBS 107.38	India	*Cuminum cyminum*	Woudenberg et al. (2015)	KP124420	JQ646305	KP125198	KP124734
A. eichhorniae	CBS 119778	Indonesia	*Eichhornia crassipes*	Woudenberg et al. (2015)	KP124426	KP125205	KP124277	KP124741
A. jacinthicola	CBS 878.95	Mauritius	*Arachis hypogaea*	Woudenberg et al. (2015)	KP124437	KP125216	KP124286	KP124753
A. longipes	CBS 540.94	USA	*Nicotiana tabacum*	Woudenberg et al. (2015)	AY278835	KC584667	AY278811	KP124758
A. tomato	CBS 103.30	–	*S. lycopersicum*	Woudenberg et al. (2015)	KP124445	KP125224	KP124294	KP124762

Solutions) into a 2 mL amber vial for subsequent chromatography analysis.

2.6. Identification of fungal secondary metabolites in pomegranate juice by UHPLC-Q-TOF-MS analysis

Extracts of pomegranate juice were injected into the UHPLC-Q-TOF-MS system for analysis, with all measurements performed in triplicate (n = 3). The analysis utilized an UHPLC system (1290 Infinity II LC, Agilent Technologies) comprising an automatic sampler, binary pump, and vacuum degasser, coupled to a quadrupole time-of-flight mass spectrometer (Agilent 6546 LC/Q-TOF) operating in positive ionization mode (Agilent Technologies, Santa Clara, CA, USA). A 5 μL injection volume was used, and each analysis required 25 min. Chromatographic separation was achieved on an Agilent Zorbax RRHD SB-C18 column (2.1 mm × 50 mm, 1.8 μm). The mobile phases were Milli-Q water (phase A) and acetonitrile (phase B), both acidified with 0.1% formic acid. The gradient elution was set as follows: 0 min, 2% B; 22 min, 95% B; 25 min, 5% B. The column was re-equilibrated for 3 min between injections, with a flow rate of 0.4 mL/min. The Dual AJS ESI source parameters were as follows: gas temperature at 325 °C, gas flow at 10 L/min, nebulizer pressure at 40 psig, sheath gas temperature at 295 °C, sheath gas flow at 12 L/min, capillary voltage at 4000 V, nozzle voltage at 500 V, fragmentor voltage at 120 V, skimmer voltage at 70 V, product ion scan range of 100–1500 Da, MS scan rate of 5 spectra/s, MS/MS scan rate of 3 spectra/s, with a maximum of 2 precursors per cycle, and collision energies of 10, 20, and 40 eV. An untargeted LC/Q-TOF metabolomics approach was applied to identify fungal secondary metabolites in pomegranate juice. Data processing, integration, and metabolite identification were conducted using MassHunter Qualitative Analysis Software B.08.00 and PCDL Manager B.08.00, as described by Dopazo et al., 2021. A score value of 95% and a Delta mass error of 1 ppm were used as confidence intervals. For the calibration of the UHPLC-Q-TOF-MS analysis method, commercial standards for the main mycotoxins produced by *Alternaria* species, including tenuazonic acid (TeA), alternariol (AOH), alternariol monomethyl ether (AME), AAL toxin TB2, and tentoxin (TEN), were used at concentrations of 0.01, 0.1, and 5 mg/L (Zwickel et al., 2016). The LOD was determined using a signal-to-noise ratio of 3:1, while the LOQ was calculated with a signal-to-noise ratio of 10:1. These values are detailed in Table S1. Precision was assessed by intra- and interday variations, with three replicate injections per day over three consecutive days at each concentration level. Recovery tests were performed on non-contaminated pomegranate juice samples, showing mean recoveries of 82.3 ± 2.1% for TeA and 87.7 ± 2.8% for AOH. The relative standard deviations (RSDs) for intraday variation ranged from 3.5% to 4.2% for TeA, AOH, and AME, and from 3.0% to 4.6% for ALT, AAL toxin TB2, and TEN. Interday RSDs ranged from 3.9% to 4.3%, confirming consistency in the detection of these mycotoxins. Matrix effects (% MEs) were evaluated and found to be minimal for pomegranate juice, with values ranging from 90% to 93% for TeA and AOH, confirming the method's suitability for detecting *Alternaria* mycotoxins in pomegranate juice samples. Quantification of all identified metabolites was performed as follows: for compounds with available commercial standards (e.g., alternariol, tenuazonic acid), concentrations were determined using calibration curves. For metabolites without standards, a semi-quantitative approach was applied using the straight-line equation of alternariol as a reference. Metabolite identification was performed according to the confidence level classification proposed by Schymanski et al. (2014). The confidence level assigned to each metabolite is reported in Supplementary Table S1. The concentrations of metabolites in juice samples from the four experimental groups (healthy and affected fruits of 'Acco' and 'Wonderful') are detailed in Table S2.

2.7. Statistical analysis

The analytical data obtained by Agilent Ultra High-Definition Accurate Mass spectrometry were log10-transformed before statistical analysis to normalize the distribution of metabolite concentrations and improve the comparability of the data. Relationships among the detected *Alternaria* secondary metabolites in two different pomegranate juice samples ('Acco' and 'Wonderful') were assessed using Pearson's correlation coefficient analysis. Heat map, Principal Component Analysis (PCA) and Random Forest (RF) analysis were conducted with MetaboAnalyst 5.0 software (Pang et al., 2021) using log10-transformed and mean-centered data. RF analysis was performed to identify key

metabolites distinguishing infected from non-infected samples. The RF model was developed using 200 trees and a variable selection strategy based on the Mean Decrease in Accuracy metric. This approach allowed for the ranking of metabolites by their importance in the classification model, highlighting specific compounds as potential biomarkers of *Alternaria* infection in pomegranate juice.

3. Results

3.1. Morphological and molecular characterization of isolates

From pomegranate fruits with heart rot symptoms in both cultivars, 'Acco' and 'Wonderful,' a total of 60 fungal isolates were obtained, all showing morphological characteristics consistent with the genus *Alternaria*. Of the total isolates, 35% originated from 'Acco' fruits and 65% from 'Wonderful' fruits showing heart rot symptoms. No *Alternaria* isolates were obtained from healthy fruits. Colonies had a flat, woolly texture and ranged in color from dark green to black when cultured on Potato Dextrose Agar (PDA), Microscopically, the isolates produced typical dark brown, club-shaped conidia arranged in branched chains, with oval-ellipsoidal shapes and 3 to 5 transverse septa; some conidia also had 0 to 5 longitudinal septa and a prominent beak. Conidial measured between (10−) 19.8 (−26) μm in length and (6−) 8.7 (−10) μm in width, aligning with previously reported dimensions for *Alternaria* species (Aloi et al., 2021; Rovetto et al., 2023; Tirrò et al., 2024). Of the 60 isolates, a subset of 25 was randomly selected for molecular analysis, including a four-gene phylogeny, based on ITS, EF-1α, GAPDH, and OPA 10-2 sequences from 24 randomly selected isolates characterized in the present study (Table 3), along with reference CBS strains (Woudenberg et al., 2015), was performed to confirm the identity of the isolates. Phylogenetic analysis grouped all isolates with *A. alternata*, clustering them with reference strains such as *A. alternata* ex-type CBS 916.96, *A. alternata* ex *citri* CBS 102.47, and CBS 112252 *A. alternata* (Fig. 2). As the isolates belonging to the same species resulted to be identical, only sequences of representative isolates were deposited in Genbank.

Table 3
Alternaria isolates from the inner tissues of pomegranate fruit (*Punica granatum* L.) affected by heart rot, characterized in this study: cultivar, geographical origin, and GenBank accession numbers for sequences of the internal transcribed spacer (ITS), translation elongation factor 1-α (EF-1α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and SCAR marker OPA 10–2.

Isolate	Cultivar	GenBank Accession Numbers			
		ITS	EF-1α	GPDH	OPA 10-2
HRA02	Acco	PQ593058	PQ609990	PQ610014	PQ610038
HRA04	Acco	PQ593059	PQ609991	PQ610015	PQ610039
HRA05	Acco	PQ593060	PQ609992	PQ610016	PQ610040
HRA08	Acco	PQ593061	PQ609993	PQ610017	PQ610041
HRA09	Acco	PQ593062	PQ609994	PQ610018	PQ610042
HRA10	Acco	PQ593063	PQ609995	PQ610019	PQ610043
HRA14	Acco	PQ593064	PQ609996	PQ610020	PQ610044
HRA17	Acco	PQ593065	PQ609997	PQ610021	PQ610045
HRA22	Acco	PQ593066	PQ609998	PQ610022	PQ610046
HRA31	Acco	PQ593067	PQ609999	PQ610023	PQ610047
HRA35	Acco	PQ593068	PQ610000	PQ610024	PQ610048
HRA45	Acco	PQ593069	PQ610001	PQ610025	PQ610049
HRW01	Wonderful	PQ593070	PQ610002	PQ610026	PQ610050
HRW05	Wonderful	PQ593071	PQ610003	PQ610027	PQ610051
HRW11	Wonderful	PQ593072	PQ610004	PQ610028	PQ610052
HRW13	Wonderful	PQ593073	PQ610005	PQ610029	PQ610053
HRW14	Wonderful	PQ593074	PQ610006	PQ610030	PQ610054
HRW15	Wonderful	PQ593075	PQ610007	PQ610031	PQ610055
HRW17	Wonderful	PQ593076	PQ610008	PQ610032	PQ610056
HRW22	Wonderful	PQ593077	PQ610009	PQ610033	PQ610057
HRW42	Wonderful	PQ593078	PQ610010	PQ610034	PQ610058
HRW48	Wonderful	PQ593079	PQ610011	PQ610035	PQ610059
HRW59	Wonderful	PQ593080	PQ610012	PQ610036	PQ610060
HRW60	Wonderful	PQ593081	PQ610013	PQ610037	PQ610061

