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# Identification and pathogenicity of *Alternaria* species causing leaf blotch and fruit spot of apple in California

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**Summary.** In late summer 2020, symptoms of leaf blotch and fruit spot were observed in two different commercial apple orchards (cultivars 'Pink Lady' and 'Modi') in San Joaquin County, California, USA. Ninety *Alternaria* isolates were obtained from symptomatic leaves and fruits collected from the orchards. Based on morphological characteristics of the colonies, sporulation patterns, and conidia, the isolates were preliminarily separated into three morphogroups, tentatively identified as *A. alternata*, *A. tenuissima* and *A. arborescens*. Multi-locus phylogenetic analyses, using nucleotide sequences of plasma membrane ATPase, calmodulin, and Alternaria major allergen genes, showed that the isolates initially identified as *A. tenuissima* clustered with strains of *A. alternata*, following the current taxonomical arrangement of the genus. Pathogenicity tests on detached wounded apple leaves and fruits, using representative isolates of the three morphogroups, fulfilled Koch's postulates. This is the first report of *A. alternata* and *A. arborescens* as causal agents of leaf blotch and fruit spot of apple in California.

**Keywords.** Etiology, foliar, apple diseases, *Malus domestica*.

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## INTRODUCTION

Apple (*Malus domestica* Borkh.) production in California covered 4,654 ha in 2021, as the sixth largest apple-producing state in the United States of America (USA) (CDFA, 2022). California produces four main apple cultivars: 'Gala', 'Fuji', 'Granny Smith', and 'Cripps Pink' ('Pink Lady®') (California Apple Commission, 2023). Several fungal diseases affect apple, including diseases of leaves and fruit (e.g. apple scab, powdery mildew, rusts), wood (e.g. European canker, Valsa canker), root and replant diseases (e.g. crown and root rots), and postharvest diseases (e.g. blue mold, gray mold and bull's-eye rot) (Sutton *et al.*, 2014).

Apple is susceptible to *Alternaria* species, which can cause different diseases, including leaf blotch, fruit spot, fruit rot, core rot, and moldy core

(Harteveld *et al*., 2013; Sutton *et al*., 2014; Gur *et al*., 2017; Elfar *et al*., 2018b). Alternaria leaf blotch is frequently observed in summer, and is characterized by the presence of small (3 to 5 mm diam.), circular gray to brown necrotic lesions on apple leaves and fruit, often with dark brown to purple margins (Elfar *et al*., 2018a). In severe cases, leaf defoliation of up to 50% can occur in susceptible cultivars (Sawamura, 2014). Symptoms on fruit are uncommon, except in highly susceptible cultivars, such as 'Golden Delicious', 'Starking Delicious', 'Indo', 'Gala', and 'Pink Lady'. Fruit spots are often small, corky, and dark, typically associated with fruit lenticels (Sawamura, 2014; Gur *et al*., 2017). Severe disease outbreaks have been reported on 'Pink Lady' apples in northern Israel, where large lesions and fruit rots have been observed. Multiple lesions on fruit, especially those adjacent to cracks around fruit calices, may coalesce to produce large dark rotted areas, with incidences up to 80% of fruit in some orchards (Gur *et al*., 2017). This disease was named Alternaria fruit rot to differentiate it from Alternaria fruit spot (Gur *et al*., 2018). The status of *Alternaria* diseases affecting apples in California is unknown. However, California is familiar with diseases caused by *Alternaria* species in other fruit and nut crops (Teviotdale *et al*., 2001; Pryor and Michailides, 2002; Zhu and Xiao, 2015; Luo *et al*., 2017; Wang *et al*., 2021).

*Alternaria mali* (syn. *A. alternata* f. sp. *mali*) is the main cited causal agent of Alternaria blotch of apple in the northern hemisphere and Australia (Filajdić and Sutton, 1991; Ozgonen and Karaca, 2006; Soleinami and Esmaizadeh, 2007; Harteveld *et al*., 2013; Sawamura, 2014; Gur *et al*., 2017). In the USA, *A. mali* was identified in the late 1980s in North Carolina, causing apple leaf blotch (Filajdić and Sutton, 1991). However, studies in in Australia, Chile, France, Italy, and Spain, have shown that several small-spored *Alternaria* species were associated with Alternaria leaf blotch and fruit spot, where *A. alternata*, *A. arborescens*, *A. infectoria*, *A. longipes*, and *A. tenuissima* were identified (Rotondo *et al*., 2012; Harteveld *et al*., 2013; Elfar *et al*., 2018a; Toome-Heller *et al*., 2018; Fontaine *et al*., 2021; Cabrefiga *et al*., 2023). Differences in virulence among *Alternaria* isolates were also detected (Harteveld *et al*., 2014b; Elfar *et al*., 2018a).

Morphological identification of the small-spored species of *Alternaria* is challenging, due to the diversity and scarcity of characteristics that allow unambiguous identification (Andrew *et al*., 2009). Use of morphological characteristics in combination with phylogenetic analyses based on multiple gene loci are essential for identification of species within small-spored *Alternaria* (Woudenberg *et al*., 2013; Lawrence *et al*., 2016). Several loci have been used in phylogenetic studies of *Alternaria*, including nuclear ribosomal regions (ITS, LSU, and SSU), and protein-coding genes. Lawrence *et al*. (2013), assessed the phylogenetic utility of ten nuclear protein coding loci, and showed that the five most phylogenetically informative loci for *Alternaria* species were ATPase, followed by calmodulin, Alternaria major allergen Alt a1, glyceraldehyde-3-phosphate dehydrogenase, and actin. The least informative loci were beta-tubulin and translation elongation factor 1-alpha. Therefore, ATPase and calmodulin have been suggested as the most appropriate loci for identification of *Alternaria* species (Lawrence *et al*., 2013, 2016).