3.2. Identification of Alternaria alternata secondary metabolites and mycotoxins in pomegranate juice

The Agilent Ultra High-Definition Accurate Mass analysis identified 22 distinct fungal secondary metabolites, including several mycotoxins, in pomegranate juice. Metabolite identification was supported by a custom database based on known secondary metabolites produced by *Alternaria* species, as reported in the literature (Table 4). The metabolites detected by UHPLC–Q-TOF-MS in each pomegranate fruit cluster are presented as a heat map (Fig. 3). Only secondary metabolites with notable abundance differences among the clusters of healthy and infected fruit of each cultivar ('Acco' and 'Wonderful') are highlighted. In the 'Wonderful affected' cluster, most secondary metabolites showed a higher relative abundance compared to 'Acco affected', specifically SMP10 (Altenusin, ALN), SMP4 (ACTG Toxin B), SMP9, SMP19, SMP16 (AMToxin III), SMP1 ((−)-Altenuene, ALT), SMP8, SMP7 (Alternariol monomethyl ether, AME), SMP21 (Tenuazonic acid, TeA), SMP2 (Alternariol 5-*O*-sulfate), and SMP5, which were approximately 2- to 3-fold more abundant. Conversely, other metabolites, including SMP17, SMP14 (Altetoxins II, ATX II), SMP11 (Alternariol, AOH), SMP15, SMP12 (Altetoxin I, ATX I), SMP6, SMP20 (Tentoxin, TEN), and SMP18, were more abundant in 'Acco affected' compared to 'Wonderful affected', with similar fold differences. Some metabolites appeared exclusively in specific clusters. SMP3 (AAL toxin TB2) was detected only in 'Wonderful affected', while SMP13 (Altetoxins III, ATX III) was unique to 'Acco affected'. Notably, SMP22 (Punicic acid) exhibited high abundance solely in the healthy fruit clusters of both cultivars. Furthermore, the dendrogram in the heat map clearly distinguishes healthy fruits from affected fruits in both varieties, indicating distinct metabolic profiles between healthy and affected samples. Principal Component Analysis (PCA) was performed on the dataset to investigate patterns of secondary metabolites from *Alternaria* in symptomatic and asymptomatic pomegranate juice samples of the two cultivars, 'Acco' and 'Wonderful'. The sum of the principal component values accounted for 92.8% of the total variance, with the first principal component (PC1) representing 85% and the second principal component (PC2) accounting for 7.8% of the total variance. In the score plot (Fig. 4A), clear clustering of each sample group was observed, with each fruit health status and cultivar showing distinct separation in specific quadrants. Specifically, Acco affected samples clustered in quadrant I, while Wonderful healthy samples grouped in quadrant IV. In contrast, Wonderful affected samples clustered in quadrant II, and Acco healthy samples were positioned in quadrant III. The loading plot (Fig. 4B) further highlighted the metabolite distributions across the clusters. Acco affected fruits, located in quadrant I, were characterized by the secondary metabolites SMP13, SMP20, SMP12, SMP17, SMP9, SMP18, SMP6, and SMP10. Meanwhile, Wonderful affected fruits clustered in quadrant IV, associated with metabolites SMP7, SMP8, SMP5, SMP19, SMP3, SMP16, and SMP21. Quadrants II and III, corresponding to the healthy samples of 'Wonderful' and 'Acco' respectively, primarily featured SMP22. These findings indicate a clear distinction in the metabolic profiles between symptomatic and asymptomatic fruits, as well as between the two pomegranate cultivars. Random forest analysis identified the secondary metabolites with the highest discriminatory influence between the four groups of pomegranate samples: Acco healthy, Acco affected, Wonderful healthy, and Wonderful affected. The metabolites with the most significant contribution to group differentiation included SMP22, SMP8, SMP7, SMP18, and SMP6, with SMP15 and SMP5 also contributing but with slightly lower relevance (Fig. 5). The model achieved an OOB (out-of-bag) error rate of 0.0833%.

The correlation matrix (Fig. 6) highlights a series of significant correlations between punicic acid (SMP22) and mycotoxins and among various mycotoxins produced by the pathogenic fungus *A. alternata* on pomegranate. In detail, the amount of SMP22 (punicic acid) is negatively correlated with the amount of all mycotoxins except for SMP3 (AAL toxin TB2). Most metabolites exhibit strong positive correlations,

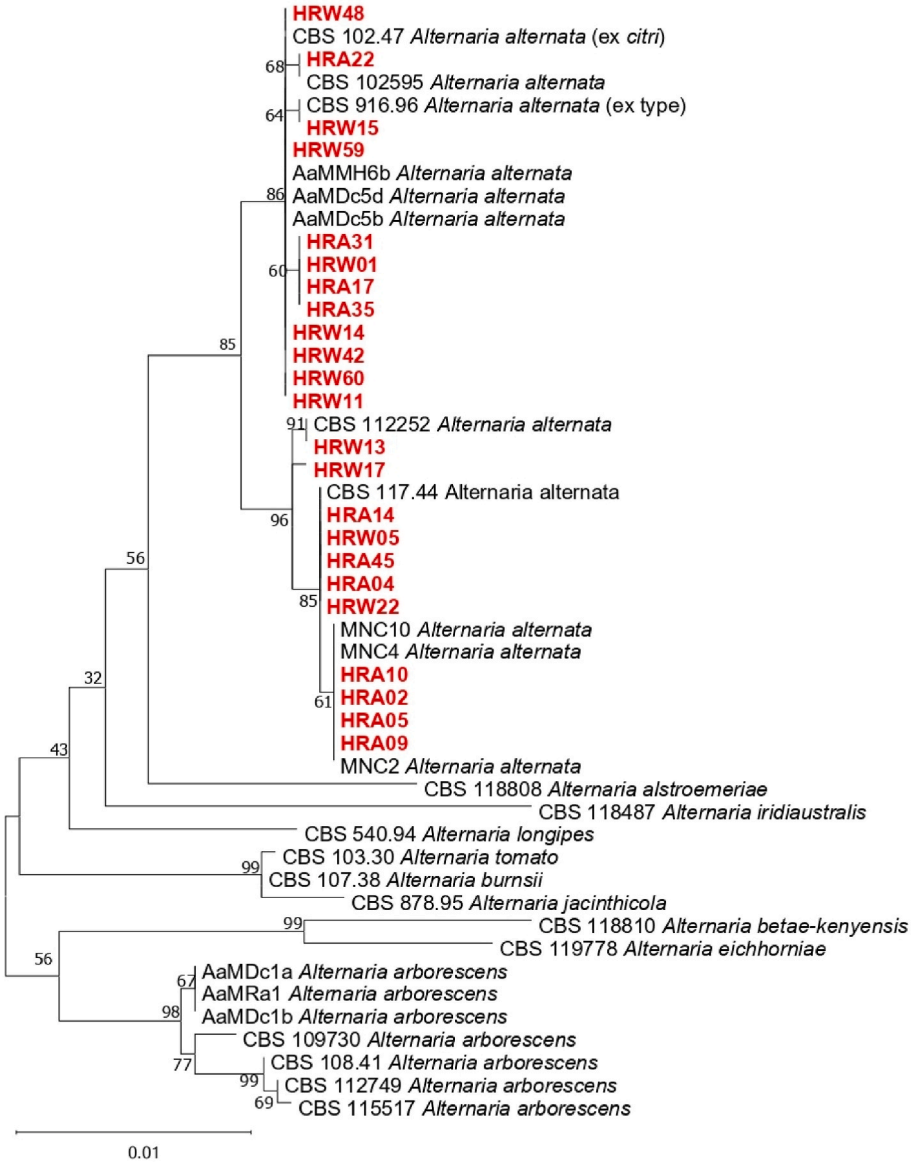

Fig. 2. Multilocus internal transcribed spacer (ITS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), translation elongation factor 1-α (EF-1α) and one SCAR marker (OPA 10–2) phylogenetic tree developed using the maximum likelihood method, based on the Tamura–Nei model. The tree with the greatest obtained log likelihood (−3882.20) is shown. Relationships between the 24 pomegranate isolates used in this study (highlighted in red), the CBS isolates of *Alternaria alternata* and other *Alternaria* species that were used as reference isolates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

except for SMP5 (alternarlactone A), which is notably elevated in "Wonderful affected" samples compared to "Acco affected". This finding suggests that SMP5 production by *A. alternata* may be more pronounced under the specific conditions associated with 'Wonderful', potentially indicating a differential response of the pathogen based on the host variety. Additionally, elevated SMP5 levels are accompanied by high levels of SMP3 (AAL toxin TB2), which appears exclusively in "Wonderful affected" samples.

4. Discussion

All fungal isolates recovered from symptomatic pomegranate fruits collected in this study were identified as *A. alternata*. The multilocus phylogenetic analysis, based on four gene regions (ITS, EF-1α, GPDH, and OPA 10–2), grouped these isolates with reference strains such as *A. alternata* ex-type CBS 916.96, *A. alternata* ex *citri* CBS 102.47, and CBS 112252 *Alternaria alternata*. Interestingly, neither ITS and EF-1α, nor GPDH, alone were able to distinguish *A. alternata* from other species of *Alternaria* that are known to infect different host plants. However, the use of multiple loci in this study allowed for the clear identification of *A. alternata* as the species responsible for the heart rot disease in the two surveyed pomegranate orchards, consistently with the findings from other studies, which also indicated *A. alternata* as the prevalent causative agent of heart rot disease in major pomegranate producing countries worldwide (Aloi et al., 2021; Ezra et al., 2015, 2019; Luo et al., 2017; Tziros et al., 2008; Vicent et al., 2016).

The main objective of this study was to identify *Alternaria* secondary metabolites in juice extracted from pomegranate fruits affected by heart rot of two renowned commercial cultivars, 'Acco' and 'Wonderful'. These two cultivars are widely cultivated in the Mediterranean region for both commercial fresh fruit production and industrial juice extraction. Analysis performed with UHPLC-Q-TOF-MS identified a total of 22 secondary metabolites of *Alternaria*, including known mycotoxins of toxicological concern. The comparison between the metabolic profiles of

Table 4
Metabolites identified in fresh pomegranate juice from symptomatic and asymptomatic fruits of 'Acco' and 'Wonderful' cultivars.

Metabolites	Chemical class	Source	ID code	Reference [a]
Altenuene (ALT)	Dibenzo-α-pyrone derivatives	*Alternaria alternata*	SMP1	Wenderoth et al. (2019)
Alternariol 5-*O*-sulfate	Dibenzo-α-pyrone derivatives	*A. alternata*	SMP2	Zwickel et al. (2016)
AAL toxin TB2	Amino polyol derivatives	*A. alternata*	SMP3	Wang et al. (2022)
ACTG Toxin B	Terpenoids	*A. alternata*	SMP4	Kono et al. (1986)
Alternarlactone A	Dibenzo-α-pyrone derivatives	*A. alternata*	SMP5	Shi et al. (2019)
Alternarienonic acid	Benzoic acid derivatives	*A. alternata*	SMP6	Aly et al. (2008)
Alternariol monomethyl ether (AME)	Dibenzo-α-pyrone derivatives	*A. alternata*	SMP7	Bensassi et al. (2011)
Altenuic acid III (AA-II)	Benzoic acid derivatives	*A. alternata*	SMP8	Puntscher et al. (2019)
Altenuisol	Dibenzo-α-pyrone derivatives	*A. alternata*	SMP9	Nemecek et al. (2012)
Altenusin (ALN)	Benzoic acid derivatives	*A. alternata*	SMP10	Escrivá et al. (2017)
Alternariol (AOH)	Dibenzo-α-pyrone derivatives	*A. alternata*	SMP11	Puntscher et al. (2019)
Altertoxin I (ATX-I)	Perylene quinones	*A. alternata*	SMP12	Puntscher et al. (2019)
Altertoxin II (ATX-II)	Perylene quinones	*A. alternata*	SMP13	Geiseler et al. (2013)
Altertoxin III (ATX-III)	Perylene quinones	*A. alternata*	SMP14	Geiseler et al. (2013)
AMToxin II	Cyclic tetradepsipeptides	*A. alternata*	SMP15	Ueno et al. (1975)
AMToxin III	Cyclic tetradepsipeptides	*A. alternata*	SMP16	Ueno et al. (1975)
AOH-3-glucoside (AOH-3-G)	Dibenzo-α-pyrone derivatives	*A. alternata*	SMP17	Scheibenzuber et al. (2020)
Desmethylaltenusin (DMA)	Benzoic acid derivatives	*A. alternata*	SMP18	Kelman et al. (2020)
Stemphyltoxin III (STTX-III)	Perylene quinones	*A. alternata*	SMP19	Puntscher et al. (2019)
Tentoxin (TEN)	Cyclic tetrapeptides	*A. alternata*	SMP20	Escrivá et al. (2017)
Tenuazonic acid (TeA)	Tetramic Acids	*A. alternata*	SMP21	Puntscher et al. (2019)
Punicic acid	Fatty acids	*Punicum granatum* L.	SMP22	Guerra-Vázquez et al. (2022)

[a] Original reports of the metabolites detected in this study.

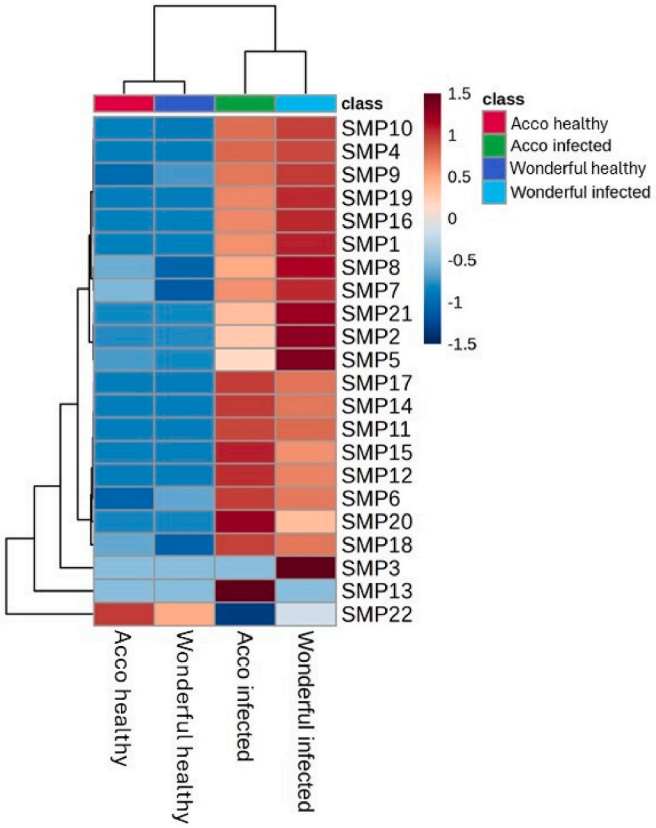

Fig. 3. Heat map representing the relative abundance of *Alternaria alternata* secondary metabolites detected in pomegranate juices. Colors are based on the relative abundance (logarithmic scale) of the secondary metabolites detected, where red represents high abundance and blue represents low abundance. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

juice from heart rot-affected and asymptomatic pomegranate fruits revealed significant differences, with juice from symptomatic fruits showing high levels of tenuazonic acid (TeA), alternariol (AOH), alternariol monomethyl ether (AME), and (−)-altenuene (ALT), consistently with a previous report which demonstrated that *Alternaria* isolates from pomegranate are able to produce these toxins *in vitro* (Aloi et al., 2021). TeA, AOH, AME and ALT, along with tentoxin (TEN), were found in tissues of diverse, taxonomically unrelated host plants infected by *A. alternata* (Rovetto et al., 2023; Thomma, 2003; Wenderoth et al., 2019).

Some of these toxins, such as TeA, AOH, and AME, have also been reported to induce toxic effects and immunosuppression in humans and animals (Pallarés et al., 2019; Saleh et al., 2024). *In vitro* tests have provided clear evidence of genotoxicity or acute toxicity in animals or rodent cells of AOH, AME, ALT, and TeA (Pallarés et al., 2019; Saleh et al., 2024; EFSA on Contaminants in the Food Chain (CONTAM), 2011). For instance, AME has been associated with immunosuppressive effects and mitochondrial apoptosis in human colon carcinoma cells (Bensassi et al., 2011). Similarly, TeA, a tetramic acid derivative, inhibits protein synthesis in mammalian cells, which makes it a concern from a public health perspective (EFSA on Contaminants in the Food Chain (CONTAM), 2011). Additionally, AOH and AME are known to be genotoxic, and AME also induces mitochondrial dysfunction, further emphasizing the toxicological relevance of these metabolites (Man et al., 2017; Wang et al., 2022). Several other biologically active metabolites, such as Altenusin (ALN), AAL-toxins, and Alternethanoxin A, were also identified in pomegranate juice in this study. Altenusin (ALN) is a multifunctional metabolite produced by *Alternaria* species, known for its antibacterial, antifungal, and antiparasitic properties (da Cruz Cabral et al., 2017). AAL-toxins, particularly AAL-toxin TE2, were also found to be prevalent in the symptomatic pomegranate fruits, specifically in the 'Wonderful' cultivar. These toxins are known to have phytotoxic, cytotoxic, and genotoxic activity (Man et al., 2017; Wang et al., 2022). AAL-toxin TE2 was recently detected at high levels in the juice of mummified fruits of two different orange cultivars (Rovetto et al., 2023). AAL-toxins are considered host-specific phytotoxins (HSTs) in several host-pathogen systems, such as *Alternaria alternata* f. sp. *lycopersici*/tomato, where they play a key role in pathogenicity (Tsuge et al., 2013; Wang et al., 2022). The presence of AAL-toxins in infected pomegranate fruit infected by *A. alternata* provides additional evidence this fungus can produce an array of toxins which varies depending on the host. In symptomatic fruits of 'Wonderful', AAL-toxins were detected in higher concentrations than in 'Acco' fruits collected in the same orchards, further confirming the host may be a major factor conditioning the ability of *A. alternata* to produce mycotoxins. Alternethanoxin A,