During late summer of 2020, an outbreak of leaf blotch and fruit spot was observed in two commercial apple orchards in San Joaquin County, California. Up to 30% of leaves and less than 1% of apples were affected by the disease. The objectives of the present study were: (i) to identify and characterize the causal agents of both of these diseases; and (ii) to test the pathogenicity of the putative pathogens on two apple cultivars ('Pink Lady' and 'Fuji').

## MATERIALS AND METHODS

### *Fungal isolations*

Symptomatic apple leaf ($n$ = 40) and fruit ($n$ = 10) samples were collected from two orchards, one of the cultivar 'Pink Lady' and the other of 'Modi', located in San Joaquin County, California. The leaves and fruit were surface disinfected by submerging in a 70% ethanol solution for 1 min. Isolations were then carried out from small pieces (2 to 5 mm length) taken from margins between diseased and healthy tissues, which were plated onto potato dextrose agar (PDA; BD Difco) acidified with 92% lactic acid 0.5 mL $L^{-1}$ (APDA). The isolation plates were incubated for 7 to 10 d at room temperature (20 to 22°C). Fungal colonies were preliminarily identified as *Alternaria* species (Simmons, 2007), using colony morphology (colour and texture) and by conidiophore and conidium characteristics. Mycelium from *Alternaria*-like colonies was transferred to fresh APDA plates, and pure cultures of 90 isolates were then obtained by plating a 50 µL of conidial suspension of each isolate on water agar. After 18 h of incubation at room temperature, a single conidium was selected under a stereomicroscope and transferred to a fresh APDA plate. Isolates were then kept on APDA at 5°C for further analyses.

*Morphological characteristics of isolates*

Colony morphology was characterized in plastic Petri dishes (90 mm diam.) containing either APDA or potato carrot agar (PCA; HiMedia) (Simmons, 2007). The plates were incubated for 7 d at 20 to 22°C, with 8 h light 16 h darkness regimes. Light was provided by daylight fluorescent tubes placed 40 cm above the culture plates. Conidia and conidiophores from three PCA plates per isolate were each mounted on colourless adhesive tape and placed on top of a drop of Shear's mounting medium (10 g potassium acetate, 200 mL glycerin, 300 mL 95% ethanol, 500 mL distilled water), and were then observed under a light microscope at 400× of magnification. Based on their morphology, the isolates were preliminarily classified into three morphogroups (A, B, and C). Nine representative isolates (group A: UCD9582, UCD9584, UCD9600; group B: UCD9588, UCD9620; group C: UCD9590, UCD9593, UCD9603, UCD9643) were selected for further study of their conidiophore and conidium features. Conidiophore ($n$ = 15 per isolate) length and width, cell numbers, and branching were determined. Conidium ($n$ = 50) shape, length, width, and number of transepta were determined. These data were compared with published descriptions of *Alternaria* species (Simmons, 2007).

*DNA extraction, PCR amplification and sequencing*

Twenty-six *Alternaria* isolates representative of the three morphological groups were selected for molecular identification (Table 1). These groups were established according to similarities in colony morphology and characteristics of their conidiophores and conidia. Total genomic DNA was extracted from 7- to 10-d-old mycelium of each isolate grown on APDA and incubated at 20 to 22°C. Mycelium of each isolate was carefully separated from the agar medium using a sterile scalpel, and was then macerated (6.0 m $sec^{-1}$ for 40 sec) in a tube containing lysis buffer and 1.0 mm glass beads, using a FastPrep-24 (MP Biomedicals). Genomic DNA was extracted using a DNA extraction kit (NucleoSpin Plant II; Macherey-Nagel GmbH & Co. KG). The Alternaria major allergen Alt a1 gene (Alt a1) was amplified using the primer pair Alt-for/Alt-rev (Hong *et al*., 2005), the plasma membrane ATPase gene (ATPase) using pair ATPDF1/ATPDR1, and the Calmodulin (CAL) gene using pair CALDF1/CALDR1 (Lawrence *et al*., 2013). Polymerase chain reactions (PCR) were carried out in a T100™ thermocycler (Bio-Rad). Each reaction had a volume of 25 mL, containing 12.5 µL of GoTaq® Green MasterMix 2X (Promega), 9.3 µL of nuclease-free water, 0.6 µL of a 10-µM solution of each primer, and 2 µL of template DNA. The amplification protocol included preheating for 2 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing for 45 s at 57°C for Alt a1, 55°C for ATPase, or 54°C for CAL, and extension at 72°C for 90 s, with a final extension for 5 min at 72°C. PCR-amplified products were visualized by electrophoresis in 1% agarose gels with 100X SYBR® Green I nucleic acid gel stain (Sigma-Aldrich), and purified using Exonuclease I and Shrimp Alkaline Phosphatase (New England BioLab), following the manufacturer's instructions. PCR products were quantified using a Quantus™ fluorometer (Promega), and were submitted to Quintara Biosciences (Hayward, CA, USA) for Sanger sequencing. Both forward and reverse sequences were assembled using Sequencher v5.4.6 (Gene Codes). A BLASTn search analysis of the consensus sequences was carried out against reference sequences in the GenBank database (https:// www.ncbi.nlm.nih.gov).

*Phylogenetic analyses*

Maximum parsimony (MP) phylogenetic analyses were carried out using MEGA v.11 (Tamura *et al*., 2021). Gaps were treated as missing data. The MP trees were obtained using the tree-bisection-reconnection branch swapping algorithm and 1,000 random sequence additions. Branch stability was estimated using bootstrap with 1,000 replicates. The alignments included sequences of Alt a1, ATPase, and CAL, from the 26 *Alternaria* isolates obtained from apple leaf blotch and fruit spot symptoms in California (Table 1) and sequences from 18 *Alternaria* isolates obtained from GenBank (Table 2). Sequences of *Stemphylium botryosum*, *S. callistephi*, and *S. vesicarium* were included as outgroups (Table 2). The phylogenetic analyses were carried out independently for each gene, and concatenated. Topology of the resulting trees was compared, and a consensus tree was selected. This tree was edited in TreeGraph v2, and visual edits were carried out in InkScape.