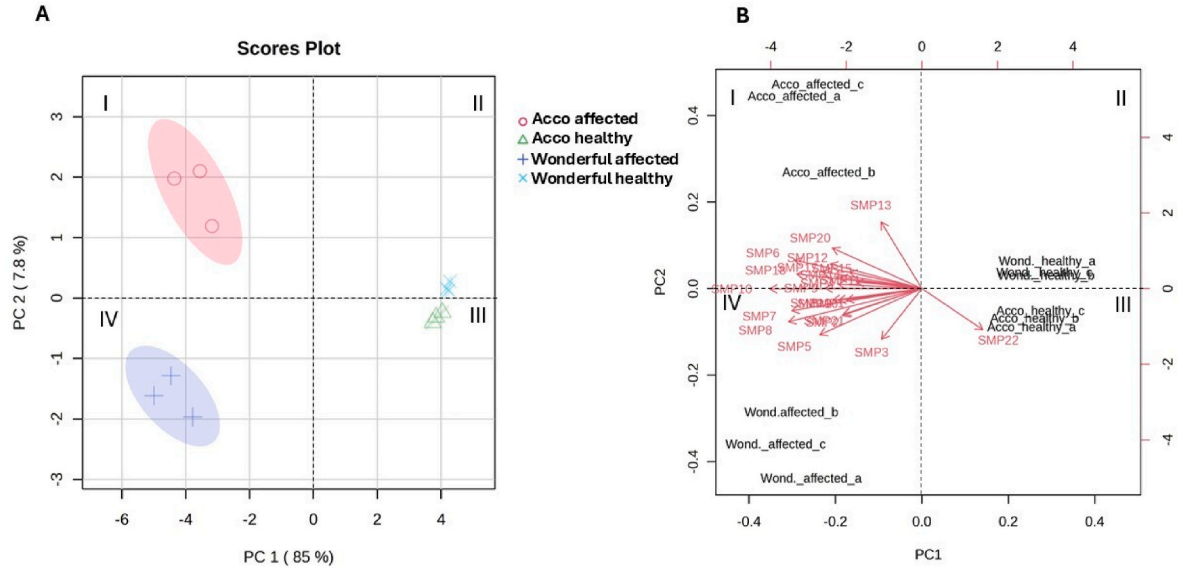

Fig. 4. Principal Component Analysis (PCA) with score plot (A) and loading plot (B) was performed based on secondary metabolites of *Alternaria alternata* detected in the juice of the 'Acco' and 'Wonderful' pomegranate cultivars. Each plot includes three distinct replicates for each fruit type: asymptomatic (healthy) and symptomatic (heart rot-affected).

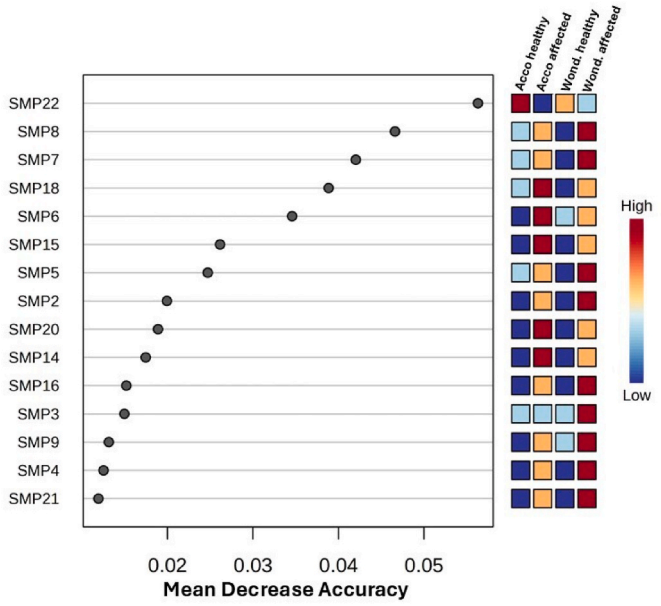

Fig. 5. Random Forest variable importance plot for the most relevant metabolites identified in this study. Higher values indicate a greater role of each metabolite in distinguishing between the different sample groups of *Punica granatum* varieties 'Acco' and 'Wonderful' (OOB error 0.0833).

another metabolite identified in this study, demonstrated inhibitory *in vitro* activity on the growth of cancer cells (Evidente et al., 2014). The cyclic tetrapeptide tentoxin (TEN), also detected in both pomegranate cultivars, has been shown to disrupt photosynthesis by selectively inhibiting chloroplast ATP synthesis, potentially contributing to tissue damage in infected fruits (Meiss et al., 2008). All these toxins were detected at high concentration in heart rot-affected pomegranate fruits. However, their role in the disease is not known. The hypothesis they can act as pathogenicity or virulence factors of *A. alternata* would deserve to be investigated. In the pathosystem *Neofusicoccum batangarum* (syn. *N. ribis*)/cactus pear it has been hypothesized that mycotoxins possessing antimicrobial activity may enhance the competitive ability of this necrotrophic fungal pathogen in colonising the plant tissues (Masi et al., 2020). Noteworthy, it was observed an inverse correlation between mycotoxin content and the concentration of punicic acid, a major bioactive compound in pomegranate seeds (Guerra-Vázquez et al., 2022; Valero-Mendoza et al., 2023). The content of punicic acid, an omega-5 polyunsaturated fatty acid classified as a conjugated linolenic acid (Goula & Adamopoulos, 2012), was substantially reduced in juice from symptomatic fruits. To explain this inverse correlation, it can be supposed that *A. alternata* mycotoxins interfere with pomegranate lipid metabolism, with a mechanism similar to that reported for fumonisins in the pathosystem *Fusarium verticillioides*/mais (Beccaccioli et al., 2021; Scala et al., 2014). The latter authors, in particular, demonstrated that oxylipins, fatty acid-derived signalling compounds, regulate negatively fumonisin synthesis. As a unique nutraceutical component of pomegranate juice, punicic acid is known for its wide spectrum of biological activities, including anti-inflammatory, anti-cancer, and hypolipidemic effects (Guerra-Vázquez et al., 2022; Kandylis & Kokkinomagoulos, 2020; Siddiqui et al., 2024; Valero-Mendoza et al., 2023). The beneficial properties of punicic acid depend on its resistance to oxidation and the critical role in regulating inflammatory and metabolic pathways, such as the activation of peroxisome proliferator-activated receptors (PPARs) (Guerra-Vázquez et al., 2022). The depletion of punicic acid in heart rot-affected fruits not only affects the nutraceutical quality of the juice, but also indicates this metabolite could be a potential biomarker for early detection of *Alternaria* infections in pomegranate fruit.

Multivariate analysis clearly separated the symptomatic and asymptomatic fruits of 'Acco' and 'Wonderful' into distinct clusters. AME and TeA were key discriminant metabolites in the profiles of juice from symptomatic fruit, suggesting they could be used, like punicic acid, as potential biomarkers for early detection of heart rot infections and mycotoxin contamination. Also, alternarlactone A, known for its antiparasitic properties (Shi et al., 2019), showed higher abundance in symptomatic fruits, suggesting a possible response of host lipid metabolism to the infection.

Overall, multivariate analysis of metabolite profiles of pomegranate fruits suggests a complex regulatory network of *A. alternata* secondary metabolism, modulated also, by the host, as shown by a markadlly higher production of AAL toxin TB2, alternarlactone A and Altertoxin II in infected fruits of 'Wonderful'. The positive correlation between Alternarlactone A and AAL toxin TB2 implies a coordinated production

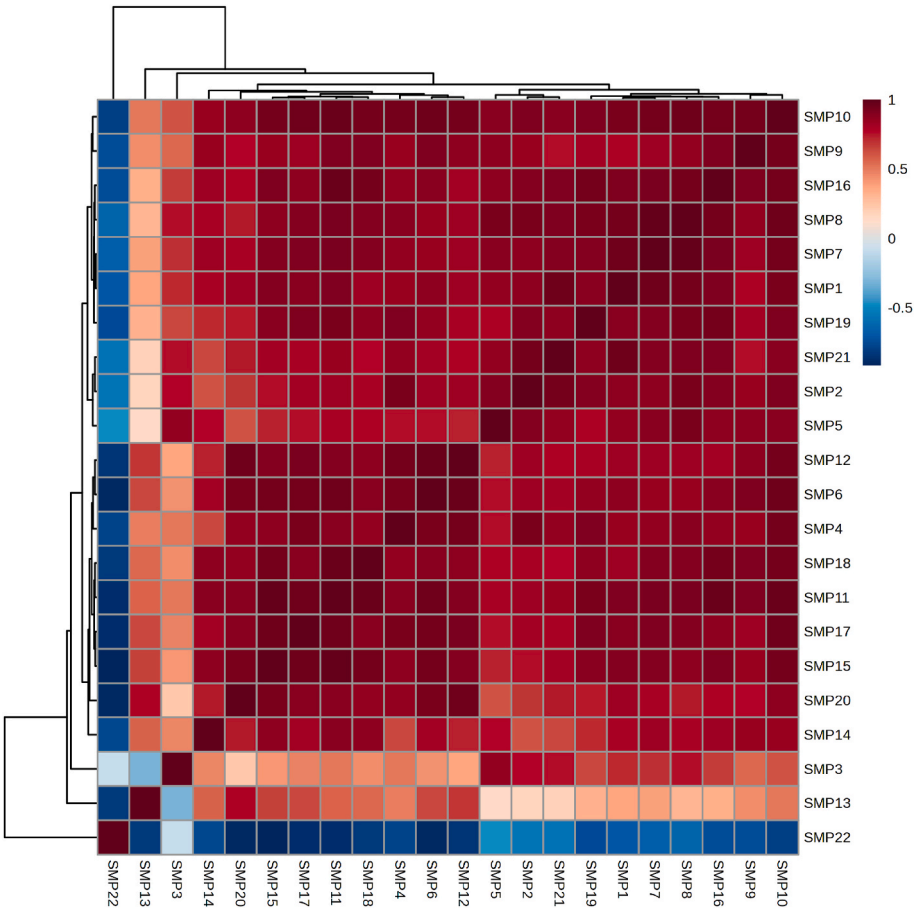

Fig. 6. Correlation matrix of the secondary metabolites produced by *Alternaria alternata* on pomegranate fruits. The heatmap shows correlation coefficients between various secondary metabolites (SMPs), with color intensity representing the strength of the correlation (blue for negative, red for positive). Distance was measured using Pearson r. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

pathway, where *A. alternata* produces Alternarlactone A in response to the presence of AAL toxin TB2, possibly as part of a broader array of toxins with similar or complementary functions. An inverse relationship between AAL toxin TB2 and Altertoxin II (ATX-II) levels was also evident. This negative correlation might reflect a resource allocation strategy by the pathogen, in which the production of one toxin inhibits the synthesis of the other. However, in this study experimental conditions were deliberately exacerbated, as each batch comprised exclusively either heart rot-affected or asymptomatic fruits, results indicate the risk of pomegranate juice contamination by *Alternaria* mycotoxins is real, also considering these toxins being heat stable are not eliminated during the industrial process of juice extraction (Aloi et al., 2021; Elhariry et al., 2016). Also the preliminary selection of fruits affected by Alternaria heart rot to reduce the risk of contamination is problematic due to the ambiguity of external disease symptoms (Aloi et al., 2021; Munera et al., 2025). Nevertheless, currently European regulations have not quantified mycotoxin limits in pomegranate juice. This issue deserves further investigation and action, also considering the consumption of pomegranate juice is expected to increase in the next years as it has been promoted for its nutritional and nutraceutical properties (Aviram et al., 2000; Bahari et al., 2024; Esposto et al., 2021; Giménez-Bastida et al., 2021; Marrone et al., 2024; Nuncio-Jáuregui et al., 2015; Onsekizoglu, 2013; Trishitman et al., 2023).

The results indicate differences in the mycotoxin profiles of fruits of the two pomegranate cultivars compared in this study. 'Acco' is considered an early-season cultivar, while 'Wonderful' ripens later (Usanmaz et al., 2014). To explain this inverse correlation profile may result, at least in part, from the ripeness grade of fruit, which implies diverse levels of sugars, acids, and other juice components and consequently may regulate the type of secondary metabolites produced by the pathogen. An additional hypothesis is that each pomegranate cultivar produces different antimicrobial compounds, either constitutive or induced by the infection, which in turn affect the pathogen secondary metabolism. The role of mycotoxins in the complex interplay between host and fungal pathogen has been investigated in detail in the pathosystem *F. verticillioides*/mais where lipid signals modulate the host response to contamination by fumonisins as well as the biology of the fungus and its ability to produce fumonisins (Righetti et al., 2021; Scala et al., 2013). As far as pomegranate heart rot is concerned, a study conducted by Ezra et al. (2019) revealed that the development of heart rot of pomegranate fruit was influenced by the cultivar and the acidity (pH) of the juice. However, as pH was not directly responsible for the inhibition of *Alternaria* conidium germination, a different juice component, likely an unidentified secondary metabolite produced by the fruit, was supposed to be the cause of the diverse disease susceptibility of pomegranate cultivars (Ezra et al., 2019).

5. Conclusions

In conclusion, this study provides new insights into the effects of *Alternaria* infection on the metabolite profile of juice extracted from the fruit of two pomegranate cultivars affected by heart rot, with a specific focus on mycotoxins. The identification of mycotoxins and cultivar-specific metabolite profiles as a response to the infection are crucial for improving food safety and quality control measures. Future research should explore innovative fruit selection and processing methods to

minimize the risk of mycotoxin contamination in pomegranate juice. Moreover, the potential role of metabolites like alternarlactone A, altenusin, and AAL-toxins as virulence factors or an adaptive response of *A. alternata* to a specific host deserves further investigation for a better understanding of the pathogen/host interactions and pathogenesis mechanisms in heart rot of pomegranate and other plant diseases caused by *Alternaria* species.

CRediT authorship contribution statement

Mario Riolo: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carlos Luz:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Cristian Bua:** Investigation, Data curation. **Salvatore Barreca:** Writing – review & editing, Validation, Data curation. **Maria Catena Tambè:** Investigation. **Jorge Calpe:** Methodology. **Marco Masi:** Writing – review & editing, Visualization, Validation, Formal analysis. **Alessio Cimmino:** Writing – review & editing, Validation, Formal analysis. **Gaetano Tirrò:** Investigation. **Giuseppe Meca:** Visualization, Supervision, Resources. **David Ezra:** Writing – review & editing, Visualization, Validation. **Santa Olga Cacciola:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships.

Acknowledgments

The authors acknowledge the projects “Nuove soluzioni tecnologiche per la filiera degli agrumi: trasferimento di in-novazione per il miglioramento degli standard qualitativi delle produzioni agrumicole e per fronteggiare la minaccia di malattie infettive di origine fungina degli agrumi nel comprensorio del GAL Eloro – NewCitrusTech” (cod. 1.3.1” - Misura 19 PSR 2014/2022 Sostegno allo sviluppo locale leader, sottomisura 19.2 “Sostegno all’esecuzione degli interventi nell’ambito della strategia di Sviluppo locale di tipo partecipativo - CLLD) and “Azioni Innovative per la Produttività del Distretto dell’Ortofrutta di Qualità – INNOVAPROD” (cod. 1.3.2” - Misura 19 PSR 2014/2022 “Sostegno allo sviluppo locale leader, sottomisura 19.2; Sostegno all’esecuzione degli interventi nell’ambito della strategia di Sviluppo locale di tipo partecipativo - CLLD).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2025.111204.

Data availability

Data will be made available on request.

References

Ali, S., Freire, L. G. D., Rezende, V. T., Noman, M., Ullah, S., Abdullah, Badshah, G., Afridi, M. S., Tonin, F. G., & de Oliveira, C. A. F. (2023). Occurrence of mycotoxins in foods: Unraveling the knowledge gaps on their persistence in food production systems. *Foods, 12*(23), 4314. https://doi.org/10.3390/foods12234314

Allen, D. R., & McWhinney, B. C. (2019). Quadrupole time-of-flight mass spectrometry: A paradigm shift in toxicology screening applications. *Clinical Biochemist Reviews, 40*(3), 135–146. https://doi.org/10.33176/AACB-19-00023

Aloi, F., Riolo, M., Sanzani, S. M., Mincuzzi, A., Ippolito, A., Siciliano, I., Pane, A., Gullino, M. L., & Cacciola, S. O. (2021). Characterization of *Alternaria* species associated with heart rot of pomegranate fruit. *Journal of Fungi, 7*(3), 1–18. https://doi.org/10.3390/jof7030172

Alshannaq, A., & Yu, J. H. (2017). Occurrence, toxicity, and analysis of major mycotoxins in food. *International Journal of Environmental Research and Public Health, 14*(6), 632. https://doi.org/10.3390/ijerph14060632

Aly, A. H., Edrada-Ebel, R. A., Indriani, I. D., Wray, V., Müller, W. E. G., Totzke, F., … Ebel, R. (2008). Cytotoxic metabolites from the fungal endophyte *Alternaria* sp. and their subsequent detection in its host plant P*olygonum senegalense*. *Journal of Natural Products, 71*(6), 972–980. https://doi.org/10.1021/np070447m.

Andrew, M., Peever, T. L., & Pryor, B. M. (2009). An expanded multilocus phylogeny does not resolve morphological species within the small-spored *Alternaria* species complex. *Mycologia, 101*, 95–109. https://doi.org/10.3852/08-135

Aviram, M., Dornfeld, L., Rosenblat, M., Volkova, N., Kaplan, M., Coleman, R., Hayek, T., Presser, D., & Fuhrman, B. (2000). Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: Studies in humans and in atherosclerotic apolipoprotein E-deficient mice. *American Journal of Clinical Nutrition, 71*, 1062–1076. https://doi.org/10.1093/ajcn/71.5.1062

Bahari, H., Ashtary-Larky, D., Goudarzi, K., Mirmohammadali, S. N., Asbaghi, O., kolbadi, H., sadat, K., Naderian, M., & Hosseini, A. (2024). The effects of pomegranate consumption on glycemic indices in adults: A systematic review and meta-analysis. *Diabetes & Metabolic Syndrome: Clinical Research Reviews, 18*, Article 102940. https://doi.org/10.1016/j.dsx.2024.102940

Bang, H. Y., Kim, Y. K., Kim, H., Baek, E. J., Na, T., Sim, K. S., & Kim, H. J. (2023). Ultra-High-Performance Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry for simultaneous pesticide analysis and method validation in sweet pepper. *Molecules, 28*(14), 5589. https://doi.org/10.3390/molecules28145589

Beccaccioli, M., Salustri, M., Scala, V., Ludovici, M., Cacciotti, A., D'angeli, S., Brown, D. W., & Reverberi, M. (2021). The effect of *Fusarium verticillioides* fumonisins on fatty acids, sphingolipids, and oxylipins in maize germlings. *International Journal of Molecular Sciences, 22*, 2435. https://doi.org/10.3390/ijms22052435