*Pathogenicity tests*

Six representative isolates were selected to test their pathogenicity, two from each of the three morphological groups: group A (isolates UCD9582 and UCD9600), B (UCD9588 and UCD9620), and C (UCD9590 and UCD9593). To stimulate sporulation, isolates were cultivated in 0.05× PDA (Pryor and Michailides, 2002) for 7 d at 20 to 22°C with cycles of 10 h of light and 14 h of darkness. Conidial suspensions were prepared from 10-

**Table 1.** Sources of isolates of *Alternaria* species from apple obtained in two commercial orchards in Stockton, California, and GenBank accession numbers for sequences of three genes (*Alternaria* major allergen Alt a1, plasma membrane ATPase, and Calmodulin) of the *Alternaria* isolates examined in this study.

| Isolate | Group | Species | Symptom [a] | Apple Cultivar | GenBank accession number [b] | | |
|---|---|---|---|---|---|---|---|
| | | | | | Alt a1 | ATPase | Calmodulin |
| UCD9582[cd] | A | *Alternaria alternata* | FS | P. Lady | MW685776 | MW685792 | MW685808 |
| UCD9584[d] | A | *A. alternata* | FS | P. Lady | MW685775 | MW685791 | MW685807 |
| UCD9598 | A | *A. alternata* | LB | P. Lady | MW685777 | MW685793 | MW685809 |
| UCD9598.2 | A | *A. alternata* | LB | P. Lady | MW685778 | MW685794 | MW685810 |
| UCD9600[cd] | A | *A. alternata* | LB | P. Lady | MW685774 | MW685790 | MW685806 |
| UCD10530 | A | *A. alternata* | LB | Modi | OQ803488 | OQ803499 | OQ803510 |
| UCD10533 | A | *A. alternata* | LB | Modi | OQ803489 | OQ803500 | OQ803511 |
| UCD10536 | A | *A. alternata* | LB | Modi | OQ803490 | OQ803501 | OQ803512 |
| UCD10539 | A | *A. alternata* | LB | Modi | OQ803491 | OQ803502 | OQ803513 |
| UCD10529 | B | *A. alternata* | LB | Modi | OQ803492 | OQ803503 | OQ803514 |
| UCD9588[cd] | B | *A. alternata* | FS | P. Lady | MW685789 | MW685805 | MW685821 |
| UCD9620[cd] | B | *A. alternata* | LB | P. Lady | MW685788 | MW685804 | MW685820 |
| UCD9590[cd] | C | *A. arborescens* | FS | P. Lady | MW685782 | MW685798 | MW685814 |
| UCD9591 | C | *A. arborescens* | FS | P. Lady | MW685783 | MW685799 | MW685815 |
| UCD9593[cd] | C | *A. arborescens* | LB | P. Lady | MW685781 | MW685797 | MW685813 |
| UCD9603[d] | C | *A. arborescens* | LB | P. Lady | MW685780 | MW685796 | MW685812 |
| UCD9643[d] | C | *A. arborescens* | LB | P. Lady | MW685779 | MW685795 | MW685811 |
| UCD9643.2 | C | *A. arborescens* | LB | P. Lady | MW685784 | MW685800 | MW685816 |
| UCD9644 | C | *A. arborescens* | LB | P. Lady | MW685785 | MW685801 | MW685817 |
| UCD9645 | C | *A. arborescens* | LB | P. Lady | MW685786 | MW685802 | MW685818 |
| UCD10531 | C | *A. arborescens* | LB | Modi | OQ803493 | OQ803504 | OQ803515 |
| UCD10532 | C | *A. arborescens* | LB | Modi | OQ803494 | OQ803505 | OQ803516 |
| UCD10534 | C | *A. arborescens* | LB | Modi | OQ803495 | OQ803506 | OQ803517 |
| UCD10535 | C | *A. arborescens* | LB | Modi | OQ803496 | OQ803507 | OQ803518 |
| UCD10537 | C | *A. arborescens* | LB | Modi | OQ803497 | OQ803508 | OQ803519 |
| UCD10538 | C | *A. arborescens* | LB | Modi | OQ803498 | OQ803509 | OQ803520 |

[a] FS = fruit spot, LB = leaf blotch.
[b] Genes: Alt a1 = Alternaria major allergen Alt a1, ATPase = plasma membrane ATPase.
[c] Isolates used for pathogenicity tests on apple fruit and leaves.
[d] Isolates used for morphological characterization.

to 14-d-old cultures. Plates were flooded with approx. 20 mL of 0.05% Tween 80 and the medium surface in each plate was scraped with a sterile scalpel. The resulting conidial suspension was filtered through four layers of gauze and the concentration was adjusted to $1 \times 10^5$ conidia $mL^{-1}$, using a haemocytometer for conidia counting.

*Apple leaves*. Detached fully expanded mature leaves from 'Pink Lady' and 'Fuji' apple ($n$ = 10 from each cultivar) were surface disinfected in 1% NaOCl for 1 min, followed by sterile distilled water for 1 min, and were then air dried inside a laminar flow hood. Nine punctures were made on each leaf, three punctures were made on the apical, basal, and middle regions, using a sterile hypodermic needle (31G). Leaves were inoculated by placing 15 µL of conidial suspension on top of each wound site. The leaves were then incubated at 20°C in humid chambers for 7 d until symptoms development. Evaluations were carried out by measuring the lesion diameters using a digital caliper. An equal number of wounded leaves treated with sterile water were included as negative controls. Re-isolations from resulting necrotic lesions were made onto APDA, and obtained colonies were identified based on the conidia morphology. The experiment was conducted twice.