Bensassi, F., Gallerne, C., Dein, O. S. el, Hajlaoui, M. R., Bacha, H., & Lemaire, C. (2011). Mechanism of Alternariol monomethyl ether-induced mitochondrial apoptosis in human colon carcinoma cells. *Toxicology, 290*, 230–240. https://doi.org/10.1016/j.tox.2011.09.087

Berbee, M. L., Pirseyedi, M., & Hubbard, S. (1999). Cochliobolus phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. *Mycologia, 91*, 964–977. https://doi.org/10.2307/3761627

Berbegal, M., López-Cortés, I., Salazar, D., Gramaje, D., Pérez-Sierra, A., García-Jiménez, J., & Armengol, J. (2014). First report of alternaria black spot of pomegranate caused by *Alternaria alternata* in Spain. *Plant Disease, 98*, 689. https://doi.org/10.1094/PDIS-07-13-0717-PDN

Bullerman, L. B., & Bianchini, A. (2007). Stability of mycotoxins during food processing. *International Journal of Food Microbiology, 119*(1–2), 140–146. https://doi.org/10.1016/j.ijfoodmicro.2007.07.035

Carballo, D., Pinheiro-Fernandes-Vieira, P., Tolosa, J., Font, G., Berrada, H., & Ferrer, E. (2018). Dietary exposure to mycotoxins through fruits juice consumption. *Reviews in Toxicology, 35*, 2–6.

Carbone, I., & Kohn, L. M. (1999). A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia, 91*, 553–556. https://doi.org/10.2307/3761358

Carbonell-Rozas, L., Albasi, V., Camardo Leggieri, M., Dall'Asta, C., & Battilani, P. (2024). Apple mycotoxins: From orchard to processed apple puree. *Fungal Biology, 128*(8PB), 2422–2430. https://doi.org/10.1016/j.funbio.2024.07.001

Castillo, N. I., Ibáñez, M., Beltrán, E., Rivera-Monroy, J., Ochoa, J. C., Páez-Castillo, M., Posada-Buitrago, M. L., Sulyok, M., & Hernández, F. (2016). Identification of mycotoxins by UHPLC-QTOF MS in airborne fungi and fungi isolated from industrial paper and antique documents from the Archive of Bogotá. *Environmental Research, 144*, 130–138. https://doi.org/10.1016/j.envres.2015.10.031

Casu, A., Camardo Leggieri, M., Toscano, P., & Battilani, P. (2024). Changing climate, shifting mycotoxins: A comprehensive review of climate change impact on mycotoxin contamination. *Comprehensive Reviews in Food Science and Food Safety, 23*(2), 1–24. https://doi.org/10.1111/1541-4337.13323

Conti Taguali, S., Riolo, M., Dopazo, V., Meca, G., & Cacciola, S. O. (2024). Characterization of mycotoxins produced by two *Fusarium* species responsible for postharvest rot of banana fruit. *Journal of Plant Pathology*. https://doi.org/10.1007/s42161-024-01751-8

Da Cruz Cabral, L., Rodriguero, M., Stenglein, S., Fog Nielsen, K., & Patriarca, A. (2017). Characterization of small-spored *Alternaria* from Argentinean crops through a polyphasic approach. *International Journal of Food Microbiology, 247*, 206–215. https://doi.org/10.1016/j.ijfoodmicro.2017.06.026

Dasí-Navarro, N., Lozano, M., Llop, S., Esplugues, A., Cimbalo, A., Font, G., Manyes, L., Mañes, J., & Vila-Donat, P. (2022). Development and validation of LC-Q-TOF-MS methodology to determine mycotoxin biomarkers in human urine. *Toxins, 14*, 651. https://doi.org/10.3390/toxins14100651

Dopazo, V., Luz, C., Calpe, J., Vila-Donat, P., Rodríguez, L., & Meca, G. (2022). Antifungal properties of whey fermented by lactic acid bacteria in films for the preservation of cheese slices. *International Journal of Dairy Technology, 75*(3), 619–629. https://doi.org/10.1111/1471-0307.12847

Dopazo, V., Luz, C., Mañes, J., Quiles, J. M., Carbonell, R., Calpe, J., & Meca, G. (2021). Bio-preservative potential of microorganisms isolated from red grape against food contaminant fungi. *Toxins, 13*, 412. https://doi.org/10.3390/toxins13060412

EFSA on Contaminants in the Food Chain (CONTAM). (2011). Scientific Opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in feed and food. *EFSA Journal, 9*, 2407. https://doi.org/10.2903/j.efsa.2011.2407

El Barnossi, A., Moussaid, F., & Iraqi Housseini, A. (2021). Tangerine, banana and pomegranate peels valorisation for sustainable environment: A review. *Biotechnology Reports, 29*, Article e00574. https://doi.org/10.1016/j.btre.2020.e00574

Elhamouly, N. A., Hewedy, O. A., Zaitoon, A., Miraples, A., Elshorbagy, O. T., Hussien, S., El-Tahan, A., & Peng, D. (2022). The hidden power of secondary metabolites in plant-fungi interactions and sustainable phytoremediation. *Frontiers in Plant Science, 13*, Article 1044896. https://doi.org/10.3389/fpls.2022.1044896

Elhariry, H. M., Khiralla, G. M., Gherbawy, Y., & Abd Elrahman, H. (2016). Natural occurrence of *Alternaria* toxins in pomegranate fruit and the influence of some technological processing on their levels in juice. *Acta Alimentaria, 45*(3), 380–389. https://doi.org/10.1556/066.2016.45.3.9

Escrivá, L., Oueslati, S., Font, G., & Manyes, L. (2017). *Alternaria* mycotoxins in food and feed: An overview. *Journal of Food Quality, 2017*, Article 1569748. https://doi.org/10.1155/2017/1569748

Esposto, S., Veneziani, G., Taticchi, A., Urbani, S., Selvaggini, R., Sordini, B., Daidone, L., Gironi, G., & Servili, M. (2021). Chemical composition, antioxidant activity, and sensory characterization of commercial pomegranate juices. *Antioxidants, 10*(9). https://doi.org/10.3390/antiox10091381

Evidente, A., Kornienko, A., Cimmino, A., Andolfi, A., Lefranc, F., Mathieu, V., & Kiss, R. (2014). Fungal metabolites with anticancer activity. *Natural Product Reports*. https://doi.org/10.1039/c3np70078j

Ezra, D., Kirshner, B., Hershcovich, M., Shtienberg, D., & Kosto, I. (2015). Heart rot of pomegranate: Disease etiology and the events leading to development of symptoms. *Plant Disease, 99*(4), 496–501. https://doi.org/10.1094/PDIS-07-14-0707-RE

Ezra, D., Shulhani, R., Bar Ya'Akov, I., Harel-Beja, R., Holland, D., & Shtienberg, D. (2019). Factors affecting the response of pomegranate fruit to *Alternaria alternata*, the causal agent of heart rot. *Plant Disease, 103*, 315–323. https://doi.org/10.1094/PDIS-01-18-0147-RE

Faedda, R., Granata, G., Massimino Cocuzza, G. E., Lo Giudice, V., Audoly, G., Pane, A., & Cacciola, S. O. (2015). First report of heart rot of pomegranate (*Punica granatum*) caused by *Alternaria alternata* in Italy. *Plant Disease, 99*, 1446. https://doi.org/10.1094/PDIS-02-15-0238-PDN

Gat, T., Liarzi, O., Skovorodnikova, Y., & Ezra, D. (2012). Characterization of *Alternaria alternata* causing black spot disease of pomegranate in Israel using a molecular marker. *Plant Disease, 96*, 1513–1518. https://doi.org/10.1094/PDIS-12-11-1041-RE

Geiseler, O., Müller, M., & Podlech, J. (2013). Synthesis of the altertoxin III framework. *Tetrahedron, 69*(18), 3683–3689. https://doi.org/10.1016/j.tet.2013.03.013.

Giménez-Bastida, J. A., Ávila-Gálvez, M.Á., Espín, J. C., & González-Sarrías, A. (2021). Evidence for health properties of pomegranate juices and extracts beyond nutrition: A critical systematic review of human studies. *Trends in Food Science and Technology, 114*, 410–423. https://doi.org/10.1016/j.tifs.2021.06.014

Goula, A. M., & Adamopoulos, K. G. (2012). A method for pomegranate seed application in food industries: Seed oil encapsulation. *Food and Bioproducts Processing, 90*, 639–652. https://doi.org/10.1016/j.fbp.2012.06.001

Guerra-Vázquez, C. M., Martínez-ávila, M., Guajardo-Flores, D., & Antunes-Ricardo, M. (2022). Punicic acid and its role in the prevention of neurological disorders: A review. *Foods, 11*(3). https://doi.org/10.3390/foods11030252

He, G., Chen, X., Hou, X., Yu, X., Han, M., Qiu, S., Li, Y., Qin, S., & Wang, F. (2023). UPLC-Q-TOF/MS-based metabolomic analysis reveals the effects of asomate on the citrus fruit. *Current Research in Food Science, 6*(May). https://doi.org/10.1016/j.crfs.2023.100523

Holland, D., & Bar-Ya'akov, I. (2018). Pomegranate (*Punica granatum* L.) breeding. *Advances in Plant Breeding Strategies: Fruits, 3*, 601–647. https://doi.org/10.1007/978-3-319-91944-7_15

Huang, Q., Guo, W., Zhao, X., Cao, H., Fan, K., Meng, J., Nie, D., Wu, Y., & Han, Z. (2022). Universal screening of 200 mycotoxins and their variations in stored cereals in Shanghai, China by UHPLC-Q-TOF MS. *Food Chemistry, 387*, Article 132869. https://doi.org/10.1016/j.foodchem.2022.132869

Kandylis, P., & Kokkinomagoulos, E. (2020). Food applications and potential health benefits of pomegranate and its derivatives. *Foods, 9*(2), 122. https://doi.org/10.3390/foods9020122

Kelman, M. J., Renaud, J. B., Seifert, K. A., Mack, J., Yeung, K. K. C., & Sumarah, M. W. (2020). Chemotaxonomic profiling of canadian *Alternaria* populations using high-resolution mass spectrometry. *Metabolites, 10*(6). https://doi.org/10.3390/metabo10060238

Khan, H., & Ali, J. (2015). UHPLC/Q-TOF-MS technique: Introduction and applications. *Letters in Organic Chemistry, 12*, 371–378. https://doi.org/10.2174/1570178612666150331204147

Kono, Y., Gardner, J. M., & Takeuchi, S. (1986). Structure of the host-selective toxins produced by a pathotype of *Alternaria citri* causing brown spot disease of mandarins. *Agricultural and Biological Chemistry, 50*(3), 801–804. https://doi.org/10.1271/bbb1961.50.801

La Bella, E., Riolo, M., Luz, C., Baglieri, A., Puglisi, I., Meca, G., & Cacciola, S. O. (2024). Fermentates of consortia of lactic acid bacteria and a cyanobacterium are effective against toxigenic fungi contaminating agricultural produces. *Biological Control, 191* (Abril), Article 105478. https://doi.org/10.1016/j.biocontrol.2024.105478

Leggieri, M. C., Toscano, P., & Battilani, P. (2021). Predicted aflatoxin b1 increase in europe due to climate change: Actions and reactions at global level. *Toxins, 13*(4), 1–21. https://doi.org/10.3390/TOXINS13040292

Logrieco, A., Moretti, A., & Solfrizzo, M. (2009). *Alternaria* toxins and plant diseases: An overview of origin, occurrence and risks. *World Mycotoxin Journal, 2*, 129–140. https://doi.org/10.3920/WMJ2009.1145

Luo, Y., Hou, L., Förster, H., Pryor, B., & Adaskaveg, J. E. (2017). Identification of *Alternaria* species causing heart rot of pomegranates in California. *Plant Disease, 101*, 421–427. https://doi.org/10.1094/PDIS-08-16-1176-RE

Man, Y., Liang, G., Li, A., & Pan, L. (2017). Analytical methods for the determination of *Alternaria* mycotoxins. *Chromatographia, 80*, 9–22. https://doi.org/10.1007/s10337-016-3186-x

Marrone, G., Basilicata, M., Di Lauro, M., Vita, C., Masci, C., Klinger, F. G., Cornali, K., Maddaloni, G., Bollero, P., De Lorenzo, A., & Noce, A. (2024). Healthy effects of pomegranate (*Punica granatum* L.) in internal medicine and dentistry. *Applied Sciences, 14*(4). https://doi.org/10.3390/app14041570

Masi, M., Aloi, F., Nocera, P., Cacciola, S. O., Surico, G., & Evidente, A. (2020). Phytotoxic metabolites isolated from *Neufusicoccum batangarum*, the causal agent of the scabby canker of cactus pear (*Opuntia ficus-indica* L.). *Toxins, 12*(2), 1–13. https://doi.org/10.3390/toxins12020126

Masiá, A., Campo, J., Blasco, C., & Picó, Y. (2014). Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry to identify contaminants in water: An insight on environmental forensics. *Journal of Chromatography A, 1345*, 86–97. https://doi.org/10.1016/j.chroma.2014.04.017

Masiello, M., Somma, S., Susca, A., Ghionna, V., Logrieco, A. F., Franzoni, M., Ravaglia, S., Meca, G., & Moretti, A. (2020). Molecular identification and mycotoxin production by *Alternaria* species occurring on durum wheat, showing black point symptoms. *Toxins, 12*, 275. https://doi.org/10.3390/toxins12040275

Meiss, E., Konno, H., Groth, G., & Hisabori, T. (2008). Molecular processes of inhibition and stimulation of ATP synthase caused by the phytotoxin tentoxin. *Journal of Biological Chemistry, 283*(36), 24594–24599. https://doi.org/10.1074/jbc.M802574200

Melgarejo, P., Martínez-Valero, R., Guillamón, J. M., Miró, M., & Amorós, A. (1997). Phenological stages of the pomegranate tree (*Punica granatum* L.). *Annals of Applied Biology, 130*, 135–140. https://doi.org/10.1111/j.1744-7348.1997.tb05789.x

Mincuzzi, A., Sanzani, S. M., Palou, L., Ragni, M., & Ippolito, A. (2022). Postharvest rot of pomegranate fruit in southern Italy: Characterization of the main pathogens. *Journal of Fungi, 8*, 475. https://doi.org/10.3390/jof8050475

Munera, S., Rodríguez-Ortega, A., Cubero, S., Aleixos, N., & Blasco, J. (2025). Automatic detection of pomegranate fruit affected by blackheart disease using X-ray imaging. *Lwt, 215*(December 2024), Article 117248. https://doi.org/10.1016/j.lwt.2024.117248

Nawrot-Chorabik, K., Sułkowska, M., & Gumulak, N. (2022). Secondary metabolites produced by trees and fungi: Achievements so far and challenges remaining. *Forests, 13*(8), 1–31. https://doi.org/10.3390/f13081338

Nemecek, G., Cudaj, J., & Podlech, J. (2012). Revision of the structure and total synthesis of altenuisol. *European Journal of Organic Chemistry, 20*, 3863–3870. https://doi.org/10.1002/ejoc.201200506

Nuncio-Jáuregui, N., Cano-Lamadrid, M., Hernández, F., Carbonell-Barrachina, Á. A., & Calín-Sánchez, Á. (2015). Comparison of fresh and commercial pomegranate juices from mollar de elche cultivar grown under conventional or organic farming practices. *Beverages, 1*(2), 34–44. https://doi.org/10.3390/beverages1020034

Onsekizoglu, P. (2013). Production of high quality clarified pomegranate juice concentrate by membrane processes. *Journal of Membrane Science, 442*, 264–271. https://doi.org/10.1016/j.memsci.2013.03.061

Oteiza, J. M., Khaneghah, A. M., Campagnollo, F. B., Granato, D., Mahmoudi, M. R., Sant'Ana, A. S., & Gianuzzi, L. (2017). Influence of production on the presence of patulin and ochratoxin A in fruit juices and wines of Argentina. *LWT, 80*, 200–207. https://doi.org/10.1016/j.lwt.2017.02.025

Pallarés, N., Carballo, D., Ferrer, E., Fernández-Franzón, M., & Berrada, H. (2019). Mycotoxin dietary exposure assessment through fruit juices consumption in children and adult population. *Toxins, 11*, 684. https://doi.org/10.3390/toxins11120684

Pang, Z., Chong, J., Zhou, G., De Lima Morais, D. A., Chang, L., Barrette, M., Gauthier, C., Jacques, P.É., Li, S., & Xia, J. (2021). MetaboAnalyst 5.0: Narrowing the gap between raw spectra and functional insights. *Nucleic Acids Research, 49*, W388–W396. https://doi.org/10.1093/nar/gkab382

Pinto, V. E. F., & Patriarca, A. (2017). Alternaria species and their associated mycotoxins. In *Methods in molecular biology* (pp. 13–32). https://doi.org/10.1007/978-1-4939-6707-0_2

Prelle, A., Spadaro, D., Garibaldi, A., & Gullino, M. L. (2013). A new method for detection of five alternaria toxins in food matrices based on LC-APCI-MS. *Food Chemistry, 140*, 161–167. https://doi.org/10.1016/j.foodchem.2012.12.065

Puntscher, H., Cobankovic, I., Marko, D., & Warth, B. (2019). Quantitation of free and modified *Alternaria* mycotoxins in European food products by LC-MS/MS. *Food Control, 102*(March), 157–165. https://doi.org/10.1016/j.foodcont.2019.03.019

Pusztahelyi, T., Holb, I. J., & Pócsi, I. (2015). Secondary metabolites in fungus-plant interactions. *Frontiers in Plant Science, 6*(AUG), 1–23. https://doi.org/10.3389/fpls.2015.00573

Righetti, L., Dall'Asta, C., Lucini, L., & Battilani, P. (2021). Lipid signaling modulates the response to fumonisin contamination and its source, *Fusarium verticillioides*, in maize. *Frontiers in Plant Science, 12*, Article 701680. https://doi.org/10.3389/fpls.2021.701680

Riolo, M., Luz, C., Santilli, E., Meca, G., & Cacciola, S. O. (2023). Secondary metabolites produced by four *Colletotrichum* species in vitro and on fruits of diverse olive cultivars. *Fungal Biology, 127*(7–8), 1118–1128. https://doi.org/10.1016/j.funbio.2023.06.003

Rovetto, E. I., La Spada, F., Aloi, F., Riolo, M., Pane, A., Garbelotto, M., & Cacciola, S. O. (2024). Green solutions and new technologies for sustainable management of fungus and oomycete diseases in the citrus fruit supply chain. *Journal of Plant Pathology*. , Article 0123456789. https://doi.org/10.1007/s42161-023-01543-6