*Apple fruit*. Mature fruit (mean total soluble solids 14.2%) of 'Pink Lady' and 'Fuji' apple ($n$ = 10 of each cultivar) were surface disinfected in 70% ethanol for 5 min and air dried inside a laminar flow hood. Each fruit was then inoculated with 20 µL of a conidial sus-

**Table 2.** Accession numbers for reference sequences of *Alternaria* isolates in GenBank used for phylogenetic analyses in this study.

| Species | Isolate[b] | GenBank accession number [a] | | |
|---|---|---|---|---|
| | | Alt a1 | ATPase | Calmodulin |
| *Alternaria alstroemeriae* | CBS 118809 | MH084526 | MH101803 | MH175185 |
| *A. alternata* | EGS 34-016 | KP275691 | JQ671874 | JQ646208 |
| *A. alternata* (=*A. angustiovoidea*) | EGS 36-172 | JQ646398 | JQ671869 | JQ646203 |
| *A. alternata* (=*A. destruens*) | EGS 46-069 | JQ646402 | JQ671873 | JQ646207 |
| *A. alternata* (=*A. dumosa*) | EGS 45-007 | AY563305 | JQ671877 | JQ646211 |
| *A. alternata* (=*A. herbiphorbicola*) | EGS 40-140 | JQ646410 | JQ671888 | JQ646222 |
| *A. alternata* (=*A. limoniasperae*) | EGS 45-100 | JQ646370 | JQ671879 | JQ646213 |
| *A. alternata* (=*A. tenuissima*) | EGS 34.015 | KP275690 | JQ811989 | JQ646209 |
| *A. arborescens* | EGS 39-128 | AY563303 | JQ671880 | JQ646214 |
| *A. arborescens* | 3.J24 | KJ921023 | KJ908244 | KJ920979 |
| *A. arborescens* (=*A. cerealis*) | EGS 43-072 | JQ646405 | JQ671883 | JQ646217 |
| *A. argyroxiphii* | EGS 35-122 | JQ646434 | JQ671926 | JQ646260 |
| *A. betae-kenyensis* | CBS 118810 | KP123966 | MH101805 | MH175189 |
| *A. eichhorniae* | CBS 489.92 | KP123973 | MH101806 | MH175190 |
| *A. gossypina* | CBS 104.32 | JQ646395 | JQ671868 | JQ646202 |
| *A. grossulariae* | CBS 100.23 | JQ646394 | JQ671867 | JQ646201 |
| *A. jacinthicola* | CBS 133751 | KP123984 | MH101793 | MH175187 |
| *A. tomato* | CBS 114.35 | JQ646389 | JQ671861 | JQ646195 |
| *Stemphylium botryosum* | ATCC 42170 | AY563274 | JQ671767 | JQ646101 |
| *S. callistephi* | EEB 1055 | AY563276 | JQ671769 | JQ646103 |
| *S. vesicarium* | ATCC 18521 | AY563275 | JQ671768 | JQ646102 |

[a] Genes: Alt a1 = Alternaria major allergen Alt a1, ATPase = plasma membrane ATPase.
[b] ATCC = American Type Culture Collection, Manassas, VA; CBS = Centraalbureau voor Schimmelcultures, Royal Netherlands Academy of Arts and Sciences, Utrecht, the Netherlands; EEB, E. E. Butler, Department of Plant Pathology, University of California, Davis, CA; EGS = E. G. Simmons, Mycological Services, Crawfordsville, IN.; 3.J = Pryor and Michailides 2002.

pension that was deposited on top of four punctures made with a sterile hypodermic needle (31G). Fruits were then incubated at 20°C inside humid chambers for 14 d until symptom development. Resulting necrotic lesions were measured using a digital caliper. An equal number of wounded fruits treated with sterile distilled water were included as negative controls. Prior to determining the necrotic lesions, each fruit was cut vertically through each wound with a sterile knife, and the necrotic lesion length inside the fruit from the wound was measured. Re-isolations from the necrotic lesions were carried out on APDA to determine fulfilment of Koch's postulates, and obtained colonies were identified based on the conidia morphology. This experiment was conducted twice.

*Experimental designs and statistical analyses*

Pathogenicity test experiments were carried out according to a 2 × 6 (apple cultivar × isolate) factorial design, with ten replicates, each of one fruit or one leaf as the experimental unit. Lesion diameters and lengths were subjected to analysis of variance (ANOVA) using generalized linear models with the corresponding R packages in InfoStat v 2008. Means were separated using Fisher's least significant difference test ($P < 0.05$).

## RESULTS

### *Symptoms and fungal isolations*

During the disease outbreak, symptoms were mainly observed on apple leaves, and were characterized by the presence of one or more circular brown necrotic lesions (each 2 to 15 mm diam.) per leaf, with each lesion enlarged in zonate circular or crescent-shaped rings, and often with a dark brown to purple margin. With time, the affected leaves turned yellow and fell prematurely. On fruits, rounded, dark-coloured, dry, corky lesions (each 2 to 30 mm diam.) were observed (Figure 1).

Colonies of isolated fungi on APDA were gray-green to dark olive green with whitish margins. All *Alternaria* isolates produced single conidiophores and catenulate brown to golden-brown conidia.


**Figure 1.** Alternaria leaf blotch and fruit spot on 'Pink Lady' apple. **A** and **B**, naturally infected leaves. **C**, A naturally infected fruit.

*Morphological characteristics of isolates*

Preliminary categorization of the isolates based on colony morphology and sporulation patterns (Simmons, 2007), placed 36% of the isolates in group A, 18% in group B, and 47% in group C.

Group A isolates were tentatively identified as *A. alternata*, with these isolates producing cottony, gray to green colonies with white margins on PDA. Conidia chains were observed along with numerous secondary chains branching on short conidiophores. Conidia were ovoid to ellipsoid, and had average dimensions of 26.8 ± 4.63 × 9.8 ± 1.3 µm (Figure 2, A, D and G).