Rovetto, E. I., Luz, C., La Spada, F., Meca, G., Riolo, M., & Cacciola, S. O. (2023). Diversity of mycotoxins and other secondary metabolites recovered from blood oranges infected by *Colletotrichum, Alternaria*, and *Penicillium* species. *Toxins, 15*(7), 407. https://doi.org/10.3390/toxins15070407

Saleh, I., Zeidan, R., & Abu-Dieyeh, M. (2024). The characteristics, occurrence, and toxicological effects of alternariol: A mycotoxin. *Archives of Toxicology, 98*(6), 1659–1683. https://doi.org/10.1007/s00204-024-03743-0

Scala, V., Camera, E., Ludovici, M., Dall'Asta, C., Cirlini, M., Giorni, P., Battilani, P., Bello, C., Fabbri, A. A., Fanelli, C., & Reverberi, M. (2013). *Fusarium verticillioides* and maize interaction *in vitro*: Relationship between oxylipin cross-talk and fumonisin synthesis. *World Mycotoxin Journal, 6*(3), 343–351. https://doi.org/10.3920/WMJ2012.1527

Scala, V., Giorni, P., Cirlini, M., Ludovici, M., Visentin, I., Cardinale, F., Fabbri, A. A., Fanelli, C., Reverberi, M., Battilani, P., Galaverna, G., & Dall'Asta, C. (2014). LDS1-produced oxylipins are negative regulators of growth, conidiation and fumonisin synthesis in the fungal maize pathogen *Fusarium verticillioides*. *Frontiers in Microbiology, 5*, 669. https://doi.org/10.3389/fmicb.2014.00669

Scheibenzuber, S., Hoffmann, T., Effenberger, I., Schwab, W., Asam, S., & Rychlik, M. (2020). Enzymatic synthesis of modified *Alternaria* mycotoxins using a whole-cell biotransformation system. *Toxins, 12*(4). https://doi.org/10.3390/toxins12040264

Schymanski, E. L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H. P., & Hollender, J. (2014). Identifying small molecules via high resolution mass spectrometry: Communicating confidence. *Environmental Science & Technology, 48*(4), 2097–2098. https://doi.org/10.1021/es5002105

Shi, H., Li, S., Bai, Y., Prates, L. L., Lei, Y., & Yu, P. (2018). Mycotoxin contamination of food and feed in China: Occurrence, detection techniques, toxicological effects and advances in mitigation technologies. *Food Control, 91*, 202–215. https://doi.org/10.1016/j.foodcont.2018.03.036

Shi, Y. N., Pusch, S., Shi, Y. M., Richter, C., Maciá-Vicente, J. G., Schwalbe, H., Kaiser, M., Opatz, T., & Bode, H. B. (2019). (±)-alternarlactones A and B, two antiparasitic alternariol-like dimers from the fungus *Alternaria alternata* P1210 isolated from the halophyte salicornia sp. *Journal of Organic Chemistry, 84*(17). https://doi.org/10.1021/acs.joc.9b01229

Siciliano, I., Ortu, G., Gilardi, G., Gullino, M. L., & Garibaldi, A. (2015). Mycotoxin production in liquid culture and on plants infected with *Alternaria* spp. Isolated from rocket and cabbage. *Toxins, 7*, 743–754. https://doi.org/10.3390/toxins7030743

Siddiqui, S. A., Singh, S., & Nayik, G. A. (2024). Bioactive compounds from pomegranate peels - biological properties, structure–function relationships, health benefits and food applications – a comprehensive review. *Journal of Functional Foods, 116* (March), Article 106132. https://doi.org/10.1016/j.jff.2024.106132

Silva, A. S., Brites, C., Pouca, A. V., Barbosa, J., & Freitas, A. (2019). UHPLC-ToF-MS method for determination of multi-mycotoxins in maize: Development and validation. *Current Research in Food Science, 31*, 1–7. https://doi.org/10.1016/j.crfs.2019.07.001

Sun, Q., Dong, Y., Wen, X., Zhang, X., Hou, S., Zhao, W., & Yin, D. (2023). A review on recent advances in mass spectrometry analysis of harmful contaminants in food. *Frontiers in Nutrition, 10*, Article 1244459. https://doi.org/10.3389/fnut.2023.1244459

Thomma, B. P. H. J. (2003). *Alternaria* spp.: From general saprophyte to specific parasite. *Molecular Plant Pathology, 4*, 225–236. https://doi.org/10.1046/j.1364-3703.2003.00173.x

Tirrò, G., Conti Taguali, S., Pane, A., Riolo, M., Ezra, D., & Cacciola, S. O. (2024). Outbreak of Alternaria Black Spot of pomegranate (*Punica granatum* L.) in Italy as a consequence of unusual climatic conditions. *Plants, 13*(14). https://doi.org/10.3390/plants13142007

Trishitman, D., Negi, P. S., & Rastogi, N. K. (2023). Concentration of pomegranate juice by forward osmosis or thermal evaporation and its shelf-life kinetic studies. *Food Chemistry, 399*(August 2022), Article 133972. https://doi.org/10.1016/j.foodchem.2022.133972

Tsuge, T., Harimoto, Y., Akimitsu, K., Ohtani, K., Kodama, M., Akagi, Y., Egusa, M., Yamamoto, M., & Otani, H. (2013). Host-selective toxins produced by the plant pathogenic fungus *Alternaria alternata*. *FEMS Microbiology Reviews, 37*, 44–66. https://doi.org/10.1111/j.1574-6976.2012.00350.x

Tziros, G. T., Lagopodi, A. L., & Tzavella-Klonari, K. (2008). *Alternaria alternata* fruit rot of pomegranate (*Punica granatum*) in Greece. *Plant Pathology, 57*, 379. https://doi.org/10.1111/j.1365-3059.2007.01668.x

Ueno, T., Nakashima, T., Hayashi, Y., & Fukami, H. (1975). Structures of Am-Toxin I and II, host specific phytotoxic metabolites produced by *Alternaria mali*. *Agricultural and Biological Chemistry, 39*(5), 1115–1122. https://doi.org/10.1271/bbb1961.39.1115

Usanmaz, S., Kahramanoğlu, I., & Yılmaz, N. (2014). Yield and pomological characteristics of three pomegranate (*Punica granatum* L.) cultivars: Wonderful, Acco and Herskovitz. *American Journal of Agriculture and Forestry, 2*(3), 61. https://doi.org/10.11648/j.ajaf.20140203.12

Valero-Mendoza, A. G., Meléndez-Rentería, N. P., Chávez-González, M. L., Flores-Gallegos, A. C., Wong-Paz, J. E., Govea-Salas, M., Zugasti-Cruz, A., & Ascacio-Valdés, J. A. (2023). The whole pomegranate (*Punica granatum* L.), biological properties and important findings: A review. *Food Chemistry Advances, 2*(July 2022), Article 100153. https://doi.org/10.1016/j.focha.2022.100153

Vicent, A., Mira, J. L., Bartual, J., Beltrán, V., Taberner, V., & Palou, L. (2016). First report of black heart of pomegranate caused by *Alternaria alternata* in Spain. *Plant Disease, 100*, 1952. https://doi.org/10.1094/PDIS-03-16-0265-PDN

Vijayalakshmi, S., Yoo, D. S., Kim, D. G., Chelliah, R., Barathikannan, K., Aloo, S. O., Tyagi, A., Yan, P., Shan, L., Gebre, T. S., & Oh, D. H. (2023). Fermented *Perilla frutescens* leaves and their untargeted metabolomics by UHPLC-QTOF-MS reveal anticancer and immunomodulatory effects. *Food Bioscience, 56*, Article 103065. https://doi.org/10.1016/j.fbio.2023.103065

Wang, H., Guo, Y., Luo, Z., Gao, L., Li, R., Zhang, Y., Kalaji, H. M., Qiang, S., & Chen, S. (2022). Recent advances in *alternaria* phytotoxins: A review of their occurrence, structure, bioactivity and biosynthesis. *Journal of Fungi, 8*, 168. https://doi.org/10.3390/jof8020168

Wenderoth, M., Garganese, F., Schmidt-Heydt, M., Soukup, S. T., Ippolito, A., Sanzani, S. M., & Fischer, R. (2019). Alternariol as virulence and colonization factor of *Alternaria alternata* during plant infection. *Molecular Microbiology, 112*(1), 131–146. https://doi.org/10.1111/mmi.14258

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: A guide to methods and applications*. San Diego: Academic Press.

Woudenberg, J. H. C., Seidl, M. F., Groenewald, J. Z., de Vries, M., Stielow, J. B., Thomma, B. P. H. J., & Crous, P. W. (2015). *Alternaria* section *Alternaria*: Species, formae speciales or pathotypes? *Studies in Mycology, 82*. https://doi.org/10.1016/j.simyco.2015.07.001

Zhang, L., & McCarthy, M. J. (2012). Black heart characterization and detection in pomegranate using NMR relaxometry and MR imaging. *Postharvest Biology and Technology, 67*, 96–101. https://doi.org/10.1016/j.postharvbio.2011.12.018

Zwickel, T., Klaffke, H., Richards, K., & Rychlik, M. (2016). Development of a high performance liquid chromatography tandem mass spectrometry based analysis for the simultaneous quantification of various *Alternaria* toxins in wine, vegetable juices and fruit juices. *Journal of Chromatography A, 1455*, 74–85. https://doi.org/10.1016/j.chroma.2016.04.066