Group B isolates were morphologically identified as *A. tenuissima*, with these isolates producing cottony, gray olive brown colonies with slight concentric growth rings and white margins on PDA. Conidia chains had between six and 14 conidia, rarely with a lateral branch. Conidia were ovoid to obclavate, each with a narrow tapered upper half, and the conidia had average dimensions of 26.7 ± 5.1 × 9.5 ± 1.2 µm (Figure 2, B, E and H).

Group C isolates were identified as *A. arborescens*. These isolates produced cottony, olive-brown colonies with concentric growth rings, often with wavy margins on PDA. The conidiophores were long and had extended secondary conidiophores. Conidia chains had two to seven conidia, and conidium development was concentrated near the apices of secondary, tertiary, and quaternary conidiophores. Conidia were ovoid to ellipsoid, and had average dimensions of 26.2 ± 6.0 × 9.0 ± 1.3 µm (Figure 2, C, F and I).

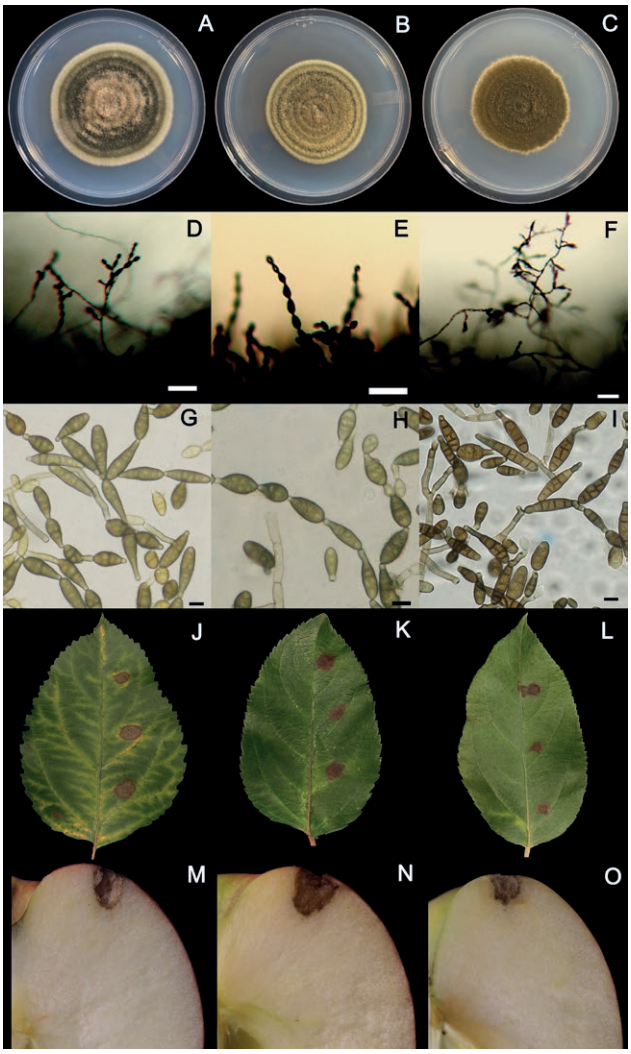


**Figure 2.** Morphological features of *Alternaria* species isolated from apple. **A** to **C**, Colony morphologies on PDA after 7 d incubation at 22°C under 8 h light/16 h dark regime; **D** to **F**, Sporulation patterns on PCA after 7 d incubation at 22°C under 8 h light/16 h dark regime; **G** to **I**, conidia; **J to L**, symptoms on inoculated 'Pink Lady' leaves; **M** to **O**, symptoms on inoculated 'Pink Lady' fruit cut vertically; **A, D, G**, **J,** and **M,** isolate of group A (UCD9582); **B, E, H**, **K, N,** isolate of group B (UCD9588); **C, F, I**, **L, O,** isolate of group C (UCD9593). White scale bars = 50 µm, black bars = 10 µm. Based on morphology and phylogeny, isolates in group A and B were identified as *A. alternata*, and isolates in group C were identified as *A. tenuissima*.

*Phylogenetic analyses*

The consensus sequence length of Alt a1 was 472 bp, of ATPase, was 1,194 to 1,197 bp, and CAL was 718 to 723 bp. The maximum parsimony analyses of Alt a1 (346 character dataset after alignment), ATPase (1,220 character dataset after alignment), and CAL (770 character dataset after alignment) combined produced a consensus tree (Figure 3) from the 25 most parsimonious trees (tree length = 731, consistency index = 0.804, retention index = 0.880, rescaled consistency index = 0.708). All the 26 *Alternaria* isolates obtained clustered with isolates that belong to the section *Alternaria*. The phylogenetic tree showed that there was clear separation of group C isolates from those of groups A and B. Isolates from group C clustered (96% bootstrap support) with the ex-type strain of *A. arborescens* (EGS 39128) and other *A. arborescens* reference isolates (Figure 3). Isolates from groups A and B, despite their morphological differences, were clustered with the *A. alternata* ex-type (EGS 40140) and other *A. alternata* reference isolates (Figure 3). Of the 26 *Alternaria* isolates obtained from apple fruits and leaves in California, 12 were identified as *A. alternata* (46%), and 14 corresponded to *A. arborescens* (54%).

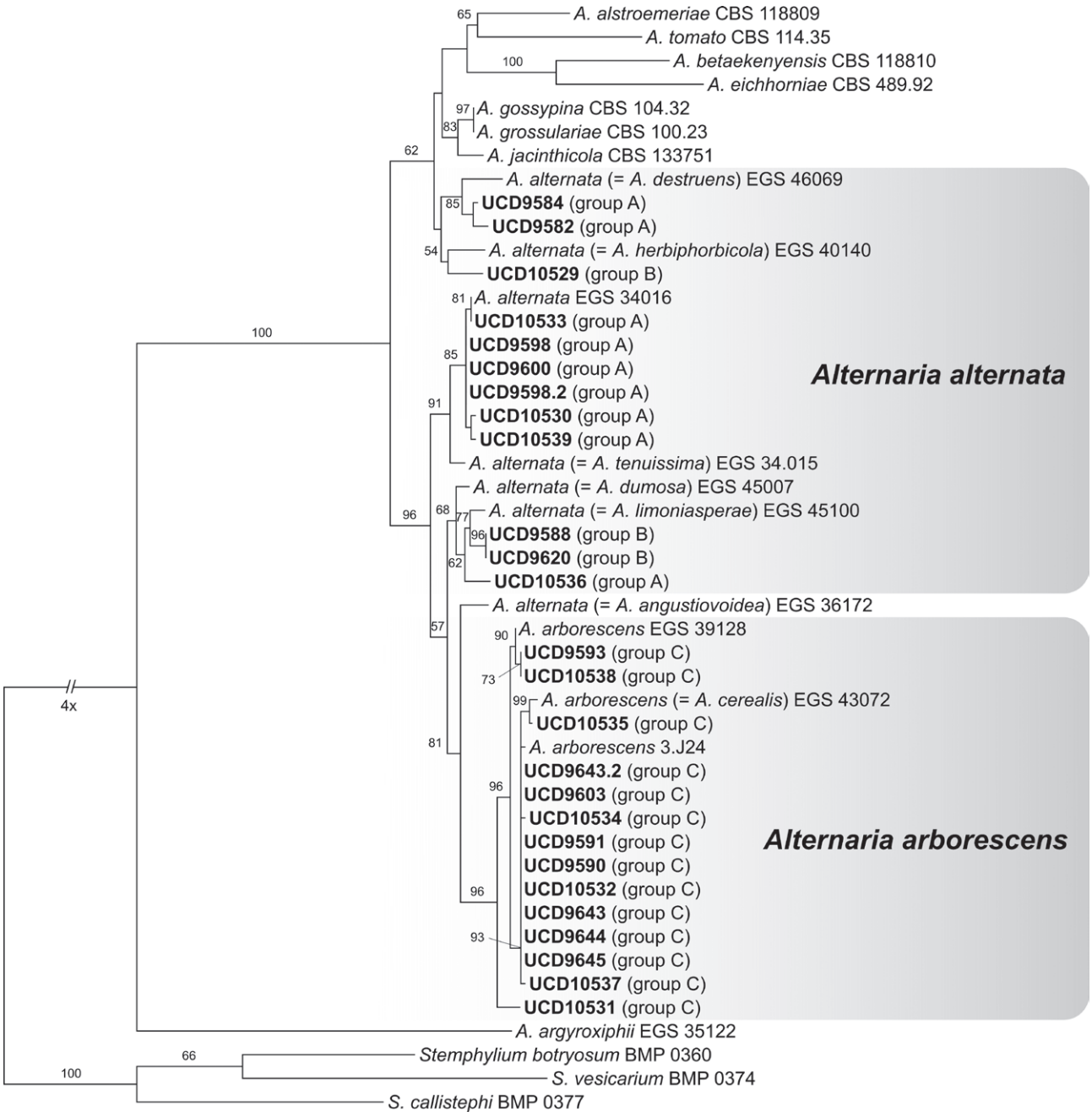


**Figure 3.** Phylogenetic tree obtained from maximum-parsimony analysis of the Alternaria major allergen Alt a1, plasma membrane ATPase, and calmodulin gene sequences of *Alternaria* species from Californian apple and sequences of ex-types in GenBank. The consensus tree shown is inferred from the 25 most parsimonious trees and bootstrap values obtained. The tree was rooted with *Stemphylium botryosum*, *S. callistephi*, and *S. vesicarium*. Tree length = 731, consistency index = 0.804, retention index = 0.880, and rescaled consistency index = 0.708. UCD and numbers (in bold) are *Alternaria* isolates from apple in California; other codes are isolates from GenBank.

*Pathogenicity tests*

All the tested isolates of *A. alternata* and *A. arborescens* were pathogenic on detached apple leaves, which developed brown necrotic lesions of 3.8 to 8.8 mm diameters after 7 d at 20 to 22°C, with the lesions developing concentric rings as they grew (Figure 2, J to L). Differences in disease severity caused by the *Alternaria* isolates were statistically significant ($P < 0.001$) for lesion diameter, but cultivar did not affect this parameter ($P =$

**Table 3.** Pathogenicity of *Alternaria* isolates studied on leaves and fruits of apple cultivars 'Pink Lady' and 'Fuji', assessed from dimensions of necrotic lesions and dry rots developed after controlled inoculations.

| Group | Species | Isolate | Leaves, mean necrotic lesion dimensions (mm) [ab] | | | | Fruit, mean dry rot lesion dimensions (mm) [ac] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 'Pink Lady' | 'Fuji' | Mean | | 'Pink Lady' | 'Fuji' | Mean | |
| A | *A. alternata* | UCD9600 | 6.6 | 8.8 | 7.7 | a | 3.9 | 9.1 | 6.5 | a |
| | | UCD9582 | 7.5 | 6.7 | 7.1 | a | 4.2 | 7.3 | 5.7 | ab |
| B | *A. alternata* | UCD9620 | 6.5 | 6.5 | 6.5 | a | 5.2 | 5.3 | 5.2 | ab |
| | | UCD9588 | 7.6 | 5.4 | 6.5 | a | 4.7 | 4.7 | 4.7 | ab |
| C | *A. arborescens* | UCD9593 | 3.8 | 4.3 | 4.0 | b | 4.1 | 4.2 | 4.1 | b |
| | | UCD9590 | 5.4 | 6.5 | 5.9 | a | 3.2 | 4.6 | 3.9 | b |
| | Mean | | 6.2 | 6.4 | | | 4.2 B | 5.8 A | | |
| *Analysis of variance* | | | | | | | | | | |
| | | | df | *F* | *P* | SED | df | *F* | *P* | SED |
| Isolate (I) | | | 5 | 5.13 | <0.001 | 0.926 | 5 | 2.37 | 0.044 | 0.849 |
| Cultivar (C) | | | 1 | 0.02 | 0.889 | 0.552 | 1 | 9.54 | 0.003 | 0.509 |
| I × C interaction | | | 5 | 1.32 | 0.259 | 1.308 | 5 | 1.77 | 0.125 | 1.089 |

[a] Non-inoculated controls remained symptomless, and these data were excluded from statistical analyses. Means (each of ten replicates) followed by the same letter in each column are not different (Fisher LSD test, $P = 0.05$). SED = standard error of the difference (standard error of the mean × √2).

[b] Leaves were inoculated with conidial suspensions ($10^5$ conidia $mL^{-1}$), and lesion diameters were determined after 7 days at 20°C in humid chambers.

[c] Fruit were each inoculated with 20 µL of conidial suspension ($10^5$ conidia $mL^{-1}$), then incubated in humid chambers at 20°C for 14 d.

0.889). The isolate × cultivar interaction was also non-significant ($P = 0.259$). The different *Alternaria* isolates had similar virulence (mean lesion diameter = 6.7 mm), except for *A. arborescens* isolate UCD9593, which was the least virulent isolate, causing the smallest lesions (mean = 4.0 mm) (Table 3).

Regardless of the *Alternaria* species, all the isolates caused dry rot on the epidermis and pulp of mature apple fruits after conidia inoculations. Symptoms consisted of dark-coloured, dry, corky lesions of lengths 3.9 to 6.5 mm (Figure 2, M to O). Significant differences ($P < 0.05$) in virulence were observed among the *Alternaria* isolates. The most virulent isolate was *A. alternata* isolate UCD9600 (mean lesion length = 6.5 mm), whereas *A. arborescens* isolate UCD9590 was the least virulent (mean = 3.9 mm). Apple cultivar had significant effects ($P < 0.01$) on lengths of the dry rot lesions, with 'Fuji' being more susceptible (mean = 5.8 mm) than 'Pink Lady' (mean = 4.2 mm). The interaction isolate × cultivar was non-significant ($P = 0.125$) (Table 3).

Re-isolations from the margins of the necrotic lesions and dry rots were accomplished from all of the inoculated leaves and fruits. Identifications of the re-isolated fungi was confirmed morphologically as those of the inoculated fungi. Non-inoculated leaves and fruits remained symptomless. These results fulfilled Koch's postulates for all the inoculated isolates.

## DISCUSSION

This study is the first to demonstrate that Alternaria leaf blotch and fruit spot are two diseases occurring in California apple orchards, and that both diseases are caused by two small-spored *Alternaria* species, *A. alternata* and *A. arborescens*. The fungi were identified by their morphological features and nucleotide sequences of three DNA barcodes (Lawrence *et al.*, 2013; Pryor and Michailides, 2002; Simmons, 2007; Woudenberg *et al.*, 2015).

There is consensus that identification of small-spored *Alternaria* species is difficult due the few morphological or molecular characteristics that allow species discrimination. Previous studies have demonstrated that host-specificity and geographic associations are not useful characters for *Alternaria* classification, and that morphological classifications are poor predictors of phylogenetic relationships among small-spored *Alternaria* taxa, especially due to high levels of morphological plasticity between and within *Alternaria* sections (Serdani *et al.*, 2002; Andrew *et al.*, 2009; Lawrence *et al.*, 2016).

Based on morphological characteristics of the colonies, sporulation patterns, and conidia, the isolates obtained in the present study from symptomatic apple leaves and fruit were grouped into three morphotypes (A, B and C). These groups were preliminarily identified, respectively, as *A. alternata*, *A. tenuissima* and *A. arborescens*. However, the multi-locus phylogenetic analyses using Alt a1, ATPase, and CAL sequences revealed that the isolates of group C formed a clear separate cluster with reference strains of *A. arborescens*. This is unlike the *A. alternata* isolates (group A) and *A. tenuissima* isolates (group B), which grouped together, despite their morphological difference in conidia chains that allow distinction between these two species (Simmons, 2007). Isolates of group B had long unbranched conidia chains, which is a key morphological characteristic for the identification of *A. tenuissima*. Similarly, in previous studies (Andrew *et al*., 2009; Wang *et al*., 2021), isolates morphologically classified as *A. alternata* and *A. tenuissima* were genetically indistinguishable using multiple molecular markers (*endoPG, OPA1-3*, and *OPA10-2*, or *ATPase*, *CAL*, and *rpb2* genes), and many other isolates were assigned as intermediates between the two groups. Based on genome and transcriptome comparisons and molecular phylogenies, Woudenberg *et al*. (2015) synonymized 35 morphospecies, which cannot be distinguished based on their multi-gene phylogenies, under *A. alternata*, including *A. tenuissima*. *Alternaria mali* was also synonymized with *A. alternata*, but *A. alternata* f. sp. *mali* is currently recognized for isolates which produce the host-specific AM-toxin. Consequently, in the present study, the isolates from group B (preliminarily as *A. tenuissima*) were then identified as *A. alternata*, along with the isolates from group A. However, there is still room for further investigation to determine presence of the AM-toxin gene and to verify their ability to produce AM-toxin.

In the last ten years, only small-spore *Alternaria* species have been described causing Alternaria leaf blotch and fruit spot of apple. *Alternaria alternata* and *A. arborescens* have been the most prevalent in different growing regions, including Australia (Harteveld *et al*., 2013), Chile (Elfar *et al*., 2018a), France (Fontaine *et al*., 2021), Italy (Rotondo *et al*., 2012), New Zealand (Toome-Heller *et al*., 2018), and Spain (Cabrefiga *et al*., 2023). These studies indicate that *A. alternata* and *A. arborescens* are the main causal agents of Alternaria leaf blotch and fruit spot in these regions, and that both species coexist in the same orchards (Fontaine *et al*., 2021). Additionally, these fungi are known to be well distributed on flowers and fruits from early season to harvest, serving as potential inoculum sources (Niem *et al*., 2007; Elfar *et al*., 2019). In the San Joaquin Valley of California, *A. alternata* and *A. arborescens* have been reported as the most prevalent species associated with Alternaria diseases in other fruit and nut crops, including Alternaria leaf spot of almond (Teviotdale *et al*., 2001), fruit rot of blueberry (Zhu and Xiao, 2015), fruit rot of mandarin (Wang *et al*., 2021), heart rot of pomegranate (Luo *et al*., 2017), and Alternaria late blight of pistachio (Pryor and Michailides, 2002). Therefore, the present study corroborates that both of these species are well adapted to the environmental conditions of the Central Valley, and that susceptible crops constitute inoculum sources of *Alternaria* species. Additionally, in the USA, specifically on the East Coast, there are reports of Alternaria leaf spots in field crops (Filajdić and Sutton, 1991) and postharvest fruit spots caused by *Alternaria* species (Jurick II *et al*., 2014; Kou *et al*., 2014).

Pathogenicity tests on detached leaves and fruits are common and efficient practices to fulfill Koch's postulates for *Alternaria* species on apples (Harterveld *et al*., 2014b; Gur *et al*., 2017; Elfar *et al*., 2018a; Toome-Heller *et al*., 2018; Fontaine *et al*., 2021). Rotondo *et al*. (2012) concluded that bioassays on detached leaf tissues were reproducible with unambiguous symptoms. Furthermore, greater proportions of lesions developed on wounded than on nonwounded leaves (Rotondo *et al*., 2012). Similar results have been observed on different crops. Examples include pistachios, where unwounded inoculated leaves did not develop substantial lesions (Pryor and Michailides, 2002), and on *Amaranthus hybridus*, where nonwounded inoculated leaves remained asymptomatic (Blodgett and Swart, 2002). However, *Alternaria* species are capable of infecting and colonizing healthy leaves, and these infections generally remain latent until leaf defenses are compromised, making them more susceptible due to injury, stress, or senescence (Blodgett and Swart, 2002; Pryor and Michailides, 2002; Rotondo *et al*., 2012).

Harteveld *et al*. (2014b) determined that regardless of the *Alternaria* species and the symptom they were originally obtained from (leaf blotch or fruit spot), all isolates were pathogenic on detached nonwounded leaves. However, not all the tested isolates caused fruit spots on attached nonwounded fruits, regardless of the symptom they were recovered from. None of the *A. arborescens* isolates they studied were pathogenic on fruits. Therefore, differential tissue specificity probably occurs across isolates. Similarly, Elfar *et al*. (2018b) found that *Alternaria* isolates obtained from leaf blotch symptoms were incapable to produce symptoms on fruits. In the present study, all the isolates of *A. alternata* and *A. arborescens* were pathogenic on leaves and fruits,

regardless of the isolate or the symptom it was obtained from. Statistically significant differences ($P < 0.001$) were detected between isolates for lesion diameters, indicating differences in virulence. Similar results have been described in previous studies, which suggest that pathogenicity is isolate-dependent rather than species-dependent (Rotondo *et al*., 2012; Harteveld *et al*., 2014b; Fontaine *et al*., 2021).

This study is the first to identify *Alternaria* species causing leaf blotch and fruit spot of apple in California, although a larger scale survey is required to establish the importance and extent of these pathogens. Based on the our results, prevalence of leaf blotch was up to 30%, which is greater than that in compared to Chile, where the observed prevalence was from 0.1 to 4.0% (Elfar *et al*., 2018a). However, the prevalence of fruit spot in the present study was less than 1%, which is similar to that reported in Australia (< 2%) (Harteveld *et al*., 2014a). In Israel, high prevalence levels have been recorded after severe outbreaks of Alternaria leaf blotch and fruit spot in 'Pink Lady' orchards, with up to 80 % of the fruit affected (Gur *et al*., 2017). Therefore, Californian isolates may be less virulent than the Israeli isolates, or the environmental conditions in California are less conducive for the development of these diseases. Epidemiological studies have shown that the diseases develop when temperatures range between 12 and 28°C, and the severity of Alternaria leaf spot increases with increasing duration of moisture (Filajdić and Sutton, 1992). These conditions coincide with the conidia release, which commences when median temperatures exceed 12.5 °C in association with precipitation events (Cabrefiga *et al*., 2023). At the optimum temperature (23.5°C) only 5.1 h of wetness were required for light infections, and 12.7 h for severe infections (Filajdić and Sutton, 1992). In the San Joaquin Valley, springs (April and May) are characterized by high rainfall and moderate temperatures (15 to 19°C), and these are followed by dry summers (June and August) with temperatures between 25 and 33°C (Mila *et al*., 2005). Therefore, the risks of severe Alternaria leaf blotch and fruit spot outbreaks are likely to be low, due to the absence of rainy days during summer. However, the presence of overhead sprinklers used by growers during the summer could be a predisposing factor for the development of Alternaria leaf blotch and fruit spot of apple.

The present study is the first to report *A. alternata* and *A. arborescens* associated with apple leaf blotch and fruit spot in California. Currently, these are considered as minor apple diseases in this state. However, the present results do not exclude the possibility that other *Alternaria* species may be associated with leaf spot and fruit spot of apple in California. Furthermore, these results serve as a starting point for understanding etiology of these diseases, and establishing disease management strategies in case outbreaks occur when predisposing conditions are present.

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